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DESENVOLUPAMENT D'UN ADENOVIRUS ONCOLÍTIC POTENT I SELECTIU COM A BASE PER A LA INCORPORACIÓ DE TRANSGENS QUE AJUDIN A L'ERADICACIÓ DELS TUMORS

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DISCUSSIÓ

1. TRACTAMENT DEL CÀNCER AMB ADENOVIRUS ONCOLÍTICS

Malgrat que durant els últims anys s'han realitzat importants avenços en el tractament del càncer, aquesta malaltia es manté com una de les principals causes de mort a nivell mundial. Els tractaments estàndard del càncer (cirurgia, radioteràpia i quimioteràpia) no ofereixen especificitat pel teixit tumoral i cal la recerca de noves modalitats terapèutiques que aprofitin les característiques moleculars úniques de les cèl·lules tumorals per obtenir un gran índex terapèutic limitat al teixit malalt. Un bon exemple d'aquestes noves teràpies són els anticossos monoclonals que bloquegen algun epítop tumoral, tals com Herceptin (Hudis, 2007) o Rituximab (Maloney *et al.*, 1997), que han arribat a ser part del tractament estàndard d'algun tipus de tumor. Malgrat això, al tractar-se d'una droga, s'han d'emprar elevades dosis per arribar a cada una de les cèl·lules d'un tumor, fet que es tradueix en greus efectes secundaris, incloent problemes de cor o reaccions autoimmunes. Alguns oncòlegs aposten per la immunoteràpia, on les cèl·lules activades del sistema immune haurien d'autoamplificar-se fins eliminar totes les cèl·lules diana. No obstant, l'èxit d'aquesta estratègia es preveu difícil, ja que els tumors creixen i evolucionen en presència d'aquestes cèl·lules i es seleccionen per evadir-les (Alemany and Cascallo, 2009). Malgrat la complexitat que presenta un virus, la viroteràpia del càncer presenta característiques pròpies que resolen aquestes limitacions, destacant la lisi directa de la cèl·lula tumoral i la capacitat d'autoamplificar la dosi inicialment administrada.

El concepte de tractar tumors amb virus és tan antic com el propi descobriment dels virus. Durant els anys 60 i 70 del passat segle, aquest concepte va adquirir el seu punt àlgid degut al descobriment de virus amb gran capacitat citolítica contra les cèl·lules tumorals (Kelly and Russell, 2007). Després de resultats decebedors en assajos clínics, la viroteràpia del càncer es va revifar durant la dècada passada quan l'enginyeria molecular es va aplicar al disseny de millors virus per al tractament de tumors. Un gran ventall de virus, tant d'ARN com d'ADN, s'han testat per a la causa (Cattaneo *et al.*, 2008), diferint entre ells en paràmetres que influencien la seva potència oncolítica, tals com la seva mida, interacció amb receptors específics cel·lulars, interacció amb factors sanguinis, eliminació per part dels macròfags, internalització i tràfic intracel·lular, velocitat de replicació, producció viral, propagació intercel·lular, sortida de la cèl·lula, inducció d'apoptosi o autofàgia i inducció de la resposta immune innata i adaptativa.

Per les seves característiques de seguretat, estabilitat *in vivo*, facilitat de manipulació i possibilitat d'obtenir títols elevats, els adenovirus oncolítics són dels virus més emprats i

prometedors dins la viroteràpia del càncer. A més, a diferència de molts altres virus, existeix un coneixement raonable de la seva biologia i permeten la incorporació de gens exògens que poden atorgar propietats úniques als virus (Cody and Douglas, 2009). Alguns adenovirus oncolítics genèticament modificats, principalment Onyx-015, ja han estat testats en assajos clínics, incloent un ampli ventall de tipus tumorals i diferents rutes d'administració. Les dades clíniques obtingudes indiquen un bon perfil de seguretat i toxicitat, exceptuant alguns efectes adversos preocupants després de l'administració d'elevades dosis (Nemunaitis *et al.*, 2001; Aghi and Martuza, 2005). Si ens fixem en l'eficàcia, la majoria de respostes observades van resultar transitòries, i el tractament no va ser capaç d'alterar significativament l'evolució de la malaltia (Habib *et al.*, 2002; Hamid *et al.*, 2003; Small *et al.*, 2006). Així, aquests resultats indicaven la necessitat d'una millora substancial dels adenovirus oncolítics per poder produir respostes efectives en humans.

Per al tractament efectiu de tumors en estat avançat, es requereix l'administració sistèmica de l'agent oncolític per tal d'arribar a tots els focus tumorals i metàstasis. A més, l'administració sistèmica permet una distribució més uniforme del virus pel tumor, ajudant a l'eradicació d'aquest (Wein *et al.*, 2003). No obstant, el virus es troba amb diverses limitacions després de ser administrat sistèmicament: primerament, el virus és eliminat ràpidament del torrent sanguini pel fetge (Alemany *et al.*, 2000) o inactivat per la unió a cèl·lules sanguínies, a anticossos neutralitzants o al complement (Parato *et al.*, 2005). Així, només una petita proporció del virus administrat arriba al tumor; un cop al tumor, una de les característiques dels adenovirus que limiten més la seva eficiència és la incapacitat de distribuir-se eficientment per tot el tumor. Algunes de les estratègies emprades en la bibliografia consisteixen en mutar certs gens virals, tals com E1B-19K o la proteïna *i-leader* (Yan *et al.*, 2003; Liu *et al.*, 2004; Subramanian *et al.*, 2006), o en sobreexpressar la proteïna ADP (Toth *et al.*, 2004) per millorar la sortida del virus de la cèl·lula, encara que aquestes modificacions s'han associat amb greus efectes adversos com la disminució de la producció viral o la pèrdua de les funcions immunomoduladores d'E3 (Pilder *et al.*, 1984; Wang *et al.*, 2003). No obstant, una de les limitacions més importants a la dispersió del virus és la matriu del tumor, que imposa barreres físiques que el virus difícilment pot travessar (Kim *et al.*, 2006). Una estratègia que s'ha mostrat molt eficient per superar aquestes barreres consisteix en aportar al virus eines per destruir-les, armant-los amb transgens tals com proteases per trencar la matriu (Kim *et al.*, 2006) o enzims convertidors de prodrogues amb efecte *bystander* per les barreres fibròtiques (Chen *et al.*, 2004). Malgrat els beneficis que aporta aquesta estratègia, la capacitat d'encapsidació del virus està limitada al 105% de la mida del genoma salvatge (Bett *et*

al., 1993), fet que limita la incorporació d'ADN exogen i pot arribar a ser incompatible amb altres modificacions dels adenovirus que confereixen selectivitat, com els promotors exògens. Com a mesura per guanyar espai per a la incorporació dels transgens, s'ha intentat delecionar la regió E3, però s'ha demostrat que la funció immunomoduladora d'aquesta regió és important per mantenir la potència oncolítica en hostes immunocompetents (Suzuki *et al.*, 2002). Al nostre grup, a més, s'ha provat de delecionar els ORF 1 i 2 o els ORF 1, 2 i 3 de la regió E4, ja que cap funció imprescindible s'ha associat amb aquestes proteïnes (Tauber and Dobner, 2001). Els virus resultants, però, quan es van armar amb diferents transgens, mostraven importants pèrdues de potència i defectes de replicació a cèl·lules tumorals comparats amb els virus que mantenien intacta la regió E4 (resultats no publicats, tesi doctoral Sonia Guedan). Per tant, és important trobar modificacions genètiques que permetin l'expressió d'aquests transgens dintre del context d'un adenovirus oncolític sense sobrepassar el límit de mida del genoma imposat per la capacitat d'encapsidació.

Els efectes adversos registrats a la clínica després de l'administració sistèmica d'adenovirus oncolítics (transaminitis, trombocitopènia, ...) (Aghi and Martuza, 2005) han assenyalat a la restricció de la replicació i expressió de proteïnes virals a cèl·lules tumorals com un dels punts claus per a l'èxit de la teràpia. L'expressió de la proteïna E1A per sí mateixa causa greus danys al fetge (Engler *et al.*, 2004), i aquesta toxicitat no es pot evitar amb delecions de funcions de gens virals, tal com la deleció d'E1B-19k present al virus Onyx-015. Per evitar aquesta toxicitat, cal limitar la transcripció d'E1A a les cèl·lules diana. La introducció de promotors exògens específics de teixit per controlar aquesta transcripció permet redirigir l'expressió d'E1A i la replicació del virus a un tipus específic de tumor, com per exemple restringir la replicació del virus a cèl·lules de càncer de pròstata utilitzant el promotor PSA (Rodriguez *et al.*, 1997) o a cèl·lules de càncer de pàncrees utilitzant el promotor uPAR (Huch *et al.*, 2009). Malgrat els avantatges d'aquesta estratègia, la utilització d'un promotor que exploti característiques comunes a la majoria de cèl·lules tumorals és més atractiu per poder tractar un rang més ampli de pacients i assegurar-se la potència del virus a totes les cèl·lules tumorals. Dintre de les característiques que presenten les cèl·lules tumorals, els defectes en la via de RB per inhibir la unió de pRB a E2F són presents en el 100% dels tumors (Sherr and McCormick, 2002). Així, un virus que aprofiti i respongui a aquests defectes presentarà una replicació selectiva a cèl·lules tumorals. La primera modificació per redirigir la replicació del virus cap a cèl·lules amb deficiències a la via de RB va ser la deleció Δ24 (dl922-947) (Fueyo *et al.*, 2000; Heise *et al.*, 2000). Aquesta modificació consisteix en la deleció del lloc d'unió a pRB del motiu CR2 de la proteïna

E1A. E1A, a més de ser la primera proteïna viral en ser sintetitzada i activar la transcripció de totes les altres proteïnes virals, també és capaç d'activar el cicle cel·lular a través de la unió a proteïnes de la família de pRB, p130 i p107 (Dyson, 1998). La formació d'aquest complex allibera E2F de pRB i permet l'activació de la transcripció dels gens virals i cel·lulars que contenen llocs d'unió d'E2F als seus promotors. Degut a la disfunció de la via de RB, la unió d'E1A a pRB no és necessària a cèl·lules tumorals. D'aquesta manera, la deleció Δ24 resulta en virus que preserven la potència oncolítica a cèl·lules tumorals, però repliquen de forma atenuada a cèl·lules normals. Malgrat això, i com ja s'ha comentat abans, aquesta mutació no evita la toxicitat hepàtica després de l'administració sistèmica, i cal regular la transcripció d'E1A. Per redirigir la transcripció d'aquesta proteïna en resposta a la desregulació de la via de RB, s'han introduït diversos promotors que responen al factor de transcripció E2F. Entre aquests promotors, en destaca el promotor E2F-1 degut a la potent expressió que indueix l'estructura única de llocs d'unió d'E2F que presenta, consistent en quatre llocs d'unió organitzats en dos palíndroms imperfectes (Neuman *et al.*, 1994, 1995). Aquesta estructura, a més, permet una activació encara més potent en el context de l'adenovirus, ja que la proteïna adenoviral E4-6/7 és capaç d'unir-se a dos molècules d'E2F i induir la unió estable d'aquest factor als palíndroms del promotor E2F-1 (Schaley *et al.*, 2000). A més, la replicació del virus es veu augmentada gràcies a la presència d'aquests mateixos llocs palindròmics al promotor salvatge dels gens virals E2 (Neill *et al.*, 1990). Com a resultat, s'aconsegueix una replicació selectiva a cèl·lules tumorals quan s'introdueix el promotor E2F-1 per controlar la transcripció d'E1A. Malgrat la cooperació que realitzen tots aquests components per activar la replicació, els resultats publicats pel nostre i altres grups amb adenovirus oncolítics que controlen E1A sota el promotor E2F-1 indiquen que existeix una important pèrdua de potència oncolítica respecte els adenovirus salvatges (Jakubczak *et al.*, 2003; Majem *et al.*, 2006). A més, si al control d'E1A s'hi afegeix un promotor addicional, com el de la telomerasa hTERT per controlar un altre gen viral, com E4 o E1B, la pèrdua de potència s'incrementa (Ryan *et al.*, 2004; Li *et al.*, 2005), descartant aquesta possibilitat i indicant la necessitat de millorar el control transcripcional d'E1A per millorar la potència dels adenovirus oncolítics.

Abans de la realització d'aquesta tesi, el nostre grup havia desenvolupat ICOVIR-5, un adenovirus oncolític que millora la potència oncolítica a cèl·lules tumorals gràcies a que inclou la seqüència òptima de reconeixement de l'inici de traducció per part del ribosoma (seqüència Kozak) davant d'E1A per millorar-ne els nivells a cèl·lules tumorals. A més, aquest virus presenta una replicació selectiva gràcies a un control d'aquesta a diferents nivells: transduccional (a través

d'un motiu RGD a la fibra per infectar independentment del receptor CAR), transcripcional (gràcies al control d'E1A mitjançant un promotor E2F-1 aïllat amb la seqüència DM-1) i a nivell d'interacció proteïna-proteïna (inclou la deleció Δ24). Aquest virus havia demostrat un potent efecte antiglioma, per sí sol (Alonso *et al.*, 2007a) o en combinació amb quimioteràpia (Alonso *et al.*, 2007b), i ser efectiu per al tractament d'un ampli rang de tipus tumorals (Cascallo *et al.*, 2007). Malgrat aquests bons resultats, ICOVIR-5 presentava encara certs nivells d'atenuació en alguns models tumorals, a més de les dificultats típiques de difusió pel tumor associades a la viroteràpia clàssica amb adenovirus. Per aquest motiu, l'objectiu d'aquesta tesi es va enfocar a la millora de la potència oncolítica d'aquest virus i a l'adaptació dels seus components genètics per poder expressar un transgen que permeti la distribució del virus per tot el tumor.

2. ICOVIR-7: MÉS E1A, MÉS POTÈNCIA ONCOLÍTICA

Degut a les pèrdues de potència oncolítica respecte els adenovirus salvatges que presenta ICOVIR-5 a certs models tumorals, era necessària alguna modificació que aconseguís que la replicació del virus i la citotoxicitat a cèl·lules tumorals es mantinguessin a nivells més similars als dels adenovirus salvatges. A més, aquesta modificació no havia de disminuir els alts nivells de selectivitat que mostra ICOVIR-5 a models de toxicitat, per millorar així la ràtio eficàcia/toxicitat d'aquest virus. Addicionalment, aquesta modificació hauria de respondre també a la desregulació de la via de RB per mantenir una bona potència oncolítica a un gran nombre i tipus de tumors. La inclusió de modificacions que responguin a altres vies o mutacions presents a les cèl·lules tumorals limitaria els tumors on el virus podria replicar de forma eficient. Degut a la cooperació tan efectiva que duen a terme E1A-Δ24 i E4-6/7 amb el palíndroms de llocs d'unió d'E2F presents al promotor E2F-1, vàrem decidir magnificar aquest mecanisme incrementant el nombre d'aquestes estructures úniques per controlar la transcripció d'E1A. Així, ICOVIR-7 incorpora vuit nous llocs d'unió d'E2F, organitzats en quatre palíndroms imperfectes, clonats al promotor E2F-1 salvatge. Aquest clonatge es va realitzar davant de la caixa CAAT del promotor per evitar interferir en l'inici de la transcripció i, a més, a prop de llocs d'unió del factor de transcripció Sp-1, ja que està descrit que Sp-1 interacciona amb E2F i ambdós cooperen per activar la transcripció (Karlseder *et al.*, 1996) (**Figura 13a**). Com a conseqüència, nivells més elevats d'E2F s'haurien d'unir al promotor que controla E1A a cèl·lules tumorals, incrementant-ne la transcripció. Això provocaria una activació més potent de la resta de promotors virals, produint nivells més elevats

d'E4-6/7 per activar un potent bucle de producció d'E1A i E2 que culminaria en una replicació més eficient de virus a cèl·lules tumorals (**Figura 13b**).

Els nostres resultats corroboren aquesta teoria. Els nous palíndroms presents a ICOVIR-7 són capaços d'incrementar la quantitat de proteïna E1A produïda (**Figura 20a**), arribant a nivells molt similars als de l'Adwt-RGD. Aquest increment d'expressió té un impacte positiu en la producció viral i en la citotoxicitat sobre cèl·lules tumorals, ja que ICOVIR-7 produeix més virions per cèl·lula que ICOVIR-5 en la majoria de línies cel·lulars testades (**Figura 20b**) i redueix el número de cèl·lules tumorals viables amb una mateixa quantitat de virus inicial (**Figura 21**). A més, aquest benefici en l'activitat antitumoral es manté en models *in vivo*: tant la mida dels tumors després de l'administració sistèmica (**Figura 22**) com la supervivència dels ratolins amb tumors subcutanis (**Figura 23**) milloren amb el tractament d'ICOVIR-7 respecte el mateix tractament amb ICOVIR-5. Així, els nostres resultats indiquen que expressar nivells elevats d'E1A, propers als nivells de l'adenovirus salvatge, és important per mantenir una bona potència oncolítica a tots els models i tipus tumorals, en contradicció amb alguns estudis publicats pel nostre i altres grups (Hitt and Graham, 1990; Nettelbeck *et al.*, 2002), on es concloïa que la pèrdua d'expressió d'E1A tenia poc efecte sobre els nivells de replicació dels virus. Malgrat aquesta millora, el nou virus ICOVIR-7 no recupera completament els nivells de potència de l'Adwt-RGD, ni en producció viral ni en citotoxicitat, indicant que encara tenim marge de millora si volem aconseguir una potència oncolítica que ens permeti obtenir bons resultats d'eficàcia antitumoral en assajos clínics.

Teòricament, als nous llocs d'unió d'E2F introduïts a ICOVIR-7 s'hi haurien d'unir també més complexes E2F-pRB-HDAC a cèl·lules normals, enfortint la associació dels nucleosomes amb l'ADN i incrementant la repressió de la transcripció (Black and Azizkhan-Clifford, 1999). Pel contrari, els nostres resultats indiquen que les selectivitat d'ICOVIR-5 i ICOVIR-7 són molt similars. La toxicitat hepàtica (**Figura 16, 18b** i **19**), així com l'hematològica (**Figura 18c** i **d**), no es redueixen amb aquesta nova modificació del promotor. Segurament, això és degut a que la combinació d'elements genètics presents a ICOVIR-5 és suficient per restringir de forma eficaç la replicació a cèl·lules normals després de l'administració sistèmica, mostrant un perfil de toxicitat que difícilment és pot millorar amb cap modificació genètica. Aquesta selectivitat és pot apreciar clarament a la **Figura 16**, on la replicació d'ambdós ICOVIRs a les seccions de fetge és molt similar a la de l'adenovirus no-replicatiu AdTL-RGD, indicant que la quantitat de virus detectada és el virus romanent de la infecció inicial i que no es produeix replicació dels ICOVIRs en aquest model.

A més, en experiments anteriors amb ICOVIR-5, es va poder observar com la toxicitat apreciada després de l'administració sistèmica de $1x10^{11}$ partícules virals d'ICOVIR-5 per ratolí era molt similar a la provocada per la mateixa quantitat de partícules d'un adenovirus inactivat amb Psolaren i llum ultraviolada (resultats no publicats). De fet, està descrit que l'administració sistèmica d'adenovirus provoca dos pics diferents de resposta inflamatòria, un a les 6 hores post-injecció i l'altre als 5 dies (Liu *et al.*, 2003). El primer pic està associat a la càpsida viral i el segon està provocat per l'expressió de proteïnes virals. Els nivells tan reduïts d'E1A observats al fetge (**Figura 19a**) juntament amb aquests experiments comparatius entre ICOVIR-5 i adenovirus inactivats, ens fan pensar que l'elevació de transaminases (**Figura 18b**) i la petita pèrdua de pes observades (**Figura 19d**) estan associades a la toxicitat causada per la càpsida adenoviral, no a l'expressió d'E1A. Complementàriament a aquests models comentats fins ara, la selectivitat d'ICOVIR-7 també es va testar a cèl·lules HUVEC i hepatòcits humans en cultiu, obtenint resultats contradictoris degut a deficiències del model, però aquestes dades es comentaran més endavant a l'apartat de discussió dels models. En conclusió, es pot dir que la combinació d'elements genètics que presenten tant ICOVIR-5 com ICOVIR-7 controla eficientment la toxicitat associada a l'expressió de la proteïna E1A i evita la replicació del virus al fetge normal.

Gràcies a una col·laboració amb el grup del doctor Akseli Hemminki de la University of Helsinki (Finlàndia), ICOVIR-7 s'ha pogut administrar en pacients humans en el marc d'un estudi basat en usos compassius (Nokisalmi *et al.*, manuscrit enviat a *Clinical Cancer Research* i adjuntat a l'annex d'aquest tesi). Aquest estudi preliminar inclou 21 pacients amb tumors metastàtics avançats i resistents a les teràpies estàndard, els quals van ser tractats amb dosis entre $2x10^{10}$ i $1x10^{12}$ partícules virals, administrades tant per injecció intratumoral com sistèmica. En general, el tractament amb ICOVIR-7 va ser ben tolerat, amb lleus efectes secundaris com febre, fatiga, transaminitis, calfreds o hiponatrèmia (**Taula 9**). A més, el virus es va poder detectar a la sang dels pacients vàries setmanes després del tractament, indicant que el virus s'havia replicat, fins i tot en presència d'anticossos neutralitzants, que es van detectar a 16 de 17 pacients analitzats. Si ens fixem en l'activitat antitumoral que va presentar el tractament amb ICOVIR-7, veiem que es va observar evidència d'activitat antitumoral a 9/17 pacients avaluables (**Taula 10**). Entre aquests, 5/12 pacients avaluables van mostrar un benefici clínic en un anàlisi radiològic, incloent una resposta parcial, dos respostes menors i dos casos de malaltia estable. Una d'aquestes respostes menors correspon a un pacient injectat sistèmicament, indicant que és possible aconseguir activitat antitumoral amb aquesta via d'administració. Complementàriament, 3/3 pacients avaluables van presentar una disminució de la densitat tumoral. A més, aquests pacients

que van presentar algun benefici clínic tenien tipus tumorals molt diversos, incloent tumors de cap i coll, càncer de pàncrees, de pròstata, o d'ovari, colangiocarcinoma i tumor de Wilms, indicant que ICOVIR-7 pot ser beneficiós per al tractament de diversos tipus de càncer. Addicionalment, és important remarcar que els pacients tractats en aquest estudi havien rebut prèviament una mitja de 5 cicles de quimioteràpia, a més de tractaments addicionals com radioteràpia o cirurgia, no responent cap d'ells als tractaments. La presència d'activitat antitumoral a 9/17 d'aquests pacients és molt remarcable, justificant la realització d'un assaig clínic randomitzat, centrat en un tipus tumoral i més ampli en número de pacients, per treure conclusions més fiables sobre el benefici que pot aportar aquest virus per al tractament del càncer. No obstant això, els resultats d'aquest estudi preliminar també indiquen que és necessari incrementar l'activitat antitumoral d'aquest virus per arribar a aconseguir respostes complertes.

Com ja s'ha comentat anteriorment, l'expressió de transgens per facilitar la distribució del virus per tot el tumor és una bona estratègia per millorar l'activitat antitumoral dels adenovirus oncolítics. Les masses tumorals, a més de per cèl·lules tumorals, també estan composades per àrees d'estroma, que inclouen fibroblasts, cèl·lules endotelials i del sistema immune, i components de la matriu extracel·lular, com col·lagen o àcid hialurònic. De fet, molts tipus tumorals es caracteritzen per petits grups de cèl·lules tumorals rodejades de grans àrees d'estroma, i això limita molt la dispersió intratumoral dels virus (Alemany, 2007; Cheng *et al.*, 2007). A la **Figura 22d** dels nostres resultats, es pot veure com a un model de tumor xenògraft que expressa grans quantitats de components de matriu extracel·lular (PC-3) (Draffin *et al.*, 2004; Ricciardelli *et al.*, 2007) l'eficàcia del tractament amb ICOVIR-7 es veu molt reduïda si la comparem amb l'eficàcia del mateix tractament a altres models. Així doncs, i amb l'objectiu d'armar ICOVIR-7, vàrem establir una col·laboració amb el grup del Dr. Peter Searle de la University of Birmingham (Regne Unit) per introduir vàries nitroreductases a mode de transgen al genoma d'ICOVIR-7. Les nitroreductases s'empren a la teràpia gènica del càncer com a convertidores de prodrogues i permetrien al virus destruir, gràcies a l'efecte *bystander* o col·lateral, un gran número de cèl·lules que ni tan sols haurien estat infectades, incloent cèl·lules de la matriu del tumor, com cèl·lules endotelials o barreres de fibroblasts (Cody and Douglas, 2009). Aquestes cèl·lules, per la seva condició de cèl·lules no tumorals, poden resultar difícils d'infectar i matar per part dels adenovirus oncolítics, fet que fa molt útil la incorporació al genoma d'aquests enzims convertidors de prodrogues.

Taula 9. Efectes adversos de l'assaig preliminar amb ICOVIR-7 a pacients humans. Modificada de *Nokisalmi et al*., 2010 (enviat a Clinical Cancer Research, annexat en aquest treball).

Efectes adversos	Grau 1 (nº pacients)	Grau 2 (nº pacients)	Grau 3 (nº pacients)	Grau 4 (nº pacients)
Hematològics				
Anèmia	6	6	1	
Transaminitis	9	4		
Hipocalèmia	7			
Hiponatrèmia	12			
Dolor				
Lloc d'injecció		1		
Pit	2			
Abdominal	5	6		
Muscular/ extremitats		5		
Esquena	1	1		
Cap	1	1		
Sistema Gastrointestinal				
Restrenyiment		4		
Diarrea	1			
Ardor d'estómac	1			
Pèrdua de la gana	3			
Nàusees, vòmits	6	2		
Inflor abdominal	3	1		
Sistema respiratori				
Disnea	3	1		
Tos	2	1		
Gola irritada	2			
Dificultat en la parla	1			
Sistema immunològic				
Febre	11	7		
Calfreds	7	3		
Altres				
Fatiga	3	13		
Sudoració	2			
Edema (extre. inferiors)		1		
Picor	2			
Sed		1		

Tipus de càncer	Dosi ($x10^{11}$ vp)	Marcadors tumorals	RECIST	Densitat tumoral (HU)	Supervivència (dies)
Mama	0,2	MR: CEA ↓ PD: CA15-6 ↑			58
Pàncrees	0,7	PD: CA19-9 ↑ CEA↑	PD		92
Tumor de Wilms	1+CPA		PR (-37%)		192
Bufeta	2+CPA		PD (+29%)	73→60 (-18%)	320
Pròstata	2	SD: PSA ↓			51
Mama	3+CPA	PD: CA15-3 ↑ CEA ↑	PD (24%)	79→57 (-28%)	142
Mama	3+CPA	PD: CA15-3 ↑			79
Cap i coll	3	PD: CEA ↑	PD		113
Ovari	3+CPA	MR: CA12-5 ↓	MR (-17%)		268*
Còlon	4		PD		62
Còlon	4				34
Estòmac	4, IV	PD: CA12-5 ↑			109
Ovari	4+CPA	PD: CA12-5 ↑			79
Ovari	4	PD: CA12-5 ↑	PD		73
Cap i coll	5+CPA				371*
Cap i coll	6+CPA, IV				49
Pàncrees	6		SD (+9%)		93
Leiomiosarcoma	6+CPA		PD (+30%)		56
Colangiosarcoma	7		SD (+13%)	93→78 (-16%)	316*
Pàncreas	7+CPA				60
Cap i coll	10, IV		MR (-10%)		167

Taula 10. Activitat antitumoral observada en l'assaig preliminar amb ICOVIR-7 a pacients humans. RECIST: Response Evaluation In Solid Tumors. HU: Hounsfield Units. MR: Mixed Response. SD: Stable Disease. PD: Progressive Disease. CEA: Carcinoembryonic Antigen. CA: Cancer Antigen. CPA: Ciclofosfamida. IV: intravenós. *: amb vida al finalitzar l'estudi. Modificada de *Nokisalmi et al.*, 2010 (enviat a Clinical Cancer Research.

Malauradament, els components genètics presents a ICOVIR-7 eleven la mida del seu genoma fins a les 37053 parells de bases, molt a prop del 105% del límit publicat d'empaquetament (Bett *et al.*, 1993). Quan es van introduir aquests transgens, els virus resultants presentaven problemes d'empaquetament i una producció viral molt reduïda en comparació amb ICOVIR-7 sense armar, fet que els feia molt difícils d'amplificar i en reduïa considerablement la seva activitat oncolítica (resultats no mostrats). Addicionalment, al nostre grup vam tenir el mateix problema quan vam intentar introduir els transgens PH-20 i GALV al genoma d'ICOVIR-5 (resultats no publicats, tesi doctoral Sònia Guedan). El genoma d'ICOVIR-5 és de mida molt similar al genoma d'ICOVIR-7, i aquests problemes amb aquests genomes armats ens indicaven que calia buscar una nova modificació que restringís l'expressió d'E1A a cèl·lules tumorals sense incrementar de forma significativa la mida del genoma de l'adenovirus salvatge.

3. ICOVIR-15: ELEMENTS MÍNIMS QUE MILLOREN LA DEPENDÈNCIA DE RB

Com ja s'ha comentat amb anterioritat, la via de RB es troba desregulada al 100% dels tumors (Sherr, 2001). Per tant, si volíem seguir en la línia de tractar un ampli rang de tipus tumorals, la nova modificació hauria de respondre també a la desregulació d'aquesta via. Si analitzem els elements genètics d'ICOVIR-5 i d'ICOVIR-7, veiem que la selectivitat ve donada, principalment, pel promotor exogen que controla E1A, però aquest element provoca un increment considerable de la mida del genoma. A més, al tractar-se de promotors exògens, cal aïllar-los dels potenciadors i llocs críptics d'inici de la transcripció presents a la senyal d'encapsidació per al correcte funcionament del promotor (Majem *et al.*, 2006). En global, el *cassette* d'ICOVIR-7 que inclou polyA, DM-1 i promotor E2F-1 modificat representa uns 1300 parells de bases addicionals, dificultant el clonatge de transgens en aquest genoma. A la bibliografia, existeixen estratègies alternatives d'aïllament de promotors exògens, com la recol·locació de la senyal d'encapsidació al braç dret del genoma (Jakubczak *et al.*, 2003) o invertir el *cassette* promotor-E1A (Hsieh *et al.*, 2002), tot i que totes aquestes estratègies presenten molts inconvenients, com inestabilitat genòmica o ineficiència d'aïllament degut a l'acció en *cis* dels potenciadors de la transcripció. Degut a aquests problemes amb l'aïllament dels promotors exògens, al nostre grup vàrem optar per una estratègia diferent, i en comptes d'anular i substituir la funció del promotor endogen d'E1A, vàrem modificar-ne els factors de transcripció

que s'hi uneixen per redirigir preferentment la transcripció d'E1A cap a cèl·lules tumorals. En l'entorn d'un adenovirus oncolític, s'havien introduït anteriorment caixes d'unió del factor de transcripció Tcf als promotors d'E1B, E2 i E3, aconseguint redirigir l'expressió d'aquests cap a cèl·lules tumorals amb la via de Wnt constitutivament activada (Brunori *et al.*, 2001). La modificació del promotor d'E1A, però, resulta més atractiva degut al control transcripcional que aquesta realitza sobre tots els altres promotors virals, aconseguint amb el control d'E1A el control de tot el cicle viral. Així doncs, i per seguir aprofitant la complexa funció dels palíndroms de llocs d'unió d'E2F dins del context d'un adenovirus, vàrem construir ICOVIR-15 introduint vuit nous llocs d'unió d'E2F, organitzats en quatre palíndroms imperfectes, al promotor endogen d'E1A, just darrera de la senyal d'encapsidació i davant de les caixes CAAT i TATA per no modificar la funció de cap d'aquestes seqüències (**Figura 25**). De fet, el promotor endogen d'E1A conté de per sí dos llocs hipotètics d'unió d'E2F, però la deleció d'ells ha demostrat que no juguen un paper important en el control d'E1A (Yoshida *et al.*, 1995). Per complementar la funció dels nous llocs E2F, darrere d'aquests també hi vàrem clonar un lloc d'unió de Sp-1 degut a la cooperació que realitzen ambdós factors de transcripció per activar la transcripció (Karlseder *et al.*, 1996). Contràriament al que succeeix amb un promotor exogen aïllat, la modificació d'ICOVIR-15 manté intacta la funció de l'element II dels potenciadors de la transcripció d'E1A, que s'ha descrit que s'encarrega d'activar tots els altres promotors virals en *cis* (Hearing and Shenk, 1986). A ICOVIR-7, la presència de la seqüència aïllant pot estar evitant aquesta funció, contribuint a que el virus perdi potència oncolítica en algun model tumoral. Per complementar la funció dels llocs d'unió d'E2F i evitar un bucle d'autoactivació en cas d'expressió residual d'E1A a cèl·lules normals, a la proteïna E1A d'ICOVIR-15 li vàrem delecionar els aminoàcids d'unió a pRB (deleció Δ24) (Fueyo *et al.*, 2000). Addicionalment, i per incrementar la infectivitat a cèl·lules tumorals, es va introduir un motiu RGD al bucle HI de la fibra (Suzuki *et al.*, 2001). Amb totes aquestes modificacions, el genoma d'ICOVIR-15 només supera al de l'Adwt-RGD en 151 parells de bases, fet que hauria de permetre una replicació eficient compatible amb una futura incorporació d'un transgen al genoma.

Aquesta modificació, en teoria, hauria de reduir la toxicitat associada a l'administració sistèmica dels adenovirus salvatges. Als nous llocs d'unió d'E2F, quan ICOVIR-15 infecta una cèl·lula quiescent, s'hi haurien d'unir complexes E2F-pRB-HDAC, que haurien d'enfortir l'associació entre els nucleosomes i l'ADN, reprimint la transcripció d'E1A (Black and Azizkhan-Clifford, 1999). Per testar aquesta teoria, es van realitzar experiments de toxicitat *in vivo*; els experiments amb cèl·lules normals en cultiu, com es comentarà més endavant, són poc

fidedignes a les condicions fisiològiques. L'únic model *in vitro* de cèl·lula normal que manté l'arrest complert de la replicació són les seccions de fetge normal humà (**Figura 17**); malgrat la fiabilitat d'aquest model, la disponibilitat de biòpsies és molt limitada. A més, entre la realització dels experiments amb ICOVIR-7 i ICOVIR-15, a l'Hospital Universitari de Bellvitge hi va haver un canvi en el protocol estàndard de tractament de la malaltia metastàtica ressecable del càncer colorectal, pacients dels quals obteníem les biòpsies de fetge normal. L'administració de quimioteràpia abans de la cirurgia limità la disponibilitat i en va alterar les condicions de la biòpsia, variant el seu aspecte i allunyant-lo d'un fetge normal sà, fet que va impedir l'avaluació de la replicació d'ICOVIR-15 *in vitro*. En l'avaluació de la selectivitat *in vivo*, l'administració sistèmica de $5x10^{10}$ partícules virals d'Adwt-RGD per ratolí provocà un increment de les transaminases en sang, degeneració del teixit hepàtic, alteracions hematològiques i una greu pèrdua de pes (**Figures 26** i **27**). De fet, aquesta dosi representa més de la LD_{50} per als virus que controlen E1A sota el promotor salvatge (Cascallo *et al.*, 2007). Pel contrari, la injecció de la mateixa dosi d'ICOVIR-15 només provocà lleugeres alteracions dels nivells d'ALT i plaquetes a dia 4 post-injecció (**Figura 26**). Aquesta toxicitat es mostrà transitòria, ja que a dia 12 post-injecció els nivells de transaminases i plaquetes recuperaren els nivells dels ratolins injectats amb PBS. Si comparem aquesta toxicitat amb la provocada per l'administració sistèmica de la mateixa dosi d'ICOVIR-7, observem com, a dia 4 post-administració, ICOVIR-15 presenta una lleugera tendència a ser més tòxic, tot i que les diferències són molt subtils. En canvi, sembla que la toxicitat associada a l'administració d'ICOVIR-15 es recupera més ràpidament, ja que, sorprenentment, els ratolins administrats amb ICOVIR-7 presenten nivells més alts de transaminases i una reducció de plaquetes encara a dia 12 post-injecció. A més del perfil de toxicitat a ratolins, també vàrem avaluar la toxicitat d'ICOVIR-15 en un model de hàmster. L'avantatge que presenta aquest model és la seva semipermissivitat a la replicació de l'adenovirus humà (Hjorth *et al.*, 1988; Lichtenstein *et al.*, 2009), fet que permet avaluar l'efecte de la replicació sobre la toxicitat. En aquest model, ICOVIR-15 és capaç de reduir de forma molt important l'elevació de transaminases produïda per l'administració d'adenovirus salvatges (**Figura 28b**) i de disminuir molt significativament l'expressió d'E1A als fetges dels hàmsters (**Figura 29**). En canvi, existeix una disminució de pes i una trombocitopènia bastant important després de l'administració d'ICOVIR-15 (**Figura 28a** i **c**), però l'administració d'un adenovirus inactivat provoca un perfil similar de toxicitat, fet que pot estar indicant que aquesta toxicitat està associada a la càpsida viral, no a l'expressió d'E1A. La toxicitat d'ICOVIR-7 no es va avaluar en aquest model, però la toxicitat similar entre ICOVIR-15 i l'adenovirus inactivat fa preveure un

perfil de toxicitat molt similar. En general, podem concloure que els nous palíndroms d'unió d'E2F dintre del promotor endogen d'E1A són capaços de reduir de manera molt important la toxicitat associada a l'expressió d'E1A després de l'administració sistèmica *in vivo*.

A més de reduir la toxicitat, els nous llocs palindròmics d'unió d'E2F són capaços d'incrementar l'activitat antitumoral dels adenovirus salvatges. La introducció d'una seqüència exògena dins del promotor d'E1A podria haver interferit en l'activació de la transcripció, reduint els nivells d'E1A a cèl·lules tumorals. No només no ocorre això, sinó que la unió d'E2F als nous llocs d'unió incrementa els nivells d'E1A, i això es tradueix en una millora de la producció viral i de la citotoxicitat a una gran varietat de cèl·lules tumorals, incloent melanoma, osteosarcoma i adenocarcinoma de pàncrees (**Figura 30** i **31**). Significativament, i al contrari del que succeeix amb ICOVIR-7 o ICOVIR-5, no existeix pèrdua de potència respecte l'adenovirus salvatge en cap línia tumoral testada, produint-se una millora en la majoria d'elles. Aquesta millora pot tenir molt a veure amb la funció dels potenciadors de la transcripció d'E1A, que als virus ICOVIR-5 i ICOVIR-7 havien perdut la seva funció. La diferència amb l'activitat antitumoral d'ICOVIR-7 és molt visible a línies com Sk-mel28 o HCT116, amb diferències de citotoxicitat de fins a 28 vegades. Així, els nostres resultats indiquen que uns alts nivells d'E1A són molt importants per aconseguir una bona activitat antitumoral, contradient altra vegada alguns treballs que refereixen que petites pèrdues d'expressió d'E1A no tenen un gran efecte sobre la potència oncolítica (Hitt and Graham, 1990; Nettelbeck *et al.*, 2002). Complementàriament, amb ICOVIR-15 es va testar un nou model provinent directament d'una biòpsia de metàstasi hepàtica d'un pacient, model que hauria de presentar característiques més semblants a les condicions fisiològiques que no pas una línia cel·lular. En aquest model (**Figura 32**), ICOVIR-15 va demostrar també la seva potència, millorant de forma important la replicació d'ICOVIR-7. Aquest increment de l'activitat antitumoral *in vitro* es va confirmar *in vivo*: l'administració intravenosa d'una sola dosi d'ICOVIR-15 reduí significativament el volum dels tumors comparat amb els ratolins tractats amb l'Adwt-RGD (**Figura 33**). Aquest experiment es va realitzar a una dosi baixa, de $2{,}5x10^{10}$ partícules virals per ratolí, ja que és la dosi màxima tolerada per injeccions sistèmiques d'adenovirus salvatges. Amb aquesta dosi, la quantitat de virus que arriba als tumors és molt reduïda, tal i com es pot observar en la poca activitat antitumoral que va presenta el tractament amb Adwt-RGD, però fins i tot en aquestes condicions ICOVIR-15 és capaç de produir un efecte antitumoral molt significatiu. Degut a que, a més, ICOVIR-15 redueix la toxicitat comparat amb l'Adwt-RGD, és va poder testar l'activitat antitumoral d'una dosi més gran, de $5x10^{10}$ partícules virals per ratolí, en un ampli rang de models tumorals xenògrafts, incloent melanoma i adenocarcinomes de pròstata, pulmó i

pàncrees. En aquestes condicions, la injecció d'ICOVIR-15 provocà una activitat antitumoral important, reduint significativament el creixement tumoral (**Figura 35**) i incrementant la supervivència dels ratolins (**Figura 36**) comparat amb el grup no tractat. En dos d'aquests models, l'activitat antitumoral d'ICOVIR-15 es va comparar amb la d'ICOVIR-7, demostrant la millora realitzada amb la nova modificació.

Tal i com està descrit a la bibliografia (Geoerger *et al.*, 2004), els anàlisis histològics realitzats al nostre grup dels tumors tractats amb adenovirus oncolítics indiquen que la presència de l'estroma del tumor limita la dispersió del virus, encofinant el virus a petites àrees envoltades de matriu i barreres fibroblàstiques. Tal i com ocorre amb ICOVIR-7, aquest efecte es fa molt evident altra vegada al model de PC-3 (**Figura 35e**), que expressa grans quantitats de matriu extracel·lular (Draffin *et al.*, 2004; Ricciardelli *et al.*, 2007). Com ja s'ha comentat anteriorment, l'expressió de transgens que ajudin a trencar aquestes barreres aportaria una eina molt útil per aconseguir l'eradicació dels tumors. És important destacar que la modificació introduïda a ICOVIR-15 no augmenta significativament la mida del genoma de l'adenovirus salvatge (només 151 parells de bases), i això no hauria d'impedir una expressió eficient de transgens, a diferència del que succeïa amb ICOVIR-7. Com a proba de concepte i per avaluar si realment la incorporació d'un transgen tenia o no efectes negatius sobre la replicació d'ICOVIR-15, vàrem construir una versió armada que incorporava un *cassette* d'expressió del transgen NfsA (**Figura 37a**). NfsA és la nitroreductasa majoritària d'*Escherichia coli* i s'ha descrit recentment que provoca un efecte *bystander* més potent que NfsB (Vass *et al.*, 2009), nitroreductasa molt utilitzada en teràpia gènica del càncer com a convertidora de prodrogues tals com CB1954, que amb l'efecte de la nitroreductasa es converteix en un potent agent alcalí bifuncional. Carregant amb aquest transgen, la replicació d'ICOVIR-15 resultà igual que la del virus no armat (**Figura 37b**), confirmant la idoneïtat d'aquest genoma per a la incorporació de transgens. Per tal de subjugar l'expressió del transgen al control d'E1A i evitar així la seva expressió a cèl·lules normals, vàrem expressar NfsA a través de la seqüència de tall i lligació 3VDE (*IIIa virus infection-dependent splicing enhancer*), sota el control del promotor major tardà. La idoneïtat d'aquest i altres mecanismes d'expressió de transgens, dintre del context d'un adenovirus oncolític, es discutirà més endavant. En el nostre cas, l'expressió de NfsA produïda per ICOVIR-15-NfsA es mostrà restringida a la fase tardana del cicle viral, a més d'acumular-se grans quantitats de transgen en aquesta fase, superant fins i tot els nivells d'expressió del promotor CMV en el context d'un adenovirus no replicatiu (**Figura 37c**). Aquest perfil d'expressió ens indica que aquest sistema d'expressió, juntament amb l'activació que provoquen els alts nivells d'E1A generats per la modificació

d'ICOVIR-15, és una bona combinació i una bona base per avaluar quin transgen pot aportar un benefici més gran per ajudar a l'eradicació dels tumors.

En resum, els nostres resultats indiquen que ICOVIR-15 presenta una ràtio eficàcia/toxicitat molt bona. Per un cantó, els palíndroms de llocs d'unió d'E2F redueixen la toxicitat causada per l'administració sistèmica d'adenovirus. Per altra banda, els mateixos llocs d'unió incrementen l'activitat antitumoral, comparat fins i tot amb els adenovirus salvatges, fet molt destacable degut als requeriments de potència que s'han fet evidents a la clínica. A més, l'aplicabilitat d'aquest virus per tractar una gran varietat de tipus tumorals, així com la reduïda mida del seu genoma, el converteixen en una base òptima per a la inserció de transgens sense haver de delecionar cap funció viral, fet que pot ajudar en gran mesura a superar les barreres que imposa l'estroma del tumor a l'èxit de la viroteràpia amb adenovirus. Malgrat que aquest virus ja suposa una excel·lent plataforma per a l'avaluació de l'efecte de diferents transgens, durant la realització d'aquesta tesi, al nostre grup es van identificat dues modificacions addicionals que podrien millorar encara més a l'activitat antitumoral d'ICOVIR-15, i en vam determinar quins beneficis aportaven i la idoneïtat de la seva inclusió a un futur candidat a un assaig clínic.

4. LA MUTACIÓ T1: EFECTES DE L'AUGMENT DE L'ALLIBERACIÓ VIRAL

La bioselecció en unes determinades condicions és un mètode de la virologia clàssica que s'ha postulat com una potent eina per incrementar l'activitat antitumoral dels adenovirus. Emprant aquesta eina, s'han identificat diverses modificacions, com mutacions a E1B-19K o a la proteïna i-líder, que augmenten la potència del virus *in vitro* (Yan *et al.*, 2003; Subramanian *et al.*, 2006). Al nostre grup, a més, es va aplicar aquest mètode *in vivo* a un model murí de tumor humà xenògraft, aportant una pressió ambiental selectiva única, amb la presència d'estroma tumoral tridimensional, molt més semblant a les condicions clíniques que la bioselecció *in vitro*. Després del procés de selecció, l'adenovirus aïllat presentava al seu genoma la mutació que es va anomenar T1, identificada com la responsable del fenotip observat (Gros *et al.*, 2008). Aquest fenotip consistia en la relocalització de la proteïna viral E3-19K a la membrana plasmàtica, perdent la senyal de retenció al reticle endoplasmàtic. Això provocava una desregulació de l'homeòstasi de calci, facilitant la sortida del virus de la cèl·lula a temps més primerencs, augmentant així la potència oncolítica a la majoria de models tumorals testats. Degut a l'hipotètic

benefici que aquest mecanisme podria aportar a l'activitat oncolítica d'ICOVIR-15, vàrem construir una versió d'aquest que incloïa la mutació T1 a la proteïna E3-19K (**Figura 38**).

Abans de la realització dels experiments de potència oncolítica, es va determinar si la inclusió d'aquesta mutació modificava la toxicitat després de l'administració sistèmica. A l'estudi ja publicat pel nostre grup, no s'havia determinat l'efecte d'aquesta mutació sobre la toxicitat hepàtica, tot i que no s'esperaven diferències significatives degut a que la causa principal de la toxicitat hepàtica és l'expressió d'E1A (Engler *et al.*, 2004). Pel contrari, en l'estudi de toxicitat és van apreciar algunes diferències, produint el virus amb la mutació T1 una menor toxicitat, destacable en la reducció d'ALT respecte ICOVIR-15 (**Figura 39**). La resposta a aquesta diferència podria estar en la interacció amb el sistema immune: Pahl i col·laboradors van descriure que E3-19K activa la via de senyalització de NF-κβ i que aquesta activació era depenent de la retenció al reticle endoplasmàtic (Pahl *et al.*, 1996). Per tant, la mutació T1 podria estar activant menys aquesta via de senyalització al fetge en cas d'una expressió residual d'E1A i E3, reduint la inflamació del parènquima hepàtic. Pel contrari, la pèrdua de retenció al reticle endoplasmàtic d'E3-19K evita la retenció dels MHC de classe I, fet que podria estar incrementant per altra banda la resposta immune contra el virus. Malgrat això, aquest increment de la resposta immune podria no donar-se degut a que E3-19K presenta altra funció per evitar la maduració dels MHC: els mutants que perden la retenció al reticle són encara capaços d'interaccionar amb TAP i interferir en la funció tapasina, evitant la incorporació d'epítops als MHC (Bennett *et al.*, 1999). Per tant, ICOVIR-15-T1 mantindria intacta encara aquesta funció, evitant en gran mesura el reconeixement dels epítops adenovirals, encara que caldria validar aquesta teoria amb experiments addicionals. De la mateixa manera, també caldria avaluar l'efecte per part de la mutació T1 de la pèrdua de la funció complementària d'E3-19K de retenir els lligands MICA i MICB al reticle, per evitar el reconeixement per part de les cèl·lules NK (McSharry *et al.*, 2008), i el paper que pot tenir aquesta pèrdua en la toxicitat hepàtica.

In vitro, sembla que ICOVIR-15-T1 reprodueix el fenotip mostrat per la mutació T1 en el context d'un adenovirus salvatge: la sortida avançada del virus de la cèl·lula provoca l'acceleració de la distribució cèl·lula-cèl·lula del virus, augmentant la mida de la calba de lisi (**Figura 40**) i millorant la citotoxicitat sobre les cèl·lules tumorals en dos dels tres models testats (**Figura 42**). La citotoxicitat d'aquest virus també es va testar a cèl·lules tumorals de hàmster, mostrant una lleugera millora (**Figura 43**); la utilitat d'aquest model es discutirà més endavant. A més, aquesta mutació no afecta a les quantitats produïdes d'E1A (**Figura 41a**), però sembla afectar a la

quantitat de virus produïda per cada cèl·lula tumoral (**Figura 41b**). Segurament, la desregulació de l'homeòstasi del calci abans que tots els virions estiguin totalment formats és el que provoca aquesta disminució de les partícules virals funcionals, tot i que la disminució és molt discreta i aquest efecte no s'havia observat en els experiments anteriors amb adenovirus salvatges amb la mutació T1. Sorprenentment, quan vàrem testar l'activitat antitumoral del nou virus *in vivo* no es va observar cap avantatge. Al treball anterior amb els adenovirus salvatges, la mutació T1 havia mostrar una disminució molt significativa dels creixement dels tumors. Pel contrari, amb ICOVIR-15-T1 no es va observar cap millora, ni de la inhibició del creixement tumoral (**Figura 44**) ni de la supervivència dels ratolins (**Figura 45**). Malgrat un augment de la citotoxicitat molt important en la línia cel·lular A549 *in vitro*, els tumors subcutanis d'aquesta mateixa línia van mostrar un perfil de creixement molt similar quan es van tractar amb ICOVIR-15 o amb ICOVIR-15-T1. Una possible explicació és que, *in vivo*, l'efecte antitumoral d'ICOVIR-15 està molt optimitzat, i amb una sortida avançada dels virions de les cèl·lules no aconseguim cap avantatge quan el que limita l'eradicació dels tumors és l'estroma. Malgrat aquest resultat negatiu, el model *in vivo* de tumor xenògraft a ratolins immunodeprimits (*nude*) en el que s'han realitzat aquests experiments no permet treure conclusions definitives. La deficiència en la maduració de les cèl·lules de llinatge T a ratolins *nude* (Segre *et al.*, 1995) evita una avaluació efectiva de l'impacte de la pèrdua de retenció dels MHC-I i MICA i MICB al reticle endoplasmàtic sobre la replicació del virus *in vivo*. Són necessaris experiments en models immunocompetents per establir les implicacions immunològiques exactes associades a la mutació T1 i decidir, en funció dels resultats obtinguts, la utilitat real que pot aportar aquest mutació.

5. RECOL·LOCACIÓ DEL MOTIU RGD: AUGMENT DE LA BIODISPONIBLITAT

Anteriorment, el nostre grup havia explorat els beneficis d'introduir el tripèptid RGD substituint el domini KKT del *shaft* de la fibra (Bayo-Puxan *et al.*, 2009). Aquest domini és el lloc hipotètic d'unió als HSG i, per tant, en principi és un lloc ben exposat que pot servir per a la presentació de tripèptids. En teoria, aquesta estratègia també es beneficia de les propietats d'eliminar el motiu KKTK, ja que la interacció amb els HSG per part de l'adenovirus s'ha postulat com un dels mecanismes d'infecció dels hepatòcits (Zhang and Bergelson, 2005). Els virus no replicatius que incorporaven aquest motiu RGDK al *shaft* de la fibra van demostrar una reducció

de 6,5 vegades en la transducció del fetge i un augment de 3 vegades en la transducció dels tumors. Per tant, es va incorporar aquest motiu al *shaft* de la fibra d'ICOVIR-15, eliminant-ne el motiu RGD del bucle HI del *knob* (**Figura 46**), per comprovar si aquests beneficis observats amb els virus no-replicatius es mantenien en el context d'un adenovirus oncolític, i si això comportava algun benefici sobre la toxicitat o la potència antitumoral.

El nou virus ICOVIR-15K es va testar en models de toxicitat murins i de hàmster. En general, els resultats comparatius amb ICOVIR-15 obtinguts van resultar contradictoris segons el paràmetre analitzat. Certa reducció dels nivells d'AST en el sèrum de hàmsters (**Figura 49b**) i un perfil més similar al grup PBS en la concentració de monòcits, neutròfils i basòfils en sang (**Figura 50**) indicaven una disminució de la toxicitat, mentre que una trombocitopènia més greu respecte la mateixa dosi del virus amb el motiu RGD al *knob* n'indicava un augment. Primerament, aquest perfil més lleu d'elevació de transaminases hepàtiques pot estar associat a la disminució de la transducció observat amb els virus no-replicatius (Bayo-Puxan *et al.*, 2009). La disminució de la transducció d'hepatòcits també pot causar la reducció dels leucòcits en sang (Engler *et al.*, 2004). L'augment de la trombocitopènia, en canvi, pot estar degut a una interacció diferencial d'ambdós càpsides amb les plaquetes. Està descrit que la causa de la trombocitopènia després de l'administració d'adenovirus en sang és la unió directe de l'adenovirus amb plaquetes, i que aquesta unió causa l'activació de les plaquetes i la retenció al sistema reticuloendotelial del fetge, destruint els adenovirus a les cèl·lules de Kupffer (Stone *et al.*, 2007). A més, també s'ha postulat que la interacció entre adenovirus i plaquetes és a través dels motius RGD i les integrines (Shimony *et al.*, 2009), de manera que la recol·locació del motiu RGD pot haver variat aquesta interacció, augmentant-la i provocant la destrucció de més plaquetes. Per altra banda, aquesta trombocitopènia també pot estar deguda a la disminució de la transducció d'hepatòcits, ja que això podria provocar un augment de la biodisponibitat de virus en sang durant les hores posteriors a l'administració, unint-se més virus a més plaquetes i provocant la destrucció d'un nombre més elevat d'aquestes. De fet, la virèmia en sang amb el nou virus sí que augmenta a les 6 hores després de la injecció, tal i com es mostra a la **Figura 52**. Per dilucidar quin d'aquests dos mecanismes és el que provoca l'augment de la trombocitopènia calen fer més experiments, com un assaig diferencial d'unió a plaquetes *in vitro*, així com experiments *in vivo* amb models animals on les plaquetes han estat deplecionades prèviament a la injecció dels adenovirus.

La millora més important amb aquest virus es produeix en l'activitat antitumoral *in vivo*. Malgrat que amb els adenovirus no-replicatius es va descriure un augment de la infectivitat sobre

cèl·lules tumorals, la citotoxicitat d'ICOVIR-15K respecte ICOVIR-15 no va experimentar cap millora (**Figura 51**). En canvi, quan el virus es va administrar a ratolins immunodeprimits que portaven tumors humans xenoimplantats, la millora va resultar molt important. A tots els models testats, amb el nou virus es va aconseguir una millora en l'estabilització del creixement dels tumors fins a dies molt avançats en els experiments, arribant fins i tot a l'eradicació d'alguns tumors en el model de la línia d'adenocarcinoma de pàncrees NP-9 (**Figura 53**). La millora va resultar també molt important en la supervivència de ratolins amb tumors subcutanis (**Figura 54**). L'explicació, altre cop, pot estar en la disminució de la transducció dels hepatòcits. Aquesta disminució augmenta la virèmia en sang a les hores posteriors a la injecció (**Figura 52**), i això provoca l'arribada de més virus al tumor, distribuint-se millor per la massa tumoral i provocant, a la llarga, un benefici molt important en l'activitat antitumoral. Al treball anterior amb els virus no-replicatius, l'augment de tres vegades en la transducció del tumor es va associar a un augment de la infectivitat sobre les cèl·lules tumorals, però també podria estar degut a l'augment de la biodisponibilitat del virus. Si, pel contrari o a més a més, existís una interacció diferencial amb les plaquetes, caldria investigar l'efecte que això pot tenir sobre l'arribada del virus al tumor, ja que s'ha descrit que les plaquetes juguen un paper important en el procés d'angiogènesi i metàstasi dels tumors (Manegold *et al.*, 2003; Falanga *et al.*, 2009). La presència de plaquetes activades a certs tipus de càncer (Verheul *et al.*, 2000), juntament amb el fet que la interacció de l'adenovirus amb les plaquetes les activa, deixa intuir una possible funció de transport cap als tumors, però calen experiments addicionals per demostrar si això es produeix o no.

6. LIMITACIONS DELS MODELS EXPERIMENTALS

Per testar les propietats de selectivitat i potència dels nous adenovirus oncolítics construïts durant la realització d'aquesta tesi, s'han emprat diferents models experimentals, tan *in vitro* com *in vivo*. En aquest apartat, s'analitzarà les característiques i la fidelitat a les condicions fisiològiques de cada un d'ells, i es discutirà la necessitat de desenvolupar nous models que ens permetin simular millor les condicions que es trobaran els adenovirus quan siguin injectats en assajos clínics.

Com a una primera aproximació després de tenir els adenovirus construïts, aquests es testen *in vitro* en cèl·lules en cultiu. L'avaluació de les conseqüències d'una infecció sobre línies

tumorals en cultiu és molt útil per determinar les característiques de potència de les noves mutacions introduïdes. La detecció d'E1A per Western-blot ens permet quantificar els efectes dels canvis en el control transcripcional d'E1A, modificacions molt útils per reduir la toxicitat dels adenovirus després de l'administració sistèmica (Alemany, 2007). La determinació de la quantitat de partícules virals produïda per cada cèl·lula tumoral indica els efectes de les noves modificacions durant un sol cicle de replicació viral. La relació entre els nivells d'E1A i la replicació del virus no és directa, i cal avaluar ambdós paràmetres per definir l'efecte de les modificacions en el control d'E1A. A línies com SCC-25, un gran canvi en les quantitats d'E1A no té un gran efecte sobre la replicació de, per exemple, ICOVIR-7 comparat amb ICOVIR-5, mentre que a línies com Saos-2 una petita millora en la quantitat d'E1A provoca una millora molt significativa de la quantitat de virus produïda (**Figura 20**), indicant que altres paràmetres segons la línia tumoral, a més d'E1A, afecten a la replicació. Per últim, la determinació de la citotoxicitat sobre un cultiu de cèl·lules tumorals infectades amb una petita quantitat de virus (baixa MOI) permet avaluar els efectes quan s'ha produït més d'un cicle de replicació, tenint en compte paràmetres com la sortida del virus de la cèl·lula o la distribució d'aquest cèl·lula-cèl·lula. L'examen d'aquests tres paràmetres en un ampli ventall de línies tumorals ens dona una idea general bastant acurada dels beneficis que poden tenir les noves modificacions sobre la potència oncolítica, encara que aquests models no inclouen certes característiques dels tumors, com l'arquitectura, que poden ser claus per a l'èxit de la teràpia.

Per testar la selectivitat dels adenovirus oncolítics, també s'utilitzen models de cèl·lula en cultiu. En aquest cas, en comptes de ser cèl·lules tumorals, són cèl·lules provinents d'un teixit sà. Malgrat la seva procedència, el creixement d'aquestes cèl·lules en cultiu implica l'activació de la replicació i, per tant, de la via de RB, fet que limita l'aplicació d'aquests models per avaluar els nostres adenovirus oncolítics, ja que la replicació d'aquests respon a l'estat replicatiu de les cèl·lules. Per induir l'estat de quiescència, la majoria de protocols de treball amb aquestes cèl·lules proposen una depleció del sèrum del medi de cultiu, però l'anàlisi de marcadors de replicació indica que aquesta depleció no és suficient (**Figura 17**). Amb aquests models, la diferència de replicació entre un adenovirus salvatge i un adenovirus de replicació condicionada a l'activació de la via de RB es mostra molt petita (**Figures 14** i **15**), segurament degut a la presència d'una gran part del cultiu en replicació. Durant la realització d'aquesta tesi, es van explorar mètodes alternatius per arrestar aquestes cèl·lules, com la infecció prèvia amb adenovirus no-replicatius que introdueixen gens supressors de tumors, com pRB o p16, que haurien d'induir l'estat de quiescència, però els resultats no van ser positius (resultats no mostrats). Per tant,

aquests models de cèl·lula normal en cultiu no reprodueixen fidedignement les condicions fisiològiques dels teixits sans, on només una part molt petita de les cèl·lules normals entren en replicació. Aquesta deficiència fa necessari testar la selectivitat dels virus a altres models.

La tecnologia *Krumdieck Tissue Slicer*, desenvolupada per Alabama Research and Development, permet la realització de les seccions de teixit (*slices*). Les condicions de tall permeten no danyar el teixit, mantenint les condicions fisiològiques durant molts dies després de la obtenció de la biòpsia. A més, es tracta de models organotípics, amb diferents tipus cel·lulars, reproduint millor les condicions originals dels teixits. Com a model de toxicitat sobre el parènquima hepàtic, es va descriure la seva utilitat com a eina per estudiar la resposta inflamatòria causada per LPS (Olinga *et al.*, 2001), i va ser proposat per algunes agències reguladores com a una bona alternativa als estudis de toxicitat amb animals. Posteriorment, el model s'ha aplicat a l'estudi de la toxicitat causada pels adenovirus oncolítics (Kirby *et al.*, 2004). En aquesta tesi, el model ha demostrat la seva valia, mostrant alts nivells de quiescència (**Figura 17**) i la possibilitat d'avaluar la replicació dels virus amb gran fidelitat a les condicions fisiològiques (**Figura 16**). Malgrat els avantatges d'aquest model, la procedència de les biòpsies directament de pacients humans limita la seva disponibilitat, tal i com s'ha comentat anteriorment, i això va impedir l'avaluació en aquest model de la majoria dels virus generats en aquest treball. Donat que el hàmster siri o daurat (*Mesocricetus auratus*) s'ha descrit com un model semireplicatiu per als adenovirus humans (Hjorth *et al.*, 1988), en aquest treball vàrem avaluar un model de seccions de fetge de hàmster per testar la replicació dels adenovirus oncolítics. La possible permissivitat d'aquest model a la replicació dels virus ens permetria solucionar els problemes de disponibilitat de les biòpsies humanes. Malauradament, i a diferència del que ocorre amb les seccions de fetge humà, de gos o de ratolí, la infectivitat dels adenovirus pels *slices* de hàmster va resultar molt reduïda, aconseguint infectar molts pocs hepatòcits, fins i tot augmentant la MOI fins a 500 unitats de transducció per cèl·lula (resultats no mostrats), fet que en descarta la seva aplicabilitat. Això pot ser degut a una mida menor de les fenestracions hepàtiques, tal i com ocorre al conill (Braet and Wisse, 2002). Paral·lelament a l'aplicació per avaluar la selectivitat, la tecnologia *Krumdieck Tissue Slicer* també permet avaluar la replicació dels virus a seccions de tumors primaris. La disponibilitat de biòpsies de tumor és molt més senzilla que la de fetge normal i no en suposa un impediment. A més, l'aplicació de quimioteràpia prèvia no suposa un desavantatge en aquest cas, ja que aquesta condició també es donarà en un possible assaig clínic, on els pacients han passat abans per varis cicles de quimioteràpia. Malgrat presentar unes condicions més properes a la dels tumors reals que les

cèl·lules en cultiu, amb presència d'altres tipus cel·lulars, com fibroblasts o cèl·lules endotelials, les poques capes cel·lulars d'aquest model no reprodueixen prou bé l'arquitectura d'un tumor, i caldran models *in vivo* per determinar paràmetres com la distribució del virus pel tumor.

El desenvolupament de nous productes farmacèutics segurs i eficaços per al tractament de malalties humanes continua tenint una forta dependència d'estudis *in vivo* en animals. Per estudis de toxicologia, existeixen protocols bastant estandarditzats, que normalment inclouen alguna espècie de rosegador i altra espècie complementaria, com gos o simi (Morton, 1998). Malgrat que amb les drogues clàssiques poden existir grans diferències en l'efecte que aquestes provoquen sobre els models animals i sobre els humans, amb els virus aquestes diferències es maximitzen. Els adenovirus humans presenten una replicació selectiva a cèl·lules humanes i el cicle viral és abortiu a espècies com, per exemple, el ratolí (Younghusband *et al.*, 1979). Per tant, els estudis de toxicitat a ratolins no permeten avaluar l'efecte de la replicació del virus sobre les cèl·lules normals. Malgrat això, aquests estudis presenten molta utilitat, sobretot amb virus on es modifica el control transcripcional d'E1A, ja que l'expressió de la proteïna E1A sí que es dóna a cèl·lules de ratolí, i sols la seva expressió ja causa una elevada toxicitat que reprodueix en gran mesura la toxicitat observada en els assajos clínics en humans (Engler *et al.*, 2004). Transaminitis, trombocitopènia i variacions en els recomptes leucocitaris es donen tant en el model murí de toxicitat (**Figura 18, 26, 39, 47** i **48**) com en els assajos clínics (Aghi and Martuza, 2005). A més, les immunohistofluorescències dels fetges dels ratolins injectats amb adenovirus permeten quantificar l'expressió d'E1A, sent un bon model per avaluar la repressió de la transcripció provocada per les noves modificacions. Així, el model de ratolí permet l'avaluació de la toxicitat causada pel virió com a partícula física, per l'expressió d'E1A i per la resposta immune que això genera, tot i que els efectes de la replicació viral i la resposta immune que aquesta pot despertar s'escapen en aquest model. A més, aquesta deficiència en la replicació no permet avaluar de forma eficient en models de potència oncolítica l'efecte del sistema immune sobre l'eficàcia antitumoral dels adenovirus. Per testar els adenovirus oncolítics en models tumorals *in vivo* en ratolins, cal implanta'ls-hi tumors humans. Per que no hi hagi rebuig de les cèl·lules humanes, cal utilitzar ratolins immunodeprimits, ja siguin *nude* o SCID. Els ratolins *nude* permeten una avaluació de l'eficàcia antitumoral en un ambient amb només una depleció parcial de sistema immune, amb la presència de cèl·lules NK i altres (Segre *et al.*, 1995), però l'absència del timus evita la funció complerta del sistema immune i deixa molts dubtes sobre els beneficis de certes modificacions, sobretot, com en el cas de la mutació T1, quan es muten proteïnes virals implicades en evadir la resposta immune. A més, les cèl·lules no tumorals presents dins d'aquests

tumors (fibroblasts, cèl·lules endotelials, ...) són murines, fet que encara complica més les condicions que es troben els adenovirus en aquests models.

Durant els darrers anys, el model de hàmster siri o daurat (*Mesocricetus auratus*) s'ha proposat com a model animal per solucionar els problemes que presenta el model murí per a l'avaluació dels adenovirus oncolítics (Thomas *et al.*, 2006; Thomas *et al.*, 2008; Lichtenstein *et al.*, 2009; Ying *et al.*, 2009). Aquesta proposta es basa en que les cèl·lules de hàmster s'han descrit com a semipermissives a la replicació dels adenovirus humans (Hjorth *et al.*, 1988). Altres models animals, com per exemple el porc o la rata del cotó, també han estat descrits com a semipermissius a la replicació dels adenovirus humans (Jogler *et al.*, 2006). Malgrat que les cèl·lules de porc han mostrat una replicació molt més eficient dels adenovirus humans que les cèl·lules de rosegadors, la major facilitat de treball amb els hàmsters l'ha portat a establir-se com a una eina essencial en qualsevol treball amb adenovirus oncolítics. Com a model de toxicitat, permetria determinar les conseqüències que suma la replicació, però, al tractar-se d'un model semireplicatiu, es fa difícil determinar el paral·lelisme que existeix entre aquest model i la toxicitat a humans. Com a model per avaluar l'activitat antitumoral, en teoria, permet el treball amb animals immunocompetents als quals se'ls implanta subcutàniament cèl·lules tumorals de hàmster sense que generin rebuig. Al nostre laboratori, però, l'anàlisi de la replicació dels adenovirus a cèl·lules tumorals de hàmster va demostrar que sí que existia replicació, però la quantitat de virus produïda restava molt lluny del rendiment a cèl·lules tumorals humanes, amb només 20 unitats de transducció produïdes per cèl·lula (resultats no mostrats, tesi doctoral Miguel Camacho). En aquestes condicions, aconseguir eficàcia sistèmica es preveu difícil. De fet, amb ICOVIR-15 s'ha realitzat un assaig d'eficàcia sistèmica a hàmsters amb tumors subcutanis Amel-3, amb indicis d'eficàcia (resultats no mostrats), però un creixement i ulceració molt ràpida dels tumors no va permetre arribar més enllà de dia 10 post-injecció. En resum, al no tractar-se d'un model completament permissiu a la replicació de l'adenovirus humà, és dubtós el seu avantatge respecte el model murí, sobretot com a model tumoral, existint a més una mancança de reactius en el mercat per avaluar qualsevol mecanisme terapèutic i sent molt més difícil l'estabulació i el maneig d'aquests animals.

El model ideal per a l'avaluació dels adenovirus oncolítics hauria de presentar una replicació efectiva dels virus, un sistema immune complert i una arquitectura i característiques dels tumors semblants als humans. Durant els últims anys, han sorgit diferents estudis que proposaven l'ús de gossos amb tumors com a model d'eficàcia i toxicitat (Hay, 2003). La

generació espontània de tumors en el gos i la semblança de les neoplàsies canines amb els càncers humans fa d'aquesta espècie animal una important eina per obtenir dades útils, ja sigui a nivell de biologia del càncer o a nivell d'efectivitat de teràpies. A més, l'existència d'un adenovirus caní amb una gran semblança amb l'adenovirus humà fa que s'hagi pensat també en aquest animal com a model de viroteràpia. L'adenovirus caní de tipus 2 (CAV-2) presenta una estructura del virió, organització genòmica, funció dels gens virals i tropisme molt semblant a l'adenovirus humà de tipus 5 (Schoehn *et al.*, 2008). Malgrat algunes diferències, com l'entrada independent d'integrines a la cèl·lula hoste (Soudais *et al.*, 2000; Chillon and Kremer, 2001), la viroteràpia dels tumors canins amb adenovirus canins, a més del benefici que pot aportar a l'oncologia veterinària, representa un model molt fidedigne a les condicions que es donen en la viroteràpia del càncer en humans, tractant-se a més de pacients reals amb tumors esporàdics i no de tumors induïts. Per al tractament de l'osteosarcoma caní, ja s'ha generat un adenovirus oncolític amb la transcripció d'E1A sota el control del promotor de l'osteocalcina (OC-CAV), mostrant resultats *in vitro* i en models de ratolí molt similars als aconseguits amb adenovirus oncolítics humans (Hemminki *et al.*, 2003). Per tal d'aplicar els avenços realitzats en aquesta tesi en el model caní, al nostre grup s'està construint una versió d'ICOVIR-15 canina (ICOCAV-15). La seva aplicació i la de futurs virus a pacients canins amb tumors de diversos orígens suposarà una eina molt potent per avaluar els beneficis de les noves modificacions en presència d'un sistema immune complert i una arquitectura tumoral complexa.

En general, els models de que disposem per avaluar les característiques dels nous adenovirus oncolítics generats són limitats, però ofereixen eines suficients per dilucidar si les modificacions oferiran o no un clar avantatge per a ser incorporades a un futur adenovirus candidat a un assaig clínic en humans.

7. ASSAIG CLÍNIC: TRIA D'UN CANDIDAT

El cost de portar un virus des del laboratori fins a la clínica pot ser molt elevat. No només s'ha de tenir en compte el cost de la realització de l'assaig en sí, sinó que tan sols la producció de 10^{15} partícules virals de l'adenovirus en condicions GMP (*Good Manufacturing Practice*) pot costar entre 600.000 i 1.000.000 d'euros. Per tant, cal una tria molt acurada de les modificacions a introduir a un possible candidat a la clínica.

Els resultats presentats en aquest treball indiquen que el control de la transcripció d'E1A present a ICOVIR-15 presenta grans avantatges respecte el control a ICOVIR-5 o ICOVIR-7. La potència oncolítica, tant *in vitro* (**Figures 30, 31** i **32**) com *in vivo* (**Figures 35 i 36**), és significativament millor a tots els models tumorals, superant fins i tot a la de l'adenovirus salvatge. La selectivitat sofreix una lleugera pèrdua amb ICOVIR-15 (**Figura 26**), però les diferències són petites i la falta d'activitat antitumoral als assajos clínics publicats amb humans porten a prioritzar la potència oncolítica (Aghi and Martuza, 2005). De fet, els resultats de toxicitat a la clínica amb adenovirus menys selectius que ICOVIR-15, com l'Onyx-015, indiquen la seguretat del tractament amb adenovirus oncolítics, fins i tot després de l'administració sistèmica. Per últim, la necessitat d'incorporar un transgen per facilitar la superació de les barreres que imposa el tumor també prioritza ICOVIR-15, que ha demostrat la compatibilitat de l'expressió de trangens amb les modificacions presents al seu genoma (**Figura 37**). Així, la base d'ICOVIR-15 es mostra com un bon començament en el disseny d'un futur candidat.

La incorporació al futur candidat de les dues modificacions descrites amb anterioritat al nostre laboratori, la mutació T1 i la fibra RGDK, és discutible. La mutació T1 sembla que presenta un lleuger avantatge reduint la toxicitat (**Figura 39**), però la manca d'un benefici en potència oncolítica *in vivo* (**Figures 44** i **45**) i d'un model immunocompetent per avaluar si el virus és neutralitzat més ràpidament pel sistema immune qüestionen la seva presència al futur candidat. Pel contrari, amb la fibra RGDK succeeix a l'inversa. L'augment de la trombocitopènia observat amb l'administració sistèmica d'aquest virus (**Figures 47** i **49**) és preocupant, mentre que l'important augment de l'activitat antitumoral *in vivo* (**Figures 53** i **54**) indica un possible gran benefici a la clínica. Experiments addicionals d'unió a plaquetes i de toxicitat ens poden ajudar a decidir-nos per aquesta fibra o per la ja clàssica amb el motiu RGD al *knob*, però donat que les diferències en el comptatge de plaquetes no són ni tan sols significatives a ratolins a dia 4, i que mostra indicis de recuperació a dia 12 (**Figura 47**), la incorporació d'aquesta modificació és molt interessant, sobretot després d'haver aconseguit eradicacions totals en un model de tumor xenògraft.

Tal i com ja s'ha discutit anteriorment, l'expressió de transgens per part dels adenovirus oncolítics pot aportar-los-hi funcions molt útils per ajudar a eradicar els tumors (Cody and Douglas, 2009). Fins a la data, els adenovirus oncolítics s'han armat amb una gran varietat de transgens, representant un ventall molt divers d'estratègies per aconseguir l'eliminació dels tumors. Entre aquestes estratègies, en destaquen les destinades a ajudar al virus a travessar les

barreres que imposa l'estroma del tumor, ja que la incapacitat per superar aquestes barreres s'ha mostrat com un dels impediments més importants per a l'èxit de la viroteràpia (Geoerger *et al.*, 2004). Amb aquest objectiu, s'han testat diverses proteïnes que degraden la matriu tumoral. La relaxina, una hormona peptídica que redueix l'expressió de col·lagen i n'augmenta la de les metal·loproteinases, s'ha introduït a dos adenovirus oncolítics diferents, mostrant en ambdós casos un increment de la distribució pel tumor i de l'activitat antitumoral (Kim *et al.*, 2006; Ganesh *et al.*, 2007). A més, l'expressió de metal·loproteinases humanes, com la 8 o la 9, que digereixen una gran quantitat de molècules de la matriu, també han demostrat incrementar l'activitat antitumoral (Cheng *et al.*, 2007; Bendrik *et al.*, 2008). Al nostre grup, la incorporació del domini extracel·lular de la hialuronidasa PH20 al genoma d'ICOVIR-15 ha mostrat una gran eficàcia (Guedan *et al.*, manuscrit acceptat a *Molecular Therapy*, adjuntat a l'annex d'aquest treball). Aquest virus, l'ICOVIR-17, va augmentar la potència oncolítica d'ICOVIR-15, induint més d'un 90% d'inhibició del creixement tumoral respecte el grup no tractat en tots els models *in vivo*. Recentment, el nostre grup està avaluant la incorporació de la metal·loproteinasa MMP-7, de mida molt reduïda (només 800 parells de bases), i que permetria la seva combinació amb altre transgen sense arribar al límit d'encapsidació. Altre tipus d'enzims que poden ajudar a una millor dispersió de l'adenovirus són els convertidors de prodrogues. Aquests enzims, a més d'augmentar la citotoxicitat sobre les cèl·lules tumorals, també són capaços d'actuar sobre les barreres fibroblàstiques i sobre les altres cèl·lules no tumorals presents als tumors gràcies a l'efecte col·lateral o *bystander*. Proteïnes com les timida-quinases (Cascante *et al.*, 2007), les citosina-deaminases (Fuerer and Iggo, 2004) o les nitroreductases (NfsA incorporada al genoma d'ICOVIR-15 en aquest treball, **Figura 37**) (Vass *et al.*, 2009) poden convertir prodrogues innòcues per altres teixits en formes actives al lloc tumoral, aconseguint multiplicar el nombre de cèl·lules tumorals afectades per la teràpia i facilitant la destrucció de les barreres dels tumors. Altres transgens, com la proteïna fusogènica GALV, incorporada al genoma d'ICOVIR-15 pel nostre grup, han demostrat un increment molt important de la potència oncolítica (resultats no publicats, tesi doctoral Sònia Guedan). Per altre costat, una estratègia diferent és l'expressió de factors que recluten cèl·lules del sistema immune al lloc de la infecció i n'indueixen l'activació i la proliferació. Aquesta estratègia, combinació de la viroteràpia i la immunoteràpia, té el potencial de destruir el tumor primari i les metàstasis a través de l'acció del sistema immune. Citoquines, com MCP-3 o GM-CSF (incorporada al genoma d'ICOVIR-15 en una col·laboració amb el grup del Dr. Akseli Hemminki de la University of Helsinki), o interleucines, com IL-4 o IL-24, s'han emprat prèviament a adenovirus oncolítics amb aquest objectiu (Bristol *et al.*, 2003; Post and Van Meir,

2003; Sarkar *et al.*, 2005; Bauzon and Hermiston, 2008). Malgrat l'aplicabilitat que tindrien totes aquestes estratègies dintre del genoma d'ICOVIR-15, l'avaluació en un assaig clínic d'un adenovirus oncolític dotat amb eines per a la destrucció de les barreres que limiten la seva eficàcia és una opció molt atractiva. Degut a que la funció de les proteïnes que degraden la matriu extracel·lular i la funció dels enzims convertidors de prodrogues ataquen parts diferents de l'estroma del tumor, la combinació d'ambdues estratègies dintre del genoma d'ICOVIR-15 podria representar un gran avanç en les respostes antitumorals. Per a la combinació d'aquestes dues estratègies, és necessària la tria de dos proteïnes que, a més de ser efectives en la seva funció, tinguin un mida petita. La combinació de la metal·loproteinasa MMP-7, de 800 parells de bases, i la nitroreductasa Nfs-A, de 720 parells de bases, dintre del genoma d'ICOVIR-15, suposaria una mida genòmica d'uns 37600 parells de bases, encara dins dels 38000 parells de bases que en suposa el límit d'encapsidació.

A més de la tria dels transgens a incorporar, també cal dilucidar els sistemes a utilitzar per a l'expressió d'aquests. En aquest treball, hem optat per la seqüència de tall i lligació 3VDE (*IIIa virus infection-dependent splicing enhancer*) per a l'expressió de NfsA. Situant el transgen darrera d'aquesta seqüència, clonat darrera de la fibra, l'expressió d'aquest respon al promotor major tardà, expressant-se en l'etapa tardana del cicle viral (**Figura 37c**). Amb aquesta estratègia, s'eviten problemes de compatibilitat entre el cicle viral i l'expressió de transgens, observat en alguns treballs (Fuerer and Iggo, 2004). A més, d'aquesta manera, i degut a que la transcripció dirigida per aquest promotor està subjugada a l'expressió d'E1A, s'aconsegueix una expressió del transgen restringida a les cèl·lules on s'expressa E1A, en el cas d'ICOVIR-15, a cèl·lules tumorals. El lloc d'inserció del transgen, darrera de la fibra, s'ha mostrat molt efectiu en estudis previs comparatius entre diferents llocs dins del genoma de l'adenovirus (Jin *et al.*, 2005; Kretschmer *et al.*, 2005). Per dirigir l'expressió des del promotor major tardà, podríem haver optat per diferents estratègies apart de les seqüències de tall i lligació. La inserció d'una seqüència IRES entre la fibra i el transgen també hauria dirigit l'expressió des del promotor major tardà gràcies a l'entrada del ribosoma per aquesta seqüència en un trànscrit compartit entre la fibra i el transgen. Malgrat els bons nivells d'expressió que s'han aconseguit amb seqüències IRES a diferents adenovirus oncolítics (Fuerer and Iggo, 2004; Lukashev *et al.*, 2005; Robinson *et al.*, 2008), la mida d'aquestes seqüències és molt gran, entre 300 i 600 parells de bases (Martinez-Salas *et al.*, 2001), descartant el seu ús dintre d'un adenovirus oncolític on cal estalviar espai. Durant els últims anys, s'han descrit unes seqüències procedents de diferents virus que provoquen que el ribosoma se salti un enllaç peptídic, produint dos proteïnes a partir d'un mateix trànscrit (Szymczak *et al.*,

2004). Aquestes seqüències *ribosome-skipping* s'han emprat ja dintre del context d'un adenovirus oncolític per expressar transgens darrera de la proteïna IX, mostrant una gran eficiència la seqüència P2A provinent del Teschovirus-1 porcí (Funston *et al.*, 2008). Aquesta seqüència és petita (66 parells de bases), però el mecanisme de funcionament d'aquesta estratègia provoca un romanent de 21 aminoàcids a la proteïna situada davant de la seqüència, en el nostre cas la fibra, que podria afectar a la funcionalitat i trimerització d'aquesta proteïna estructural. Entre les diferents seqüències de tall i lligació, la seqüència 3VDE és la seqüència que dirigeix l'expressió de la proteïna IIIa de l'adenovirus, i ha demostrat estar subjecte a una regulació molt estricte durant el cicle viral (Delsert *et al.*, 1989; Muhlemann *et al.*, 2000). A més dels resultats d'aquest treball, mostrant alts nivells d'expressió de NfsA limitats a la fase tardana del cicle viral (**Figura 37c**), aquesta seqüència ja s'ha emprat amb anterioritat al nostre grup per a l'expressió de diferents transgens, mostrant una gran eficiència amb tots ells (Garcia-Castro *et al.*, 2005; Guedan *et al.*, 2008). Així, l'ús de la seqüència 3VDE darrera de la fibra sembla una bona opció per a l'expressió de transgens, tot i que cal buscar una segona estratègia si es vol expressar un segon transgen. En aquest cas, una bona opció seria enllaçar ambdós transgens amb una seqüència P2A de *ribosome-skipping*, expressant els dos transgens en un sol trànscrit, en quantitats similars i amb una seqüència de mida petita, encara que caldria avaluar si la cua d'aminoàcids no afecta a la funció del primer transgen.

Així, el genoma d'un possible candidat a un assaig clínic que incorpori les modificacions testades en aquest treball es mostra a la **Figura 55**. En ell, es combina els beneficis que atorguen la modificació del promotor d'E1A d'ICOVIR-15 i la deleció Δ24 amb l'expressió de transgens per a destruir l'estroma del tumor, restringint l'expressió d'aquests a cèl·lules tumorals. La combinació de dos transgens com MMP-7 i NfsA, tots dos expressats gràcies a la seqüència 3VDE i enllaçats amb la seqüència P2A, permetria actuar sobre l'estroma per dos fronts d'acció diferents, ampliant a més l'efecte sobre les cèl·lules tumorals gràcies a l'efecte *bystander* de la NfsA. Provisionalment, el virus incorporaria la fibra modificada amb el motiu RGD al *shaft* de la fibra, a l'espera d'experiments que permetin aclarir l'efecte d'aquesta modificació sobre les plaquetes.

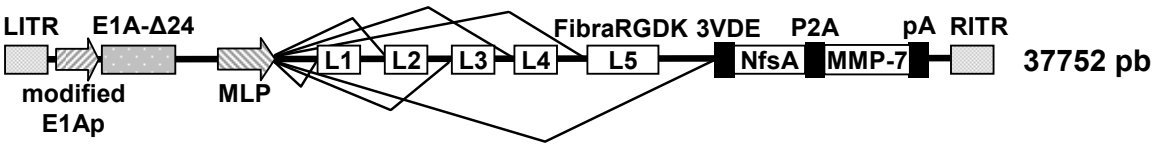

Figura 55. Representació esquemàtica dels components genètics d'un possible candidat a assajos clínics. El virus model incorpora la modificació del promotor d'E1A introduïda a ICOVIR-15, la deleció Δ24 d'E1A i la modificació RGDK del *shaft* de la fibra. Sobre la base d'ICOVIR-15K, el virus incorpora darrera de la fibra dos transgens diferents, NfsA i MMP-7. Per dirigir l'expressió d'aquests transgens sota el control del promotor principal tardà, s'incorpora davant dels transgens l'acceptor de tall i lligació 3VDE, i darrere d'aquests una seqüència poliA (pA). Per separar els dos transgens en dos proteïnes diferents a partir d'un mateix trànscrit, entre ells s'incorpora la seqüència de *ribosome-skipping* P2A. La mida del genoma resultant es detalla a la figura.

En resum, el treball realitzat durant el curs d'aquesta tesi ens ha permès trobar una bona plataforma sobre la qual treballar en un futur immediat per solucionar les dificultats que presenta la teràpia viral del càncer amb adenovirus i poder obtenir respostes antitumorals efectives a la clínica.

CONCLUSIONS

1. La inserció de llocs palindròmics d'unió del factor de transcripció E2F al promotor E2F-1 millora la potència oncolítica de l'adenovirus de replicació condicionada ICOVIR-5, tant *in vitro* com *in vivo*, però no en millora la selectivitat i n'augmenta la mida del genoma fins molt a prop del límit d'encapsidació.

2. La inserció de llocs palindròmics d'unió d'E2F al promotor endogen d'E1A redueix l'expressió d'E1A al fetge i la toxicitat associada a l'administració sistèmica d'adenovirus salvatges.

3. La inserció de llocs E2F al promotor endogen d'E1A és capaç d'augmentar l'expressió d'E1A a cèl·lules tumorals respecte els adenovirus salvatges, fet que es tradueix en un augment de l'activitat antitumoral en models *in vitro* i *in vivo*.

4. La inserció de llocs palindròmics E2F al promotor endogen d'E1A augmenta la mida del genoma en només 151 parells de bases, fet que permet la incorporació del transgen NfsA sense que la replicació del virus se'n vegi afectada.

5. Un virus que combina la inserció de llocs E2F al promotor endogen d'E1A amb la mutació T1 accelera la sortida del virus de la cèl·lula, tot i que aquest efecte no es veu reflectit en una millora de la potència oncolítica en models *in vivo* immunodeprimits.

6. Un virus que combina la inserció de llocs E2F al promotor endogen d'E1A amb una fibra amb el motiu RGDK inserit al *shaft* augmenta l'eficàcia antitumoral *in vivo* gràcies a un increment de la biodisponibilitat del virus, augmentant la virèmia en sang hores després de l'administració.

7. Un virus que combina la inserció de llocs E2F al promotor endogen d'E1A amb una fibra amb el motiu RGDK inserit al *shaft* provoca un augment de la trombocitopènia a dia 4 i dia 12, tant en el model de ratolí com en el de hàmster.

8. En global, la combinació de llocs d'unió d'E2F al promotor endogen d'E1A amb la mutació Δ24 i la inserció del motiu RGDK al *shaft* de la fibra representa un disseny d'adenovirus oncolític depenent de la via de RB amb gran potència i selectivitat, un ampli espectre d'aplicació antitumoral i capacitat per acomodar transgens dins del seu genoma sense afectació de la replicació viral.

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ANNEX

Systemic Toxicity–Efficacy Profile of ICOVIR-5, a Potent and Selective Oncolytic Adenovirus Based on the pRB Pathway

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E2F acts as a transcriptional repressor when bound to unphosphorylated RB during the G_1 or G_0 phase. Upon phosphorylation, E2F is released from the E2F–RB complexes to activate transcription. Tumor cells are characterized by an increase in the level of "free" E2F as a consequence of the absence or hyperphosphorylation of RB. The E2F-1 promoter is a well-characterized E2F-responsive promoter, and it can be used to control adenovirus E1a gene expression as a strategy to achieve tumor-selective expression and replication of an adenovirus. ICOVIR-5 (Ad-DM-E2F-K-Δ24RGD) is an optimized oncolytic adenovirus that combines E1a transcriptional control by an insulated form of the E2F promoter with the Δ24 mutation of *E1a* to improve the therapeutic index of AdΔ24RGD. ICOVIR-5 also contains the Kozak sequence at the E1a start codon, which is important to restore E1a expression and viral replication to AdwtRGD levels in tumor cells. The unique combination of genetic elements in ICOVIR-5 allows the selectivity for cells with a deregulated E2F–RB pathway to be increased and potent anti-tumoral activity to be maintained. Dose–response toxicological and efficacy studies after a single systemic administration in pre-clinical models in mice are presented to demonstrate that this virus holds promise for treatment of disseminated cancer.

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INTRODUCTION

E2F transcription factors (E2F-1 to E2F-6) form heterodimeric complexes with dimerization partner (DP) proteins to activate promoters containing E2F-binding sites. In this way, E2F activates the transcription of genes with a pivotal role in G_1/S cell cycle transition, such as cyclin A, cyclin E, dihydrofolate reductase, and thymidine kinase. In quiescent cells, E2F/DP heterodimers act as repressors of those genes by complexing with hypophosphorylated forms of proteins of the pRB family that in turn dock histone deacetylases to the complex. The blockade of the E2F transactivation domain and the deacetylation of histones lead to the down-regulation of gene transcription. pRB phosphorylation causes the dissociation of pRB from E2F and the disassembly of this quaternary complex, with the subsequent activation of the genes required for S-phase entry by free E2F–DP. The phosphorylation of pRB is controlled during the cell cycle through the activity of cyclin-dependent kinases (CDKs) that are in turn inhibited by CDK inhibitors. Cyclin D-dependent kinases CDK4 and CDK6 are activated by mitogenic signals and inhibited by p15, p16, p18, and p19 CDK inhibitors. The functional connection of pRB, cyclin D, CDK4, and p16 and related proteins to move through the G_1/S transition point is known as the pRB pathway. Deregulation of this pathway leading to the loss of pRB binding to E2F is necessary, although not sufficient, to cause cancer.[1] Therefore, the pRB pathway is altered in 100% of tumors and it should be an ideal target for anti-tumor drugs. However, the pathway is often activated as a result of the loss of pRB or CDK inhibitors. In this case the oncogenic defect becomes difficult to target with small chemical drugs because the target is missing. Adenovirus molecular interaction with the pRB pathway offers a unique opportunity to target this pathway using oncolytic adenoviruses.

Our initial approach to designing oncolytic adenoviruses that target the pRB pathway was to delete the pRB-binding site of E1a.[2,3] This deletion rendered a mutant adenovirus unable to dissociate pRB from E2F in quiescent normal cells. Without free E2F, adenovirus *E2* genes are not transcribed and replication does not proceed. In subsequent work we increased the potency of this mutant oncolytic virus through the insertion of RGD peptide at the HI loop and the restoration of the E3 region in the virus backbone.[4,5] Nevertheless, the resulting virus AdΔ24RDG contains the endogenous *E1a* promoter and in turn expresses the mutated E1a ubiquitously, which precludes its proper systemic use to treat disseminated cancer. Toward this treatment goal, we inserted the E2F-1 promoter following others' work.[6] This promoter contains two E2F-binding sites, and it is activated by E2F–DP heterodimers and repressed by E2F–DP–pRB–HDAC quaternary complexes. In addition to the applicability of this promoter to all tumor types, its dual activity (*i.e.*, activation in tumor cells and repression in normal cells) makes it especially appropriate to control replication

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of oncolytic adenoviruses. To maintain proper regulation of the E2F-1 promoter in the virus genome, we insulated it using translation termination signals and a CTCF-binding insulator from the myotonic dystrophy locus DM-1.[7] Despite a good level of E1a repression in normal quiescent cells, this oncolytic adenovirus expressed less E1a than AdwtRGD in three out of six tumor cell lines tested (SCC-25, FaDu, and SKMel-28 cells).

Now we present an additional modification that aims to boost the translation of the E2F-1-regulated E1a-Δ24 protein. This alleviates the loss of potency observed in some tumor cell lines. With this optimized virus, we have performed the dose–response study of toxicity and efficacy presented here.

RESULTS

ICOVIR-5 replication depends on the abrogation of the pRB pathway

ICOVIR-5 is a new AdΔ24RGD-derivative oncolytic adenovirus that has been designed to increase replication potency in tumor cells compared with ICOVIR-2.[7] ICOVIR-5 controls the expression of the *E1a-Δ24* gene under an E2F-1 promoter that has been insulated with the myotonic dystrophy locus insulator DM-1. In contrast to ICOVIR-2, ICOVIR-5 contains the sequence CCACC (Kozak's sequence) immediately before the first codon of the *E1a* gene. This element is known to optimize the translation of messenger RNA (mRNA) by eukaryotic ribosomes,[8] and we hypothesize that the insertion of this sequence in an insulator/promoter-regulated oncolytic adenovirus can counteract the loss of potency that we have previously observed in some tumor cells. **Figure 1a** shows how genetic elements presents in ICOVIR-5 work in cells with a functional pRB pathway (normal cells) and in cancer cells.

To assess how the modulation of the retinoblastoma pathway affects the composition of the protein complexes that physically interact with the E2F-1 promoter in the ICOVIR-5 genome, we performed chromatin immunoprecipitation assays. The human lung carcinoma cell line A549 presents a disrupted pRB pathway by p16 homozygous deletion.[9] After cells were infected with ICOVIR-5, the Ad genome was detected only when immunoprecipitated with an antibody against E2F-1, and not with an anti-pRB antibody (**Figure 1b**), which indicates that the *E1a* gene promoter in ICOVIR-5 is occupied by free E2F-1 in cancer cells. Pre-treatment of A549 cells with a replication-deficient adenoviral vector expressing retinoblastoma protein (AdCMV-pRB) changes the occupancy of the ICOVIR-5 E1a promoter. In this case the ICOVIR-5 genome can be pulled down with both anti-E2F-1 and anti-pRB antibodies (**Figure 1c**). Thus, in these pRB-transduced cells, the E2F-1 responsible elements along the ICOVIR-5 genome are associated with pRB/E2F-1 complexes.

In agreement with these data, the levels of E1a mRNA increased 30-fold in A549 cells after co-infection with an empty vector (AdCMV-pA) and ICOVIR-5 compared with infection with the pRB-expressing vector alone. However, the combination of AdCMV-pRB and ICOVIR-5 resulted in E1a mRNA levels close to control (**Figure 1d**). A similar profile was obtained with Fiber mRNA. Further confirmation of the repression of ICOVIR-5 activity in pRB-expressing cells was gained from viral production experiments using the same model: pre-infection with AdCMV-pRB reduced ICOVIR-5 viral yield by several logs (**Figure 1e**).

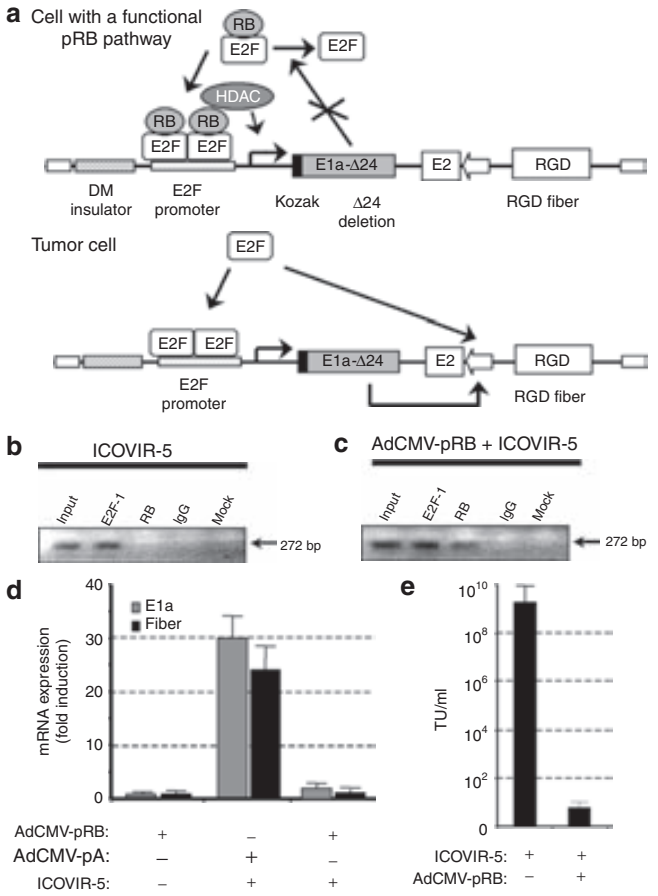

Figure 1 Dependency of ICOVIR-5 on the abrogation of retinoblastoma function. (**a**) Diagram of the components of ICOVIR-5 that confer selective replication. E1a-Δ24 is unable to disrupt the pRB–E2F complexes, avoiding a positive-feedback loop if the promoter repression by pRB–E2F complexes leaks. Deregulation of the pRB pathway in tumor cells releases free E2F that activate the E2F responsive elements in ICOVIR-5. The presence of the Kozak sequence increases the efficacy of E1a-Δ24 expression. The RGD-modified fiber increases the virus infectivity. (**b**) Characterization of E2F-1 promoter occupancy of the ICOVIR-5 genome during viral infection. Bands correspond to E2F-1 promoter–specific polymerase chain reaction (PCR) products detected after immunoprecipitating chromatin of A549 infected cells using anti-E2F-1, anti-pRB, or non-specific antibodies. (**c**) Restoration of pRB activity changes the profile of ICOVIR-5 genome occupancy. The same assay as in **b** was performed but after prior transduction of cells with AdCMV-pRB. (**d**) Dependence of E1a and fiber messenger RNA (mRNA) expression on pRB presence. A549 cells were subjected to the same treatments as in chromatin immunoprecipitation experiments, and mRNA levels were assessed by quantitative real-time PCR. (**e**) Replication of ICOVIR-5 depends on the presence of pRB activity. A549 cells were infected with AdCMV-pRB and ICOVIR-5 or with AdCMV-pA and ICOVIR-5 as in **b**. At day 4 after ICOVIR-5 infection cell extracts were collected and viral production determined. bp, base pairs; HDAC, histone deacetylase; IgG, immunoglobulin G; RB, retinoblastoma protein; TU, transduction units.

ICOVIR-5 neither expresses viral proteins nor replicates in normal cells

Quiescent normal fibroblasts and primary cultures of hepatocytes were infected with equivalent doses of ICOVIR-5 and various control viruses, and the viral replication profile was analyzed. **Figure 2a** is a schematic representation of the different viral genomes used in this study. **Figure 2b** shows that E1a expression was markedly reduced after ICOVIR-5 infection in contrast

to infection with AdwtRGD or AdΔ24RGD in both cell types. Of note, ICOVIR-5 abolishes E1a expression as efficiently or even better than ICOVIR-2 in human hepatocytes, which indicates that the introduction of the Kozak sequence in the ICOVIR-5 genome does not modify the selectivity achieved with ICOVIR-2.

These data correlate with the replication profile obtained in liver biopsies. Precision-cut human liver slices allow the replication specificity of oncolytic viruses to be tested in a pre-clinical setting of systemic administration.[10] By using such a system, we could quantify a 38-fold decrease in viral production with respect to its non-selective replicating adenoviral counterpart, AdwtRGD, in normal cells (**Figure 2c**).

In vivo toxicity profile of systemic ICOVIR-5 in Balb/C immunocompetent mice

ICOVIR-5-mediated toxicity was assessed after a single intravenous injection. Weight loss, overall survival, liver enzymes (aspartate aminotransferase and alanine aminotransferase), and hematological profile were determined at day 5 after injection of increasing virus doses. Whereas an intravenous dose of 5×10^{10} viral particles (vp) represented more than the LD_{50} value for wild-type adenovirus (Adwt)–injected mice, the LD_{10} of ICOVIR-5 was 1×10^{11} vp (**Table 1**). At 5×10^{10} vp, ICOVIR-5 did not reduce body weight and only slightly increased transaminases in contrast to Adwt (**Table 1**; **Figure 3a**). Moreover, whereas a significant decrease in platelets and lymphocytes was associated with a single intravenous injection of 5×10^{10} vp of Adwt, no hematological alterations were detected with ICOVIR-5 at this dose or at a higher dose (1×10^{11} vp) (**Figure 3b** and **c**).

In addition, E1a expression was evaluated by immunostaining of the liver, the main target organ after systemic adenovirus injection. Expression of E1a was efficiently restricted in ICOVIR-5-injected mice even at the highest intravenous dose (1×10^{11} vp) compared with samples from mice injected with Adwt at 5×10^{10} vp (**Figure 4b** versus **c** and **d**). Histological analysis of liver samples obtained at day 3 after systemic administration (**Figure 4e–h**) showed that a single 5×10^{10}-vp dose of Adwt

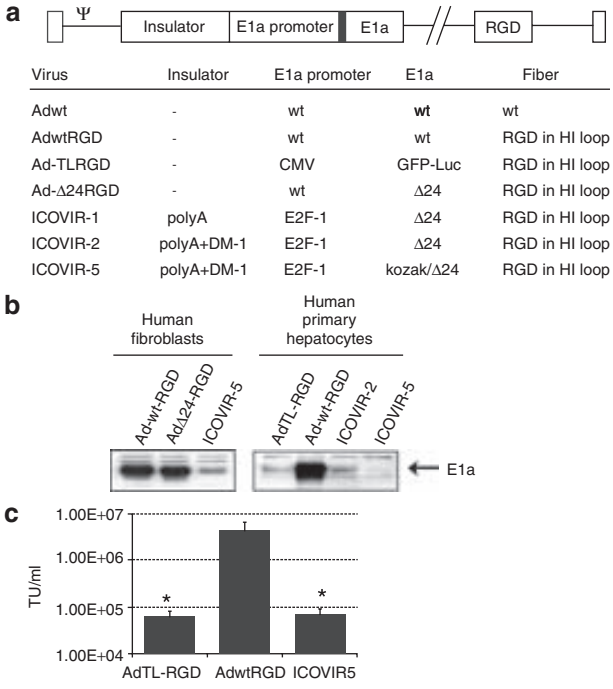

Figure 2 Selectivity of ICOVIR-5 in normal human cell models. (**a**) Structure of the viruses used in this study. (**b**) Restriction of E1a protein expression in normal cells infected with ICOVIR-5. E1a was analyzed by western blotting in human fibroblasts arrested by serum starvation and primary human hepatocytes infected with the indicated viruses at multiplicities of infection of 25 or 5. (**c**) Virus production was measured in slice extracts in triplicate on day 6 after infection. The *P*-value refers to the difference from the AdwtRGD-infected group according to a two-tailed unpaired Student's *t*-test. *$P < 0.02$. Adwt, wild-type adenovirus; CMV, cytomegalovirus; GFP, green fluorescent protein; TU, transduction units.

Table 1 Toxicity of systemic ICOVIR-5 in Balb/C immunocompetent mice

Treatment	Dose (vp/mouse)	Survival[a]	Body weight variation[a,b]
PBS	—	10/10	3.3 ± 2.0
Adwt	2.5×10^{10}	5/5	2.5 ± 1.1
	5×10^{10}	3/5	−11.0 ± 1.2
	1×10^{11}	0/5	−14.8[c]
ICOVIR-5	2.5×10^{10}	10/10	3.2 ± 3.0
	5×10^{10}	15/15	0.9 ± 0.9
.	1×10^{11}	9/10	−3.2 ± 3.7

Abbreviations: Adwt, wild-type adenovirus; PBS, phosphate-buffered saline; vp, viral particles.
[a]Parameters determined at day 5 after viral administration. [b]Expressed as mean ± SD. [c]Measured at day 4 after administration for two live animals.

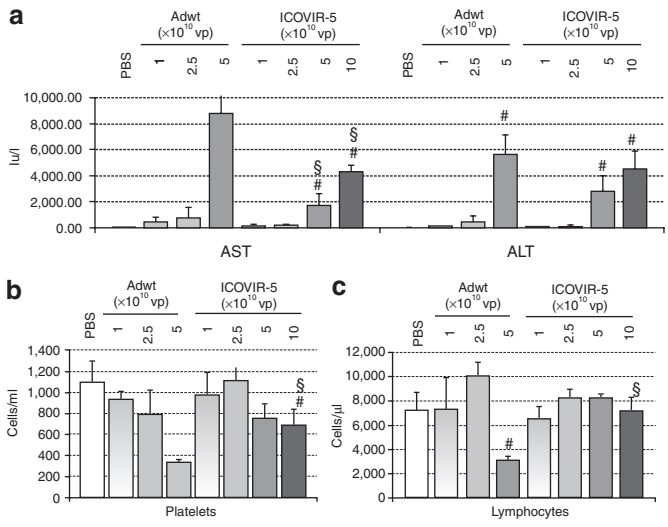

Figure 3 Hepatic and hematological toxicity profile after systemic ICOVIR-5 administration in immunocompetent mice. The average values for (**a**) serum transaminases and (**b**) platelet or (**c**) lymphocyte concentrations in Balb/C mouse peripheral blood at day 5 after intravenous administration of increasing single doses of Adwt or ICOVIR-5. Phosphate-buffered saline (PBS) administration was used in the control group. Mean values ± SD of 5–10 mice/group are depicted. #, significant ($P < 0.05$) by two-tailed unpaired Student's *t*-test compared with the PBS group; §, significant ($P < 0.05$) by two-tailed unpaired Student's *t*-test compared with the group of mice injected with 5×10^{10} vp of Adwt; Adwt, wild-type adenovirus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Iu/l, International units per liter; vp, viral particles.

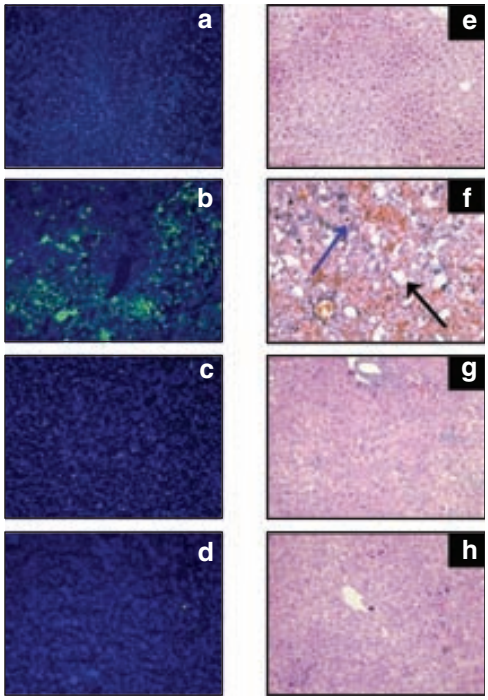

Figure 4 ICOVIR-5 effectively controls E1a hepatic expression *in vivo*. Liver E1a expression assessed by (**a–d**) immunohistochemistry in frozen sections or (**e–h**) eosin–hematoxylin staining of equivalent paraffin-embedded liver sections. Mice livers were collected at day 3 after intravenous administration of (**a, e**) phosphate-buffered saline (PBS), (**b, f**) 5×10^{10} vector particles (vp) of wild-type adenovirus (Adwt), or (**c, g**) 5×10^{10} vp or (**d, h**) 1×10^{11} vp of ICOVIR-5. Livers from mice injected with Adwt presented intense E1a expression that correlated with evident symptoms of degenerative cirrhosis, such as macrosteatosis (black arrow) or large necrotic areas with Councilman bodies (blue arrow).

resulted in evident signs of hepatitis (macrosteatosis, presence of Councilman bodies, and large necrotic areas) (**Figure 4f**). In contrast, livers from mice treated with ICOVIR-5 at the same dose or a twofold higher dose displayed a practically normal phenotype, with only marginal Councilman bodies in the more superficial areas (**Figure 4g** and **h**). The analysis of ICOVIR-5-injected livers at later time points (day 7) demonstrated the presence of mitotic nuclei and reduced necrotic areas, which suggests the existence of regeneration processes (data not shown).

The Kozak sequence in ICOVIR-5 rescues of E1a expression and virus replication in tumor cells

Given the genetic elements of ICOVIR-5, the loss of activity displayed in normal cells should be effectively rescued when the retinoblastoma pathway is impaired and cellular levels of free E2F-1 are high. To confirm this hypothesis we evaluated the effects of ICOVIR-5 infection in a panel of tumor cell lines of different origin, including lung, melanoma, head and neck, cervix, and prostate. As shown in **Figure 5a**, E1a protein levels after infection with ICOVIR-5 are as high as the parental AdwtRGD in all assayed cell lines when analyzed by western blot. Importantly, the levels of expression of E1a achieved were higher in ICOVIR-5-infected cells than in ICOVIR-2-infected cells in those cell lines where ICOVIR-2 had demonstrated partial attenuation (SKMel-28 and FaDu cells). These data suggest that the enhancement in transcription from the heterologous E2F-1 promoter induced by the insertion of the Kozak sequence in ICOVIR-5 results in improved potency.

Additional confirmation of the increased oncolytic potency of ICOVIR-5 was obtained from cytotoxicity and replication studies. The amount of ICOVIR-5 required to cause a 50% reduction in cell viability (IC_{50}) is equivalent to the IC_{50} for AdwtRGD in a

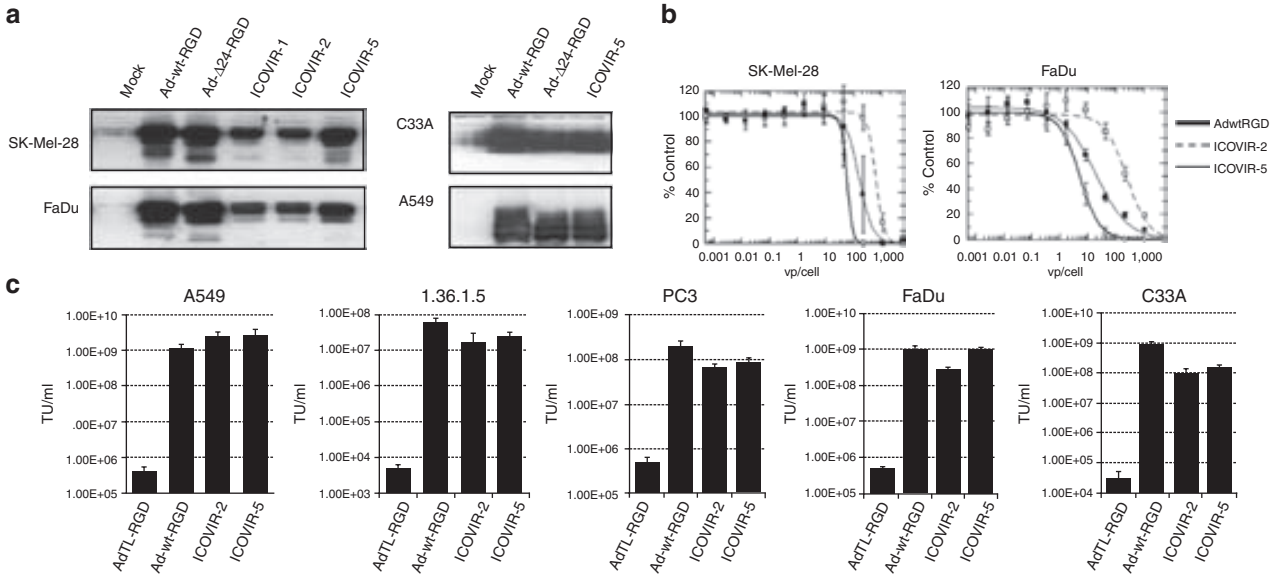

Figure 5 Anti-tumoral activity of ICOVIR-5 *in vitro*. (**a**) ICOVIR-5 expresses E1a at the level of AdwtRGD in different tumor cell lines. Anti-E1a western blot 22 hours after infection with a dose of each virus that allowed more than 80% of transduction (multiplicity of infection (MOI) of 25 for SKMel-28 and FaDu cells; MOI of 10 for C33A and A549 cells). (**b**) Comparative cytotoxicity of ICOVIR-5. Cells were infected with the indicated viruses at doses ranging from 50,000 to 0.001 viral particles (vp)/cell. At days 5–8 after infection the amount of viral particles/cell that produced 50% growth inhibition (IC_{50} value) was estimated. (**c**) Replication of ICOVIR-5 in tumor cells. Different tumor cell lines were infected with the indicated viruses as in **a** or at an MOI of 20 (PC3 cells) or 40 (SCC25). Virus production was measured 4 days after infection. To normalize the bioactivity of different virus preparations, HEK293 cells were used. Adwt, wild-type adenovirus; TU, transduction units.

Table 2 Potency of ICOVIR-5 in different tumor cell lines *in vitro*

Cell line	Origin	IC_{50} Adwt-RGD (vp/cell)	IC_{50} ICOVIR-5 (vp/cell)	Deficiency[a] versus Adwt-RGD
SKMel-28	Melanoma	57.9 ± 12.7	54.4 ± 3.2	0.9×
FaDu	Head and neck	5.2 ± 0.5	12.9 ± 0.4	2.4×
A549	Lung	0.020 ± 0.007	0.020 ± 0.006	1.0×
NP-9	Pancreas	16.0 ± 4.4	7.0 ± 2.6	0.4×
A375P	Melanoma	33.3 ± 7.6	65.1 ± 34.4	2×

Abbreviations: Adwt, wild-type adenovirus; vp, viral particles.
[a]"Deficiency" is defined as the ratio between IC_{50} values obtained for ICOVIR-5 and Adwt-RGD.

panel of human tumor cell lines (**Table 2**). Again, the cytotoxicity of ICOVIR-5 was enhanced compared with that of ICOVIR-2 in FaDu and SKMel-28 cell lines. IC_{50} values obtained in FaDu and SKMel-28 for ICOVIR-5 were 12.9 and 54.4 vp/cell respectively, whereas the IC_{50} values for ICOVIR-2 were 247.4 and 610.4 vp/cell, respectively (**Figure 5b**). Viral progeny studies for ICOVIR-5 also indicated that its production was not reduced with respect to AdwtRGD (**Figure 5c**). Altogether, the results indicate that ICOVIR-5 displays an improved therapeutic index (*i.e.*, phenotype of ICOVIR-5 in tumor cells versus normal cells) compared with ICOVIR-2 and warrants therapeutic testing by systemic administration.

Systemic anti-tumoral efficacy of ICOVIR-5

Mice carrying C33A (cervix), SKMel-28 (melanoma), or A549 (lung carcinoma) tumors were treated with a single intravenous dose of ICOVIR-5 at 1×10^{11} vp/mouse. No significant toxicity was observed in these efficacy studies, probably owing to the reduced impact of the immune system in the nude mice model. ICOVIR-5 was able to induce significant tumor growth inhibition in all three models (**Figure 6**). At day 32, untreated mice carrying C33A tumors had to be killed due to uncontrolled tumor growth. At this time point, ICOVIR-5 treatment induced a reduction of more than 67% in the growth of C33A tumors with respect to the control group, which was statistically significant ($P < 0.05$) (**Figure 6a**). In the SKMel-28 model, where the tumors grew more slowly, ICOVIR-5 dosed at 1×10^{11} vp/mouse was able to stop tumor growth from day 20 after administration, and at day 35 the percentage of inhibition compared with untreated tumors was 51% (ratio of percentages expressed as a new percentage; $P < 0.03$) (**Figure 6b**). At the same time point, the growth of A549 tumors was 48.5% lower than that of non-treated tumors (**Figure 6c**).

To study whether the limited efficacy in this latter model was related to the distribution profile of ICOVIR-5 in the tumor mass, we carried out immunohistochemical staining for adenoviral capsid proteins in A549 tumor sections. The results from samples obtained at day 27 after treatment revealed patches of positive cells within the tumor mass that were not present in untreated tumors (**Figure 6d**, upper left versus right panel), indicating that the anti-tumoral activity was associated with the presence of ICOVIR-5. Interestingly, such infected/degenerated regions appeared to be surrounded by fibrotic tissue (**Figure 6d**, lower left panel). To confirm that such areas correspond to connective tissue, equivalent

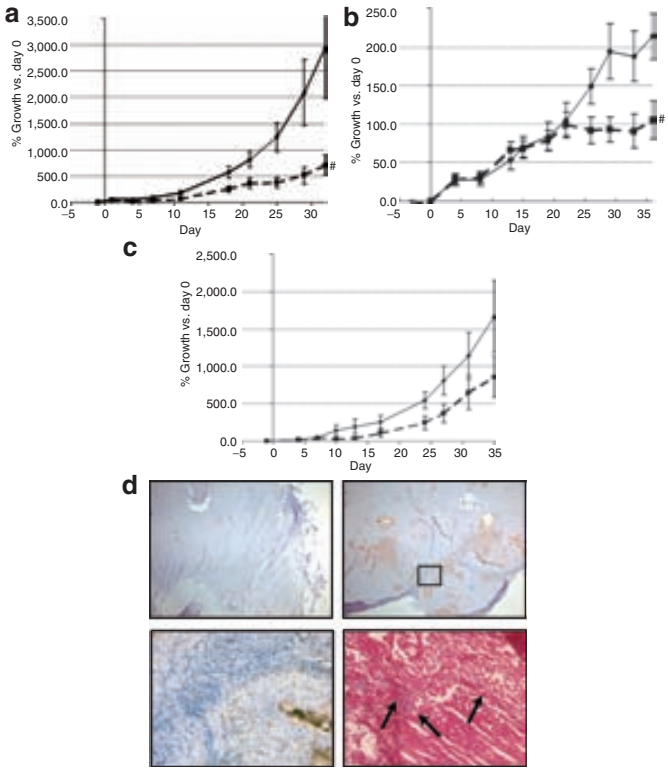

Figure 6 Efficacy after intravenous administration of ICOVIR-5 in different tumor models *in vivo*. Nude mice with subcutaneous xenografts of (**a**) C33A, (**b**) SKMel-28, or (**c**) A549 (n = 10–12 tumors/group) were randomized and treated with a single intravenous dose of 1×10^{11} vector particles (vp) of ICOVIR-5 (solid squares) or phosphate-buffered saline (PBS) (solid diamonds). Percentage of tumor growth ± SE is plotted. #, significant ($P < 0.05$) by two-tailed unpaired Student's t-test compared with mice injected with PBS. (**d**) Anti-adenovirus immunohistochemistry of SKMel-28 xenograft tumors obtained at day 27 after intravenous administration of PBS (upper left panel) or 1×10^{11} vp of ICOVIR-5 by tail-vein (upper right and lower left panels). Paraffin-embedded sections were incubated with an anti-adenovirus antibody and counterstained with hematoxylin (upper left and right panels, ×4 magnification; lower left panel, ×40 magnification). Lower right panel: Masson staining was performed to detect collagen fibers bordering the adenovirus-positive cells in the tumors treated with ICOVIR-5 intravenously at 1×10^{11} vp (arrows), ×40 magnification.

sections were stained using Masson's trichromic technique. As shown in **Figure 6d** (lower right panel), blue dye (which reveals the presence of collagen fibrils) localizes around the infected areas. Both the non-human (murine) and non-tumor phenotype of the cells and the high content of extracellular matrix in those areas behave as physical barriers to intra-tumoral virus dissemination, which could account for the incomplete tumor response.

DISCUSSION

Toxicity and efficacy are the main parameters of a pre-clinical study. Although presented separately, these are parts of a unity. A very low toxicity allows for a dose increase that can yield efficacy. High efficacy allows dosing at no toxicity. At a clinical level, most information on systemic oncolytic adenoviruses was obtained with Onyx-015 in different tumor types.[11,12] The results indicate that the major obstacle to clinical development was low efficacy, not toxicity. It is clear, then, that oncolytic adenoviruses

that keep the replication potency of wild-type adenoviruses in tumors should be used. However, a recent clinical study with a more potent oncolytic adenovirus has indicated that toxicity needs to be monitored carefully when systemic administration is considered.[13] Even viruses with more cytopathic potency than the wild-type adenovirus are being proposed in the search for greater anti-tumor efficacy.[14,15] Those viruses need to be highly selective to be safe.

The Δ24 deletion of the pRB-binding site in conserved region 2 (CR2) of E1a was a first step toward creating the potent and selective virus presented here. This deletion has been taken as a basis to develop more potent oncolytic adenoviruses by several groups.[5,16–23] Although the deletion is broadly used, it is worth discussing the role of this CR2 mutation and other *E1a* mutations to confer tumor-selective replication. CR1 and CR2 are two CRs of E1a essential to bind pRB, p130, and p107 family protein members. In addition, the carboxy-terminal half of CR1 and amino-terminal domain of E1a bind p300 to stimulate E2F transcriptional activity, and this could partially compensate the lack of free E2F upon infection with CR2 mutants. However, it is important to note that although either p300 (bound by the N-terminus and CR1) or pRB (bound by CR1 and CR2) binding is enough to induce cellular DNA synthesis, binding to both is essential to pass G_2/M. With regard to oncolytic adenoviruses, single CR1 mutants such as dl1101 are barely selective and have an undesired attenuation in tumor cells.[24] In contrast, single CR2 mutants such as AdΔ24 preserve the oncolytic potency in tumor cells and are attenuated in normal quiescent cells.[2,3] Double CR1/CR2 mutants, such as dl01/07, may increase the pRB-dependent selectivity at the expense of reducing potency.[24,25] Finally, as E4-orf6/7 binds also to pRB, the combination of this deletion with the E1a-CR2 deletion seemed necessary to achieve better selectivity. However, so far this has not been the case.[26] Taking all these considerations into account, we decided not to include the N-terminal/CR1 E1a deletions and E4 deletions in ICOVIR-5. Instead, and given that E1a expression is a major factor that affects the hepatic toxicity of an adenovirus upon systemic administration,[27] we considered the use of a tumor-selective promoter to control E1a-Δ24.

Several groups have used the E2F-1 promoter to control E1a expression.[28–31] The importance of the Δ24 (CR2) deletion in allowing for the proper control of such a promoter in an oncolytic adenovirus has not been formally tested. However, the expression of wild-type E1a overcomes the pRB regulation of the E2F-1 promoter inserted in the oncolytic virus genome, indicating that the Δ24 mutation is advantageous when this promoter is used.[29] Theoretically, the use of wild-type E1a would engage a positive-feedback loop if any promoter leakage occurs. In this regard, our results show that it is difficult to obtain a complete promoter shut-off in normal cells. The use of two E2F promoters in two different adenovirus genes has been reported as a way to increase the selectivity of replication. However, promoter repetition has also led to genome instability in viruses such as Onyx-411, which contains E2F-1 promoters in E1a and in E4. Genomic instability has been observed even when E4 was regulated by a different promoter or when the packaging signal of the virus was moved next to E4 at the right end of the genome, suggesting that any E4 modification can decrease stability.[26] For this reason, and to save space for the potential insertion of transgenes, we focused on optimizing the E1a regulation by keeping the packaging signal at its native left end. In a previous work we demonstrated that in an oncolytic adenovirus context, insulation of the E2F-1 is required for the proper control of E1a expression.[7] However, the potency of this insulated promoter proved to be heterogeneous in a panel of tumor cell lines. A decrease in potency has been observed with E2F–E1a, an oncolytic adenovirus with wild-type E1a under the E2F-1 promoter.[30] Although we did not study the reason behind this behavior, it could be related to the different levels of free E2F present in tumor cells. Bauerschmitz *et al.* observed that when the cox2 promoter was used to control *E1a-Δ24* expression in an oncolytic adenovirus, its replication was attenuated in cell lines with low cox2 expression.[25] To compensate for the lower transcriptional activity of the E2F promoter in certain tumor cells, we decided to increase the translation of the E1a mRNA using the optimal ribomose recognition sequence described by M. Kozak.[8] This approach is used commonly in gene expression vectors. As ICOVIR-2 and ICOVIR-5 differ only in this sequence, we can conclude that it is responsible for the observed oncolytic potency rescue in ICOVIR-5.

Systemic administration of human adenovirus in mice leads to a toxicity characterized by increased serum levels of transaminases, degeneration of liver tissue, and severe weight loss.[32] At a systemic level, we could not see a differential toxicity between high doses of AdΔ24RGD and Adwt.[7] This was expected as AdΔ24RGD does not preclude the toxicity associated with E1a expression, and in mice the toxicity associated with virus replication is not readily seen. The first dose at which toxicity could be observed with ICOVIR-5 was 5×10^{10} vp/mouse. This dose caused a tenfold lower increase in transaminases than was observed with Adwt. These results with ICOVIR-5 are similar to our previous results with ICOVIR-2.[7] This was expected, as the addition of the Kozak sequence should improve the translation of E1a-Δ24 only when the promoter is active in tumor cells. We can conclude that ICOVIR-5 retains the selectivity of ICOVIR-2 but gains anti-tumor potency.

In comparison with similar oncolytic viruses that target the pRB pathway, ICOVIR-5 toxicity is similar to that reported for OAS403, an oncolytic adenovirus with *E1a* under the E2F-1 promoter and E4 under the human telomerase reverse transcriptase promoter, in terms of both transaminases and hematology.[30] A complete blood cell count performed at different time points after systemic administration of 1×10^{11} vp of OAS403 per mouse showed a 50% decrease in platelets as the only hematology parameter affected.[30] However, in contrast to ICOVIR-5, OAS403 depends on the up-regulation of telomerase expression and it is attenuated in tumor cells compared with the single promoter virus E2F–E1a.

Despite ICOVIR-5 having a replication efficiency similar to AdwtRGD, tumors with actively replicating ICOVIR-5 do not regress. Previous data on the biodistribution of AdΔ24RGD after intravenous administration have demonstrated that RGD-modified viruses effectively reach subcutaneous tumor masses.[33] We have obtained similar data with ICOVIR-5 (data not shown). However, histology analysis reveals that the presence of tumor stroma precludes a complete intra-tumoral spread, as also reported previously.[34] Such interference of tumor architecture with virus

spread has been postulated as a putative explanation of the lack of correlation between *in vitro* cytotoxicity and *in vivo* anti-tumor efficacy.[30] A rational solution will have to consider the amount and type of stroma in the tumor, which varies with tumor type. Stroma is composed of fibroblasts, endothelial and immune cells, and matrix components such as collagen. To break through the extracellular matrix barriers, the virus could be armed to express specific proteases such as relaxin.[35] Similarly, the virus could disrupt cell barriers by expressing pro-drug converting enzymes with bystander effect on normal cells.

ICOVIR-5 combines five different genetic modifications to achieve a selective and potent anti-tumor effect. The E2F-1 promoter, insulating elements, and Δ24 deletion are designed to avoid E1a expression and subsequent virus replication in normal cells. The Kozak and RGD insertions are designed to provide efficient replication and infectivity in tumor cells. Future improvements may be pursued by arming the virus with transgenes and by increasing the selectivity at the level of re-targeting. In this regard, blood factors play a major role in targeting adenovirus to hepatic cells,[36] and capsid mutations that abrogate the binding to these blood factors will likely reduce the toxicity that results from the interaction with Kupffer cells and hepatocytes.[37] In summary, a combination of genetic modifications such as those present in ICOVIR-5 can yield a high level of selective and efficient replication, but major hurdles associated with biodistribution and intratumoral spread need to be tackled.

MATERIALS AND METHODS

Cell culture and liver slice preparation. HEK293 human embryonic kidney cells, A549 (human lung adenocarcinoma), FaDu (squamous head and neck carcinoma), SkMel-28 and A375P (melanoma), C33A (cervix carcinoma), and PC-3 (prostate carcinoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). 1.36.1.5 (a clone of SKMel-131) was a kind gift from Francesc X. Real (Institut Municipal d'Investigació Mèdica, Barcelona, Spain). The NP-9 human pancreatic tumor cell line was established in our laboratory.[38] All cell lines were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum. Human fibroblasts were provided by Antonio Tugores (Almirall, Barcelona, Spain).

Human liver samples were obtained from the Department of Surgery of the Hospital Universitari de Bellvitge (L'Hospitalet, Spain), with approval from the medical committee. Primary cultures of human hepatocytes were obtained by a two-perfusion technique and seeded in collagen-coated plates.[39] Liver slices were prepared as described.[40]

Viruses. Adwt was obtained from the American Type Culture Collection (Manassas, VA); AdwtRGD was created by digesting pVK503 plasmid (which contains the complete Ad5 genome with RGD-modified fiber)[41] with *Pac*I and further transfection in 293 cells. AdΔ24RGD and ICOVIR-2 have been previously described.[5,7,42] All these viruses were propagated in A549 cells. Replication-deficient AdTL-RGD, AdCMV-pA, and AdCMV-pRB have also been described[42,43] and were propagated in HEK293 cells. ICOVIR-5 was constructed by inserting a consensus Kozak sequence in the genetic backbone of ICOVIR-2. To this end, a *Kpn*I fragment from pShuttle-DM-E2F-Δ24 plasmid[7] containing the insulated version of the E2F-1 promoter was subcloned into pGEM-3Z (Promega, Southampton, UK). The resulting plasmid was used to replace the E1a translation start site using oligonucleotides with the Kozak sequence. The *Kpn*I fragment containing the modified version of the E2F–E1a construct was returned to pShuttle-DM-E2F-Δ24 to generate pShuttle-DM-E2F-K-Δ24. As a final step, pShuttle-DM-E2F-K-Δ24 was recombined with pVK503 by homologous recombination to construct pICOVIR-5. Virus ICOVIR-5 was obtained after digestion of this plasmid with *Pac*I and transfection in HEK293 cells. ICOVIR-5 was plaque-purified and amplified in A549 cells, and purified using a CsCl gradient. The viral genomic structure was verified by restriction analysis. The DM-1 insulator, E2F-1 promoter, Kozak sequence, E1a-Δ24 deletion, and RGD fiber were sequenced.

Chromatin immunoprecipitation assay. A549 cells were seeded in 6-well plates, and 20 hours later the cultures were infected with the replication-deficient AdCMV-pRB or with the control adenoviral vector AdCMV-pA at a multiplicity of infection (MOI) of 10 at 37 °C for 30 minutes. After 48 hours, the cultures were treated with ICOVIR-5 at an MOI of 2 at 37 °C for 24 hours. Cells were then fixed by exposure to 1% formaldehyde for 10 minutes at 37 °C, washed twice with phosphate-buffered saline (PBS) containing a mixture of protease inhibitors (Sigma, St. Louis, MO), and re-suspended in 200 µl of lysis buffer (1% sodium dodecyl sulfate, 10 mmol/l EDTA, 50 mmol/l Tris–HCl, pH 8.1) with protease inhibitors. Chromatin was sheared by sonicating five times for 10 seconds each using a 60 Sonic Dismembrator (Fischer Scientific, Strasbourg, France) followed by centrifugation for 10 minutes at 14,000 rpm. Of the resulting supernatant, 20 µl was set aside as input chromatin. The subsequent immunoprecipitation and extraction steps were carried out using a commercially available chromatin immunoprecipitation assay kit (Upstate Biotech, Lake Placid, NY) following the manufacturer's instructions. E2F-1 (KH-95), pRB (C15), or mouse immunoglobulin G antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to immunoprecipitate the cross-linked chromatin. The following primers were used to amplify a 272-bp fragment in the E2F-1 promoter and the adjacent genome of ICOVIR-5: 5′-TGTCTGTCCCCACCTAG GAC-3′ and 5′-GCGGTTCCTATTGGCTTTAAC-3′.

E1a and fiber mRNA expression by TaqMan analysis. Quantitative polymerase chain reaction was performed as described.[29] The cycling conditions for were 10 minutes at 95 °C for 1 cycle; 15 seconds at 95 °C and 1 minute at 60 °C for 40 cycles. Relative gene expression was compared with the comparative threshold cycle method.[44]

E1a protein expression analysis. Cell cultures (~2 × 10^5 cells) were infected at an MOI that allowed at least 80% infectivity, and whole-cell protein extracts were prepared 22 hours after infection by incubation in lysis buffer (400 mmol/l NaCl, 1 mmol/l EDTA, 5 mmol/l NaF, 10% glycerol, 1 mmol/l sodium orthovanadate, 0.5% Nonidet NP-40, and a mixture of protease inhibitors (Sigma, St. Louis, MO) in 10 mmol/l Tris–HCl pH 7.4) for 1 hour at 4 °C. Clarified samples (15 µg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 10% gels and transferred to nitrocellulose membranes (GE Healthcare, Arlington Heights, IL). Membranes were immunoblotted with a polyclonal anti-E1a (Clone 13S-5) (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody.

Virus progeny production determination and* in vitro *cytopathic effect. Cell cultures were grown to 60–80% confluency in 24-well plates and subsequently infected at an MOI that allowed at least 80% infectivity. Liver slices were infected at an MOI of 50 after 2 hours of pre-incubation in 6-well plates. Virus was removed 2 hours after infection, and cells were washed twice with PBS and incubated in fresh virus-free medium. On day 4 after infection (cell lines and primary cell cultures) or day 6 after infection (liver slices), cells and medium were harvested and freeze–thawed three times. Viral titers in cell extracts were determined according to an anti-hexon staining-based method.[7,45] Cytopathic effect assays were performed as previously described.[46]

In vivo toxicity studies. Animals for toxicological and efficacy studies were maintained in the facility of the Institut de Recerca Oncologica–Institut d'Investigació Biomèdica de Bellvitge (Barcelona), AAALAC unit 1155. All animal studies have been approved by the Institut d'Investigació

Biomèdica de Bellvitge Ethical Comitee for Animal Experimentation. Increasing doses of viruses (Adwt or ICOVIR-5) were injected intravenously into the tail vein in 6-week-old immunocompetent Balb/C male mice at a dose of 10 ml/kg in PBS (n = 5–10). Daily observations for body weight, morbidity, and moribundity were performed. At days 3, 5, and 7 after infection, mice were killed and various samples were collected. Blood samples were obtained by intracardiac punction, and clinical biochemistry for creatinin and transaminase levels and hematological determinations were performed by the Clinical Biochemistry and Hematological Services of the Veterinary Faculty at the Autonomous University of Barcelona. Mice livers were collected and portions were fixed in 4% formaldehyde for 24 hours at room temperature or frozen in O.C.T. (Sakura Finetek, Zoeterwoude, The Netherlands). E1a-immunodetection was performed by incubating O.C.T.-embedded liver sections with a primary polyclonal antibody anti-adenovirus-2 E1a (clone 13 S-5, Santa Cruz Biotechnology) and a AlexaFluor 488-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Slides were counterstained with 4′,6-diamino-2-phenylindole and visualized under a fluorescent microscope (Olympus BX51; Olympus Optical Company, Hamburg, Germany).

Evaluation of* in vivo *antitumoral efficacy. Subcutaneous C33A cervical, SKMel-28 melanoma, or A549 lung carcinoma tumors were established by injection of 1×10^7 cells into the flanks of 6-week-old male Balb/C *nu/nu* mice. Once tumors reached 100 mm^3 (day 0), mice were randomized (n = 10–12 per group) and a single intravenous injection of PBS or 1×10^{11} vp of ICOVIR-5 at a dose volume of 10 ml/kg in PBS was given via the tail vein. Tumor size, as well as mice status, was recorded three times weekly, and tumor volume was calculated according to the equation V (mm^3) = $\pi/6 \times W \times L^2$, where W and L are the width and the length of the tumor, respectively. The percentage of growth was defined as $[(V - V_0)/V_0] \times 100$, where V_0 is the tumor volume on day 0. The significance of differences in tumor growth rate between treatment groups was assessed by a two-tailed Student's unpaired t-test. The statistical significance was set at $P < 0.05$.

To detect adenoviral hexon proteins in the tumor xenografts, paraffin-embedded sections of tumors obtained at day 27 after treatment were de-paraffinized and rehydrated with xylene and ethanol according to standard procedures. Immunodetection was performed after endogenous peroxidase activity and heat antigen retrieval, using polyclonal anti-adenovirus (ab6982; Abcam Cambridge, UK) as primary antibody. Rabbit immunoglobulin G was used as control. Detection was performed using the DAKO LSAB2 system kit (DAKO, Carpinteria, CA) and slides were developed with diaminobenzidine tetrahydrochloride (DAKO, Carpinteria, CA) and counter-stained with hematoxylin. Masson trichromic stain was used to reveal connective tissue, as it allows the detection of collagen fibers, which are stained blue.

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ORIGINAL ARTICLE

A modified E2F-1 promoter improves the efficacy to toxicity ratio of oncolytic adenoviruses

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The E2F-1 promoter has been used to confer tumor-selective E1A expression in oncolytic adenoviruses. Tumor specificity is mainly conferred by a unique structure of E2F-responsive sites organized in palindromes. Binding of the E2F–pRb complex to these palindromes results in repression of transcription in normal cells. Owing to deregulation of the Rb/p16 pathway in tumor cells, binding of free E2F activates transcription and initiates an auto-activation loop involving E1A and E4-6/7. ICOVIR-7 is a new oncolytic adenovirus designed to increase the E2F dependency of E1A gene expression. It incorporates additional palindromes of E2F-responsive sites in an insulated E2F-1 promoter controlling E1A-Δ24. The E2F palindromes inhibited replication in normal cells, resulting in a low systemic toxicity at high doses in immunocompetent mice. The Δ24 deletion avoids a loop of E2F-mediated self-activation in nontumor cells. Importantly, the additional E2F-binding hairpins boost the positive feedback loop on the basis of E1A-mediated transcriptional regulation of E4-6/7 turned on in cancer cells and increased antitumoral potency as shown in murine subcutaneous xenograft models treated by intravenous injection. These results suggest that the unique genetic combination featured in ICOVIR-7 may be promising for treating disseminated neoplasias.
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Keywords: *oncolytic adenovirus; E1A; E2F-1 promoter; E2F-binding sites; Rb pathway*

Introduction

Genetic engineering techniques and a better understanding of the interactions between viruses and cells result in an improved design of tumor-selective adenoviruses for cancer treatment.[1] Among the modifications introduced into oncolytic adenoviruses to confer tumor selectivity, promoters for E1A transcription control are essential if systemic use is desired, as they can prevent E1A expression in nontarget tissues. In this regard, tissue-specific promoters have been tested to target cancers arising from specific tissues.[2,3] However, promoters active in a wider range of tumor types increase the patient population potentially benefiting from a new treatment agent. A tight and potent promoter regulated by repression in normal cells and activation in tumor cells might be an ideal option for reaching a high antitumor effect with low toxicity.

Deregulation of the retinoblastoma (Rb/p16) pathway is a hallmark of cancer cells.[4] Aberrations of this pathway lead to the release of E2F transcription factors for the activation of the E2F site containing promoters. Most E2F-responsive genes are involved in the control of cell cycle or in DNA synthesis and its activation leads to an S-phase entry. It is interesting that silencing of these promoters is also carried out by the same E2F-binding sites in quiescent cells, when E2F is bound to pRb. The formation of a complex involving E2F-pRb and histone deacetylase represses transcription by binding to E2F sites.[5] One of the promoters controlled by E2F is the E2F-1 promoter. It is subject to a strict activation loop of transcription because of its unique roles in apoptosis and DNA repair.[6] Transcriptional control of this promoter depends on a unique structure consisting in four E2F-binding sites organized as two imperfect palindromes.[7,8] Importantly, a similar palindromic pattern controls the expression of the E2a promoter of adenovirus type 5,[9] suggesting the optimal structure of this arrangement in terms of DNA length to confer E2F responsiveness.

S-phase induction is a requisite for adenoviral life cycle. Therefore, adenoviruses have evolved two different mechanisms to induce an expression of the *E2F-1* gene. First, the E1A protein displaces pRb from the E2F–pRb complex to release E2F and activate E2F-1 and viral promoters.[10] Second, the E4-6/7 protein complements this function by binding to free E2F and forming a complex that induces the cooperative and stable binding of E2F to the inverted binding sites present in the E2F-1 and E2a promoter.[11] Disruption of the Rb pathway in tumor cells can functionally substitute E1A-mediated E2F release and, therefore, selective replication in tumor cells can be achieved by deleting the pRb-binding site of E1A. This has been used in Δ24 and dl922–947 oncolytic adenoviruses.[12,13] However, a constitutive expression of mutated E1A protein in every infected cell causes high

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toxicity by itself and compromises the systemic administration of these viruses for treatment of disseminated cancer. With the aim of overcoming this limitation, the E2F-1 promoter has been used to control E1A transcription because of its E2F addiction.[14] In normal cells, binding of the E2F–pRb complex to the promoter restricts E1A transcription and the viral life cycle is aborted. In cancer cells, E1A transcription is activated by free E2F and a loop of autoactivation is triggered by E1A and E4-6/7, leading to the replication of the virus and lysis of cancer cells.

In this study, we modify the E2F-1 promoter to increase the dependency of virus replication on free E2F. Our group has previously described ICOVIR-5, an oncolytic adenovirus controlling selective replication at different levels: transduction (integrin-mediated adenoviral infection), transcription (insulated E2F-1 promoter, which contains two palindromic E2F-responsive sites, controlling E1A), optimized ribosome recognition (Kozak sequence) and protein–protein interaction (E1A-Δ24 unable to bind pRb). ICOVIR-5 has shown a potent antiglioma effect alone[15] or in combination with chemotherapy[16] and antitumor efficacy in a wide range of subcutaneous tumor models after systemic administration with a low toxicity profile.[17] In this study, we improved the transcriptional control of E1A using an E2F-1 promoter modified by the insertion of four extra E2F-palindrome sites. Viral replication is improved in cancer cells and an important enhancement of antitumoral potency *in vivo* is achieved in all cancer models tested. Importantly, replication in normal cells and toxicity in mice are not compromised by this promoter modification. These results are potentially applicable to improve the selectivity and potency of other oncolytic adenoviruses currently based on promoters controlled by E2F transcription factors or even on promoters without E2F sites.

Results

ICOVIR-7 replication is restricted in normal cells

The palindromic E2F-binding sites located between positions −36 and −6 of the E2F-1 promoter confer the potency and selectivity of this promoter.[7,8] ICOVIR-5 is an oncolytic adenovirus controlling E1A-Δ24 transcription under the E2F-1 promoter.[15–17] To enhance the dual role of E2F on transcription control, an E2F-responsive promoter was constructed by inserting four new E2F-responsive palindromes into the E2F-1 promoter. ICOVIR-7 is a novel oncolytic adenovirus designed to increase selective replication in tumor cells by placing this modified E2F-responsive promoter to control E1A-Δ24 transcription. As E1A expression determines toxicity,[18] a stricter control of E1A transcription may allow the systemic administration of oncolytic adenoviruses for the treatment of disseminated neoplasias. Figure 1a represents how genetic modifications of ICOVIR-7 work together to abrogate replication in normal cells.

Treatment of disseminated cancer requires a systemic injection of the oncolytic agent. As a large proportion of the virus administered systemically ends up in the liver, analysis of virus replication in precision-cut human liver slices is an important tool for estimating liver toxicity.[19] Liver biopsy samples were infected and cell extracts were titrated at day 6 after infection. We reached a complete inhibition of replication with ICOVIR-7, detecting the same progeny production as with the nonreplicative negative control AdTL-RGD (Figure 1b). Replication inhibition with ICOVIR-5 was also complete. Another tissue type relevant in adenoviral toxicity is endothelium. Vascular endothelium injury is reported to be a prominent abnormality after a high-dose injection of adenovirus.[20] Human umbilical vein endothelial cells (HUVECs) were infected and replication after 4 days was analyzed. It was of interest that ICOVIR-7 replicated almost sevenfold less than AdwtRGD and was even more selective than ICOVIR-5, reducing by twofold the replication of this previous version (Figure 1c, $P<0.05$).

Hepatic and hematological toxicity after systemic ICOVIR-7 administration in vivo

To assess *in vivo* systemic toxicity after ICOVIR-7 administration, immunocompetent mice were injected with increasing doses of ICOVIR-7 or control viruses by a single intravenous injection. Animals were weighed daily and liver enzymes (AST and ALT) and hematological parameters were determined at day 5 after injection. For positive control AdwtRGD, samples were collected at day 3 after injection because of much higher toxicity. No casualties were observed after administration of ICOVIRs.

Transaminase elevation is an indicator of liver injury after systemic administration of adenoviruses,[21] and it has been reported after a high-dose injection of oncolytic adenovirus in clinical trials.[22,23] A dose of 5×10^{10} viral particles of AdwtRGD caused up to an 80-fold elevation of both AST and ALT compared with non-treated animals (Figure 2a). In contrast, the injection of the same dose of ICOVIR-7 did not increase transaminase levels significantly. Even at a higher dose of ICOVIR-7 (1×10^{11} viral particles per mouse), only a slight increase in transaminase levels was observed. Hematological alterations are also frequently observed after systemic administration of large doses of adenoviruses.[20,23,24] We observed significant thrombocytopenia and lymphopenia after AdwtRGD injection, but not after ICOVIR-7 (Figures 2b and c). In addition, although intravenous AdwtRGD at 5×10^{10} viral particles decreased the body weight of mice significantly, indicating high toxicity, ICOVIR-7 at the same dose did not differ from phosphate-buffered saline (PBS) in this regard (Figure 2d). A dose of 1×10^{11} viral particles of ICOVIR-5 or ICOVIR-7 per mouse slightly reduced the body weight of mice.

Owing to the inability of human adenoviruses to productively replicate in mouse cells, the main toxicity in murine livers is associated with E1A expression.[18] Strong immunostaining was detected throughout livers from AdwtRGD-treated mice. In contrast, even at the highest dose of ICOVIR-7 (1×10^{11} viral particles per mouse), little E1A was detected, indicating that the E2F-responsive promoter effectively restricts the expression of E1A in liver (Figure 3a). A histological analysis of livers from animals treated with AdwtRGD revealed evident symptoms of degenerative cirrhosis (macrosteatosis, presence of Councilman bodies and large necrotic areas), symptoms that were absent in livers from ICOVIR-7-treated animals (Figure 3b). Importantly, the *in vivo* toxicity profile of ICOVIR-7 was similar to that of ICOVIR-5,

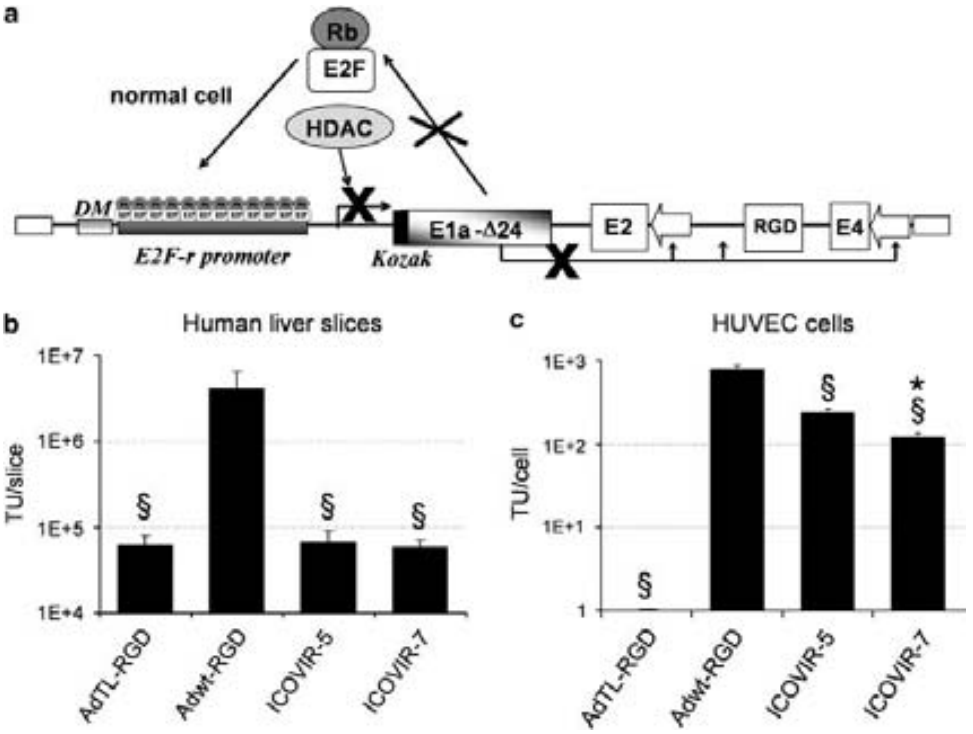


Figure 1 Abrogation of ICOVIR-7 replication in normal cells. (**a**) Diagram of ICOVIR-7 components that repress replication in normal cells. ICOVIR-7 incorporates four extra palindromes of E2F-responsive sites in the E2F-responsive promoter controlling E1A-Δ24. In quiescent cells, the E2F transcription factor forms a complex with functional pRb. The binding of the complex to these hairpins of E2F-responsive sites avoids E1A-Δ24 transcription by the docking of histone deacetylase to the complex. The Δ24-deleted E1A cannot disrupt the pRb–E2F complex, which prevents a vicious circle of autoactivation by E2F in case of promoter leakage. Furthermore, the DM-1 insulator avoids selectivity loss by the endogenous E1A enhancer. (**b**) Viral replication in human liver slices. Human liver slices were infected at 50 transduction units per cell (TU per cell) and viral yield (TU per slice) was determined in slice extracts at day 6 after infection. AdTL-RGD is a replication-deficient control, AdwtRGD is a nonselective positive control, whereas ICOVIR-5 is an older oncolytic virus featuring a prototype E2F-1 promoter for controlling E1A-Δ24 transcription. (**c**) Viral replication in normal human umbilical vein endothelial cells (HUVECs). HUVECs were infected with a nonreplicative virus (AdTL-RGD), with AdwtRGD, ICOVIR-5 or ICOVIR-7 at a multiplicity of infection of 40 and virus production was measured in cell extracts 5 days after infection as indicated in Materials and methods. The means of four independent replicas are shown and two independent experiments were carried out. +s.d. error bars are plotted. §Significant $P<0.05$ by two-tailed unpaired Student's *t*-test, compared with that of the AdwtRGD-infected group. *Significant $P<0.05$ by two-tailed unpaired Student's *t*-test, compared with that of the ICOVIR-5-infected group.

which is a highly selective virus, but also somewhat abrogated in terms of potency in tumor cells.[17]

The E2F-binding hairpins in ICOVIR-7 rescue oncolytic potency in vitro

E2F-responsive sites have a double role in controlling transcription.[5] Besides repressing transcription when E2F is bound to pRb, they also activate transcription when E2F is released, as occurs in cancer cells. Figure 4a depicts the manner in which new E2F-responsive palindromes introduced in the ICOVIR-7 E1A-Δ24-controlling promoter cooperate with the E4-6/7 protein to activate replication and overcome the attenuation that ICOVIR-5 presents in some cancer cell lines. To test oncolytic potency *in vitro*, a panel of tumor cell lines, including lung, cervix, prostate, colon, head and neck carcinoma, melanoma and osteosarcoma, was infected. E1A expression 20 h after infection and virus production 4 days after infection were analyzed for ICOVIR-7 and control viruses.

E1A is the first gene expressed from the adenovirus genome and controls the expression of other virus genes.[25,26] The enhancement of its selective expression in tumor cells may result in an increase of oncolytic potency. In this regard, ICOVIR-7 was able to restore E1A levels similar to AdwtRGD on all tested cancer cell lines, as analyzed by western blot (Figure 4b). A densitometric quantification of E1A bands revealed an increase in E1A levels by 17% in A549, by 152% in Saos-2, by 119% in 1.36.1.5 and by 254% in SCC-25 cells with respect to ICOVIR-5. It is significant that this restoration of expression was more evident in the cancer cell lines in which the E1A expression of ICOVIR-5-infected cells was reduced (Saos-2, 1.36.1.5 and SCC-25).

A near wild-type level of E1A expression is important to maintain an efficient virus replication.[27] ICOVIR-5 progeny production was attenuated in some cancer cell lines and ICOVIR-7 was able to increase its replication in most of them, namely, in Saos-2, Sk-mel28, 1.36.1.5, C33A and Isrec-01 (Figure 4c). Significantly, this improvement was most apparent in Saos-2 and Sk-mel28, in which ICOVIR-5 replication was weakened compared with that of AdwtRGD. In addition, cytopathic effect assays were carried out to determine IC_{50} in different tumor cell lines. The amount of ICOVIR-7 needed to kill 50% of cells was lower in all tumor cell lines tested (Supplementary Table 1). These data indicate that the insertion of

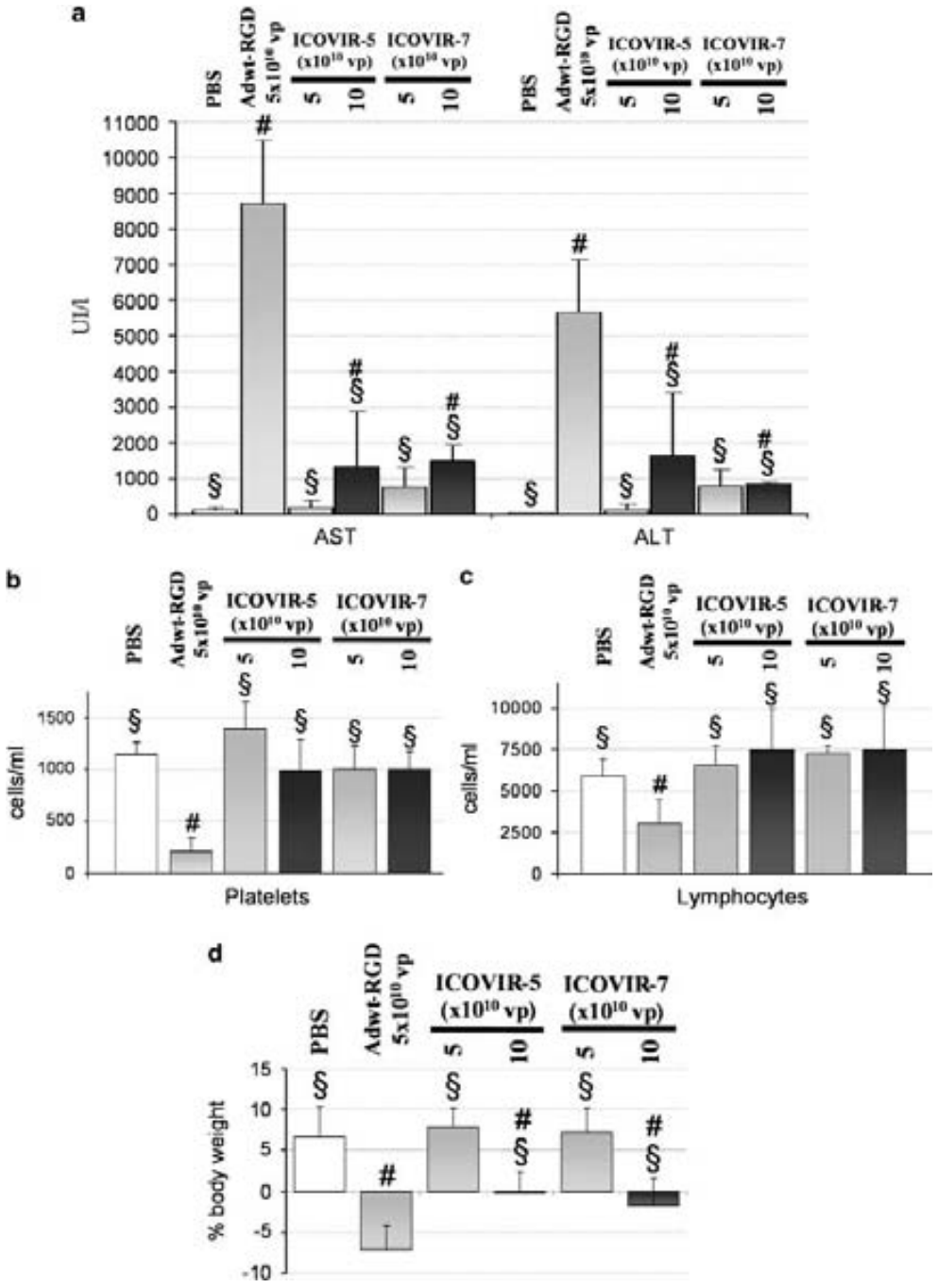


Figure 2 *In vivo* toxicity profile of ICOVIR-7. Body weight variation (**a**) and serum transaminase (**b**), platelet (**c**) and lymphocyte (**d**) concentrations in Balb/C mouse peripheral blood at day 5 after intravenous administration of phosphate-buffered saline (PBS) or ICOVIRs; the AdwtRGD-injected group was analyzed at day 3 after administration because this dose represented more than the LD_{50} at day 4 and it was analyzed only at the lower dose because it is lethal at day 3 after administration with the higher dose. §Significant $P<0.05$ by two-tailed unpaired Student's *t*-test, compared with that of the AdwtRGD group. #Significant $P<0.05$ by two-tailed unpaired Student's *t*-test, compared with that of the PBS group.

additional E2F-responsive sites in E1A-controlling promoter results in improved oncolytic potency *in vitro* and may involve a more efficient antitumor treatment *in vivo*.

Antitumor efficacy of ICOVIR-7 after systemic administration

Subcutaneous models were selected to represent a wide range of tumor types. As disseminated disease is the most relevant clinical situation, mice bearing C33A (cervix), Sk-mel28 (melanoma), NP-18 (pancreatic adenocarcinoma) or PC-3 (prostate) tumors were injected with a single intravenous dose of ICOVIR-7, ICOVIR-5 or PBS. AdwtRGD could not be tested in these experiments because of its high toxicity at treatment doses, as shown in Figure 2.

When animals with C33A tumors were treated with PBS, tumor size at day 32 was 7.3-fold larger than in mice

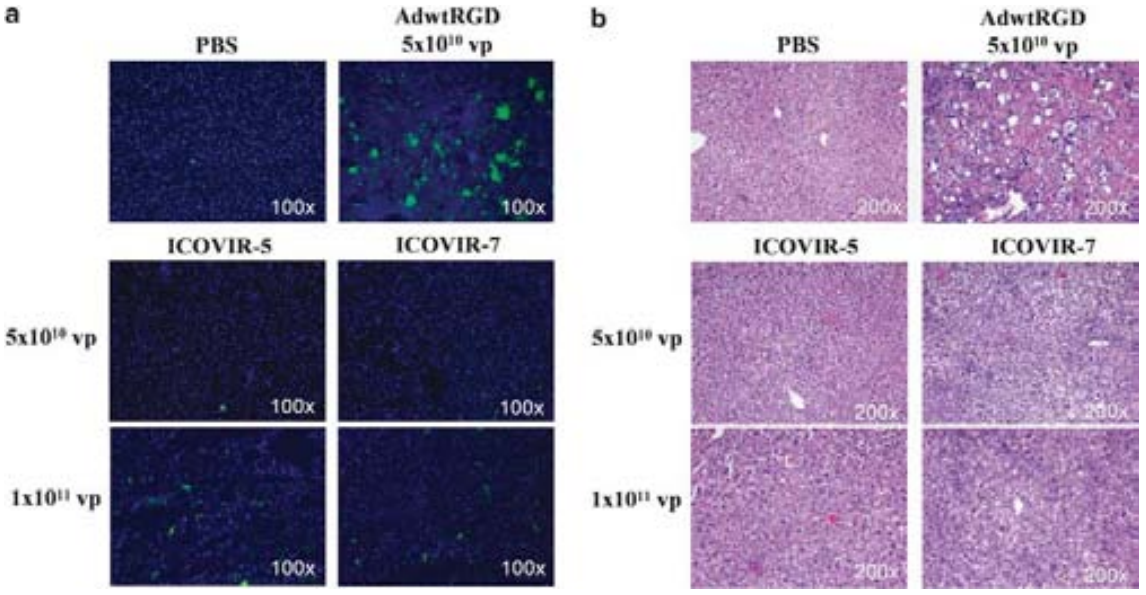


Figure 3 ICOVIR-7 retains the high selectivity of ICOVIR-5 with regard to hepatic toxicity and expression of E1A *in vivo*. Liver E1A expression was assessed by immunohistofluorescence in frozen sections (**a**) and hematoxylin–eosin staining of equivalent paraffin-embedded liver sections was performed (**b**). Mouse livers were collected at day 3 after intravenous administration of phosphate-buffered saline or viruses. Livers from mice injected with ICOVIR-5 or ICOVIR-7 at both doses were close to negative for E1A expression and hepatic toxicity, whereas livers from mice injected at 5×10^{10} viral particles (vp) present intense E1A expression and evident signs of hepatitis, such as macrosteatosis, for the presence of Councilman bodies and large necrotic areas.

treated with ICOVIR-7 ($P < 0.05$) (Figure 5a). Furthermore, ICOVIR-7 seemed more effective than ICOVIR-5, as tumor size was 1.75-fold larger in the latter group (not significant). In mice with Sk-mel28 tumors, tumor size in PBS-treated mice was 2.6-fold higher than in the ICOVIR-7 group ($P < 0.0009$) (Figure 5b). Moreover, tumors in mice treated with ICOVIR-5 were 1.7-fold larger than those in mice treated with ICOVIR-7 ($P < 0.05$). In mice bearing NP-18 tumors, an aggressive model of pancreatic adenocarcinoma, tumor size in PBS-treated animals was twofold larger than that in the ICOVIR-7 group ($P < 0.00002$) (Figure 5c). Tumors treated with ICOVIR-5 at the end of the experiment were 1.5-fold larger than those treated with ICOVIR-7 ($P < 0.01$). In mice with PC-3 xenografts, a single intravenous treatment was not as effective as in the other two models perhaps because of higher amounts of hyaluronic acid and other matrix components.[28,29] However, tumors in PBS-treated animals were nevertheless 1.7-fold larger than in ICOVIR-7-treated animals ($P < 0.05$) (Figure 5d) and tumors in ICOVIR-5-treated animals were 1.2-fold bigger than those in the ICOVIR-7 group (not significant).

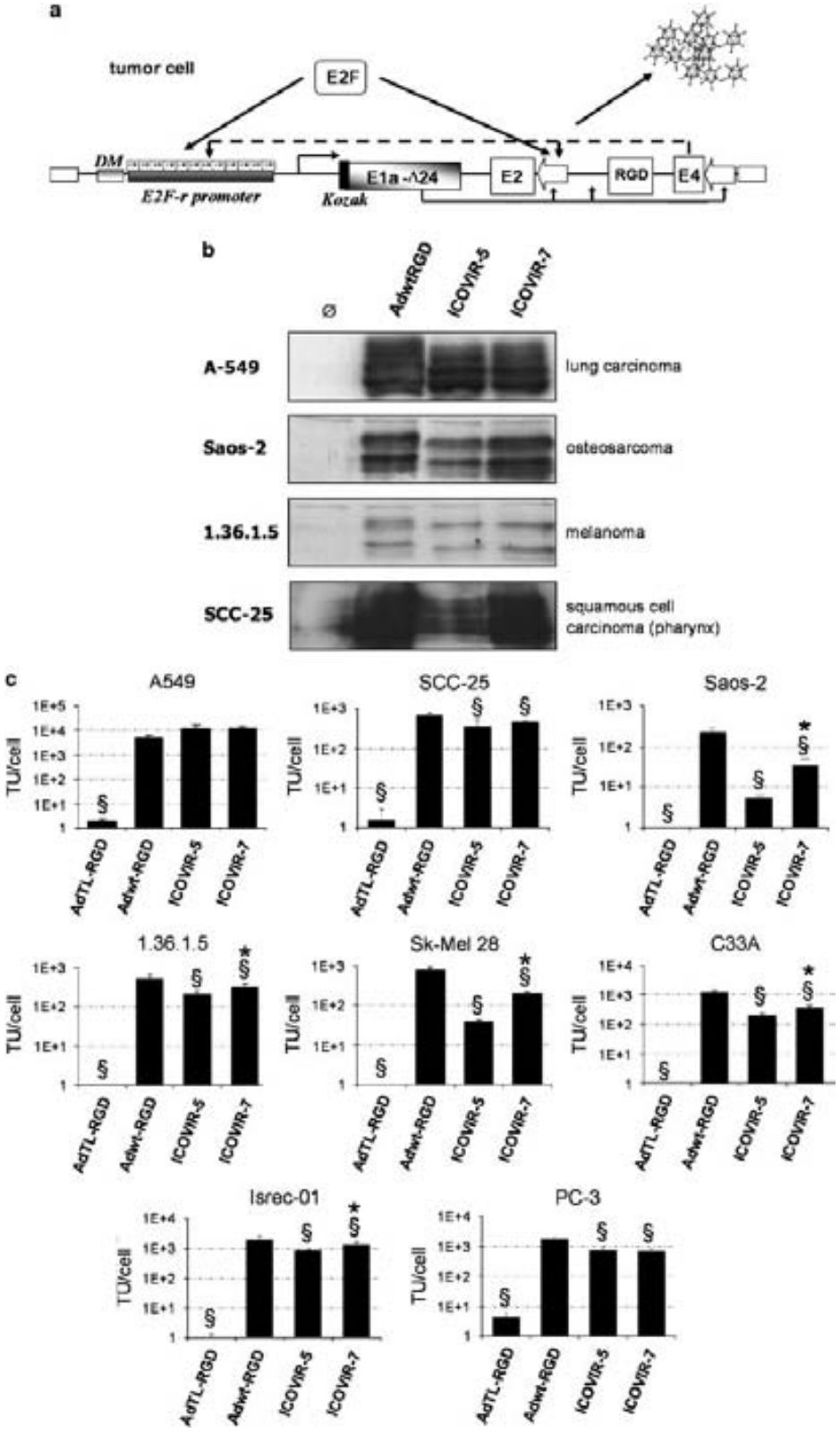
Viral replication in tumors after systemic injection of ICOVIR-7 was shown by immunohistofluorescence for adenoviral capsid proteins. Tumors were collected at day 25 after injection. No capsid proteins were observed in untreated tumors, whereas positive cells were observed in ICOVIR-7-treated tumors (Figure 5e).

Discussion

Treating tumors at an advanced stage requires systemic delivery. Clinical data with oncolytic adenoviruses injected systemically indicate the need for more potent and selective viruses. Those tested in patients have proven to be relatively safe, showing evidence of efficacy.[22,30–32] However, some potentially concerning adverse effects were observed at high doses and most responses detected were transient, suggesting the need for enhancing efficacy. Therefore, further research is required to improve selectivity and, above all, potency of these viruses.

Tumors cells are characterized by presenting defects in the Rb pathway, which inhibit pRb binding to E2F.[4] An oncolytic adenovirus that takes advantage of this deregulation may acquire a selective replication for tumor cells. The first modification aiming conditional replication in Rb pathway-deficient cells was the Δ24 (dl922–947) deletion.[12,13] This modification consists of deleting the pRb-binding site of the conserved region 2 (CR2) of E1A proteins. E1A proteins, besides being the first viral proteins synthesized from the adenovirus genome and capable of activating the transcription of other virus genes, activate the cell cycle through binding to the pRb, p130 and p107 family of proteins.[33] The formation of this complex releases E2F from pRb to allow the transcription activation of viral and cellular genes containing E2F-responsive sites. As the Rb pathway is dysfunctional in virtually all tumor cells,[4] E1A binding to pRb is not necessary in malignant cells. Therefore, Δ24 deletion results in attenuated replication in normal cells while preserving oncolytic potency. Furthermore, a virus combining this deletion with the insertion of an RGD motif at the HI loop of the fiber has shown improved oncolytic potency.[34] Despite this inability to release E2F, hepatic toxicity is observed when this virus is injected systemically, as E1A transcription is not regulated and takes place in normal cells.[18] For the systemic treatment of disseminated cancer, E1A transcription control is required. In this regard, the E2F-1 promoter has previously been used to direct E1A transcription in response to Rb pathway deregulation.[35–37] Among

several cellular E2F-responsive promoters, E2F-1 is a popular choice because of its efficient E2F-binding structure, which consists of four binding sites organized as two imperfect palindromes.[7,8] Besides the E2F release by E1A, the adenoviral E4-6/7 protein has also evolved to increase free E2F levels. This protein interacts directly with different members of the E2F family and mediates the stable binding and activation of the E2F-1 promoter.[11] As a result, we reach an efficient and important positive feedback loop in tumor cells when this promoter is



placed to control E1A transcription. Furthermore, the loop is increased by the presence of a similar E2F-binding structure in the E2a promoter of adenovirus 5.[38] Previously, our group has shown the importance of combining Δ24 deletion with an insulated E2F-1 promoter that controls E1A transcription as a safety measure in case of promoter leakage.[27] The binding of zinc-finger protein CTCF to the insulator DM-1 prevents transcription from E1A enhancers and the Δ24 deletion abrogates a feedback loop in normal cells. Furthermore, we also showed that oncolytic potency can be enhanced by using the optimal ribosome recognition sequence upstream of E1A.[17]

Taking into consideration the sophisticated cooperation of E1A-Δ24 and E4-6/7 with the unique structure of E2F sites present in E2F-1 promoter to activate/repress replication, we decided to magnify this mechanism by multiplying the number of these unique structures in the E1A-controlling promoter. Thus, ICOVIR-7 incorporates eight new E2F-binding sites organized as four palindromes in addition to the endogenous ones present in the wild-type E2F-1 promoter. The new sites were inserted upstream of CAAT boxes to avoid interfering on transcription initiation. As a result, higher levels of free E2F binding may increase E1A transcription in cancer cells. This may lead to a higher activation of viral promoters, resulting in higher levels of the E4-6/7 protein and activating a potent loop of E1A and E2a production that may culminate in a more efficient production of virus in cancer cells. Our results corrobo-

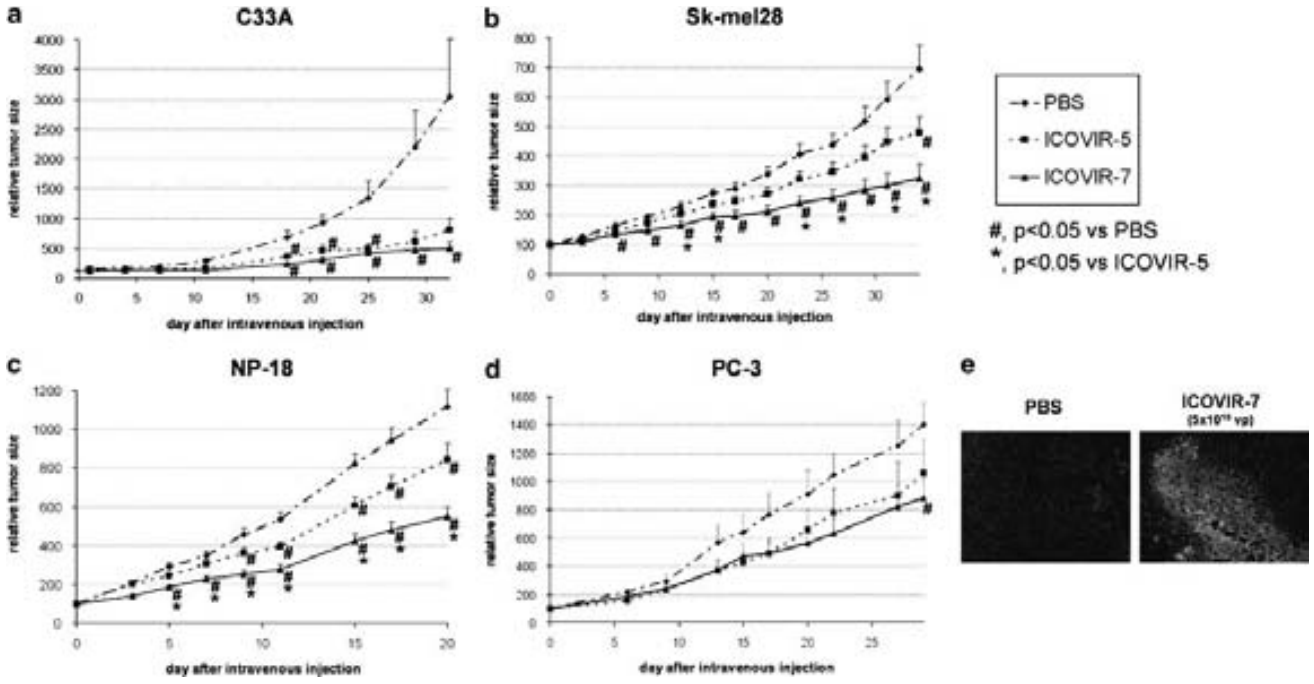


Figure 5 Systemic antitumor efficacy *in vivo*. Nude mice with subcutaneous xenografts (~100 mm³) of C33A (**a**), Sk-mel28 (**b**), NP-18 (**c**) or PC-3 (**d**) were randomized into groups and treated with a single intravenous dose of phosphate-buffered saline (PBS) (solid diamonds), ICOVIR-5 (solid squares) or ICOVIR-7 (solid triangles). $n = 10$–16 tumors per group. Dose is 1×10^{11} viral particles (vp) in panel **a** and 5×10^{10} vp in panels **b**–**d**). The percentage of tumor growth ± s.e. is plotted. #Significant $P < 0.05$ by two-tailed unpaired Student's *t*-test compared with that of mice injected with PBS. *Significant $P < 0.05$ by two-tailed unpaired Student's *t*-test compared with that of mice injected with ICOVIR-5. (**e**) Detection of ICOVIR-7 replication in Sk-mel28 tumors at day 25 after intravenous administration. Antiadenovirus immunohistofluorescence was carried out and counterstained with 4′,6-diamino-2-phenylindole to reveal replication of ICOVIR-7 throughout the treated tumors.

Figure 4 Potency of ICOVIR-7 in tumor cells. (**a**) Mechanism of activation of ICOVIR-7 replication in tumor cells. Deregulation of the pRb pathway releases the E2F transcription factor, which activates E1A-Δ24 transcription by binding to E2F-palindrome sites present in the E2F-responsive promoter of ICOVIR-7. The Kozak sequence increases E1A-Δ24 translation efficacy, which activates the other adenoviral promoters. The E4-6/7 protein forms a complex with E2F and increases the transcription of E1A-Δ24 and E2 in an efficient circle of autoactivation and lysis of cancer cells. The RGD-modified fiber increases virus infectivity. (**b**) The modified E2F-responsive promoter of ICOVIR-7 increases E1A expression in tumor cell lines in comparison with the prototype promoter in ICOVIR-5. Anti-E1A western blots were performed on cell extracts obtained 24 h after infection with a dose of each virus, which allowed more than 80% of transduction (40 multiplicity of infection (MOI) for SCC-25; 20 MOI for Saos-2 and 1.36.1.5; and 10 MOI for A549 cells). (**c**) Replication of ICOVIR-7 in tumor cells. Different tumor cell lines were infected as indicated in (**b**) or with an MOI of 20 for Sk-mel28, Isrec-01 and PC-3 cells and with an MOI of 10 for C33A cells. Virus production was measured 4 days after infection as indicated in Materials and methods. A previous infection in 293 cells (which allows E1a-independent adenoviral replication) was used to normalize the different virus preparations. Viral yield was evaluated in quadruplicate for each cell line, by carrying out two independent experiments. +s.d. error bars are plotted. §Significant $P < 0.05$ by two-tailed unpaired Student's *t*-test, compared with that of the AdwtRGD group. *$P < 0.02$ versus that of the ICOVIR-5-infected group according to a two-tailed unpaired Student's *t*-test.

rate this hypothesis. E1A production was enhanced in all cancer lines tested (Figure 4b) and this had a positive effect on virus production, increasing the number of virions produced by most of the tumor cell lines tested (Figure 4c). Importantly, this enhancement was maintained when viruses were tested in subcutaneous mouse models. A substantial benefit in the control of tumor growth was observed after a systemic injection of ICOVIR-7 compared with ICOVIR-5 (Figures 5a–d). Therefore, our data suggest that high levels of E1A are important to maintain oncolytic potency in all tumor cell models, contrary to some reports in which reduced E1A expression had little effect on replication.[39,40]

Theoretically, the new E2F-binding sites may attach a higher number of E2F–pRb–histone deacetylase complexes in normal cells and this may strengthen the association of nucleosomes with DNA, increasing transcription repression.[5] However, our results indicate that selectivities of ICOVIR-5 and ICOVIR-7 are similar. Liver (Figures 1b, 2a and 3) and hematological toxicity (Figures 2b and c) is not reduced by this promoter modification. This is probably because of the fact that the genetic combination present in ICOVIR-5 already efficiently restricts replication in normal cells when injected systemically, showing selectivity levels difficult to improve. This is clearly observed in Figure 1b, in which replication of both ICOVIRs in liver slices was similar to that of a nonreplicating virus (AdTL-RGD), indicating the detection of remaining viruses and discarding replication in that model. After the administration of adenoviruses, there is an induction of two distinct peaks of inflammatory response occurring at 6 h and 5 days.[41] The early phase is capsid mediated and the second peak is dependent on the transcription of viral proteins. The low levels of E1A expression in the liver driven by both ICOVIRs (Figure 1a) and previous toxicity experiments injecting 1×10^{11} UV-psoralen-inactivated viral particles (data not shown) suggest that the transaminase elevation and the slight loss of weight observed after injection of 1×10^{11} viral particles of ICOVIRs are probably because of the toxicity caused by adenoviral capsids, not by E1A. In addition, replication in HUVECs (Figure 1c) shows an advantage of ICOVIR-7 in terms of selectivity, but cultured cells arrested by serum deprivation are less reliable as a normal cell model because of the difficulty to be completely arrested. In fact, we detected proliferation markers (cyclin E and Ki-67) in HUVECs or in human hepatocyte extracts by western blot analysis (data not shown), even after serum deprivation. This proliferating status might explain the small difference in terms of replication in HUVECs between wild-type virus and ICOVIRs. Importantly, western blot analysis did not reveal detection for proliferation markers in human liver slice extracts. Thus, we can conclude that the genetic combination present in the ICOVIR backbone properly controls E1A-mediated toxicity.

Despite the potential enhancement of antitumor potency, complete regression of tumors was not achieved in mice models. Tumor masses, besides cancer cells, are composed of stromal areas, including fibroblasts, endothelial and immune cells and matrix components such as collagen or hyaluronan acid. Many tumor types are characterized by small groups of tumor cells surrounded by large areas of stroma and this may limit the intratumoral spread of viruses, especially when replication is restricted to cancer cells.[42,43] Figure 5d shows how the use of a xenograft model (PC-3) expressing high amounts of matrix components[28,29] reduces the efficacy of ICOVIR-7 with respect to other models. Arming adenoviruses with therapeutic transgenes is a rational approach to circumvent these barriers. The coadministration of soluble hyaluronidase with oncolytic adenoviruses[44] or the expression of relaxin in the context of an oncolytic adenovirus[45] seems promising. Alternatively, the use of prodrug-converting enzymes may represent a useful tool to breach endothelial and fibroblastic cell barriers.[46–48] Thus, incorporation of different transgenes into the ICOVIR-7 backbone is under consideration. Restriction of the expression of these transgenes in normal cells and their compatibility with the viral life cycle are important issues to be considered. Late expression has been postulated as a good solution to avoid such problems, as major late promoter transcription is subjugated to E1A expression.[49] The tight control of E1A exerted by the ICOVIR-7 backbone implies that incorporation of a transgene expressed late in the viral cycle may contribute to tumor eradication without increasing toxicity in normal cells.

In summary, our data point toward ICOVIR-7 being a potent oncolytic agent against a broad range of tumors that present limited toxicity when injected systemically. However, further research needs to be carried out to achieve total tumor regression with a single intravenous adenovirus dose.

Materials and methods

Cell culture

HEK293 (human embryonic kidney cells), A549 (human lung adenocarcinoma), SkMel-28 (melanoma), SCC-25 (head and neck squamous carcinoma), Saos-2 (osteosarcoma) and C33A (cervix carcinoma) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). NP-18 (pancreatic adenocarcinoma) was obtained from our laboratory.[50] The 1.36.1.5 (a clone of SkMel-131, melanoma) cell line was a kind gift from FX Real (Institut Municipal d'Investigació Mèdica, Barcelona, Spain). Isrec-01 (colon cancer cell line) was a kind gift from R Iggo (University of St Andrews, Scotland, UK). HUVECs (human umbilical vein endothelial cells) were purchased from Cambrex Bio Science Baltimore Inc. (Baltimore, MD, USA). All tumor cell lines, excluding Isrec-01, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum at 37 °C, 5% CO_2. The DMEM of Isrec-01 cells was supplemented with 10% fetal bovine serum. HUVECs were cultured in endothelial cell medium.

Viruses

AdwtRGD, AdTL-RGD and ICOVIR-5 have been previously described.[17,51,52] All these viruses were propagated in A549 cells, excluding the replication-deficient AdTL-RGD, which was propagated in HEK293 cells. ICOVIR-7 was created by inserting two E2F-binding site hairpins in the E2F-1 promoter of ICOVIR-5. To this aim, a unique *BsiWI* site was created in the E2F-1 promoter of pGEM-E2F-K-E1A-Δ24 by site-directed mutagenesis. Using this plasmid digested with *BsiWI* and oligonucleo-

tides E2FF2 (5′-GTACGTCGGCGGCTCGTGGCTCTTT CGCGCAAAAAGGATTTGGCGCGTAAAAGTGGTTCGA A-3′) and E2FR2 (5′-GTACTTCGAACCACTTTTACGCG CCAAATCCTTTTTGCCGCGAAAGAGCCACGAGCCG CCGAC-3′) annealed, pGEM-E2F3-K-E1A-Δ24 was created. The *KpnI* fragment containing the modified version of the E2F-1 promoter was inserted into pShuttle-DM-E2F-K-E1A-Δ24 to generate pShuttle-DM-E2F3-K-E1A-Δ24. Finally, this plasmid was recombined with pVK503 by homologous recombination in *Escherichia coli BJ* to construct pICOVIR-7. ICOVIR-7 was obtained by transfection of the *PacI* fragment of pICOVIR-7 in HEK293 cells. The virus was plaque purified, amplified in A549 cells and purified using a CsCl gradient. The viral genome was verified by restriction analysis and by sequencing the DM-1 insulator, E2F-1 promoter, Kozak sequence, E1A-Δ24 and RGD-modified fiber using oligonucleotides oligo22 (5′-AAGTGTGATGTTGCAAG TGT-3′), KozakR (5′-CTGGCGGCCATTCTTCGGTAATA ACACCTCCGTGGCAGATAATATGT-3′), 1R (5′-CCTCC GGTGATAATGACAAG-3′) and FiberUp (5′-CAAACGC TGTTGGATTTATG-3′).

Human liver slice preparation

Human liver samples were obtained from the Department of Surgery of the Hospital Universitari de Bellvitge (L'Hospitalet de Llobregat, Spain), with approval from the Research Ethics Committee. Liver slices were prepared using a Krumdieck Tissue Slicer (Alabama R&D, Munford, AL, USA) as described.[53,19] The slices were washed and incubated for 2 h before infection at 37 °C with Williams' E medium saturated with 95% O_2/ 5% CO_2 and supplemented with 10% fetal bovine serum, insulin (0.1 μM) and D-glucose (25 mM).

E1A protein expression analysis

Cell cultures ($\sim 2 \times 10^5$ cells seeded in 6-well plates) were infected at a multiplicity of infection that allowed at least 80% infectivity (multiplicity of infection (MOI) of 40 for SCC25 cells, MOI of 20 for 1.36.1.5 and Saos-2 cells and MOI of 10 for A549 cells). Whole-cell protein extracts were prepared 24 h after infection by incubation in lysis buffer (400 mM NaCl, 1 mM EDTA, 5 mM NaF, 10% glycerol, 1 mM sodium orthovanadate, 0.5% Nonidet NP-40 and a mixture of protease inhibitors (Sigma, St Louis, MO, USA) in 10 mM Tris–HCl pH 7.4) for 1 h at 4 °C. Clarified samples (15 μg per lane) were separated by a 10% SDS polyacrylamide gel electrophoresis and were transferred to a nitrocellulose membrane (GE Healthcare, Arlington Heights, IL, USA). Detection was carried out by immunoblotting membranes using a polyclonal anti-E1A primary antibody (Rabbit, Clone 13S-5) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a polyclonal anti-rabbit conjugated with horseradish peroxidase (goat, DakoCytomation, Glostrup, Denmark). E1A bands from western blot were quantified using a BIO-RAD GS-800 densitometer (Bio-Rad, Hercules, CA, USA).

Virus progeny production

Cell cultures ($\sim 2 \times 10^5$ cells seeded in 6-well plates) were infected at an MOI that allowed at least 80% infectivity (MOI of 40 for SCC25 and HUVECs, MOI of 20 for Skmel28, PC-3, Isrec-01, 1.36.1.5 and Saos-2 cells and MOI of 10 for C33A and A549 cells). Human liver slices were infected at an MOI of 50, with an estimated cell number of 1×10^6 cells per slice on the basis of a 10-cell-thick slice (~ 250 μm) and an 8-mm-slice diameter.[19] At 2 h after infection, cultures were washed twice and incubated in a fresh virus-free medium. At day 4 after infection for cell cultures or at day 6 for human liver slices, cells and medium were harvested together and freeze thawed thrice. Viral titers were determined by an antihexon staining-based method.[27]

In vivo *toxicity study*

Mice for toxicological and efficacy studies were maintained in the facility of the Institut de Recerca Oncològica-IDIBELL (Barcelona), AAALAC unit 1155. All animal studies were approved by the Institut d'Investigacio Biomedica de Bellvitge Ethical Committee for Animal Experimentation. Purified viral particles (5×10^{10} or 1×10^{11}) were injected intravenously into the tail vein of 6-week-old immunocompetent Balb/C male mice in a volume of 10 ml kg^{-1} in PBS ($n = 5$). Daily observations for body weight, morbidity and moribundity were carried out. At day 3 after injection for AdwtRGD-injected mice and at day 5 after injection for ICOVIRs- and PBS-treated animals, the mice were killed and different samples were collected. Blood samples were collected by intracardiac punction, and clinical biochemical and hematological determinations were carried out by the Clinical Biochemistry and Hematological Services of the Veterinary Faculty at the Autonomous University of Barcelona. The significance of differences in biochemical and hematological rates between treatment groups was assessed by a two-tailed Student's unpaired *t*-test. Mice livers were resected and portions were fixed in 4% formaldehyde for 24 h at room temperature (for paraffin embedding and further hematoxylin–eosin staining) or frozen in OCT. E1A-immunodetection was performed by incubating OCT-embedded liver sections with a primary polyclonal antibody antiadenovirus-2 E1A (clone 13 S-5, Santa Cruz Biotechnology) and an AlexaFluor 488-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA). Slides were counterstained with 4′,6-diamino-2-phenylindole and visualized under a fluorescent microscope (Olympus BX51, Hamburg, Germany).

In vivo *antitumoral efficacy*

Subcutaneous C33A cervical, SkMel-28 melanoma, NP-18 pancreatic adenocarcinoma or PC-3 prostate carcinoma tumors were infused by injection of 1×10^7 cells into the flanks of 6-week-old male Balb/C *nu/nu* mice. When tumors reached 100 mm^3 (experimental day 0), mice were randomized ($n = 10$–16 per group) and were injected by a single intravenous injection of PBS, with 5×10^{10} (for SkMel-28, NP-18 and PC-3 model) or 1×10^{11} (for C33A model) viral particles of ICOVIR-5 or with 5×10^{10} (for SkMel-28, NP-18 and PC-3 model) or 1×10^{11} (for C33A model) viral particles of ICOVIR-7 in a volume of 10 ml kg^{-1} in PBS through the tail vein. Tumor size and mice status were monitored thrice a week. Tumor volume was defined by the equation V (mm^3) = $\pi/6 \times W \times L^2$, where W and L are the width and length of the tumor, respectively. The percentage of growth was calculated as $((V - V_0)/ V_0) \times 100$, where V_0 is the tumor volume on day 0. The significance of differences in tumor growth rate between treatment groups was assessed by a two-tailed Student's unpaired *t*-test.

OCT-embedded sections of tumors obtained at day 25 after treatment were used to detect adenoviral proteins. Immunodetection was performed using polyclonal anti-adenovirus (ab6982; Abcam, Cambridge, UK) as the primary antibody and an AlexaFluor 488-labeled goat anti-rabbit antibody (Molecular Probes). Rabbit immunoglobulin G was used as control. Slides were counterstained with 4′,6-diamino-2-phenylindole and visualized under a fluorescent microscope (Olympus BX51). Representative photographs of each slide were taken.

Conflict of interest

The authors declare no conflict of interest.

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Verapamil Enhances the Antitumoral Efficacy of Oncolytic Adenoviruses

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The therapeutic potential of oncolytic adenoviruses is limited by the rate of adenovirus release. Based on the observation that several viruses induce cell death and progeny release by disrupting intracellular calcium homeostasis, we hypothesized that the alteration in intracellular calcium concentration induced by verapamil could improve the rate of virus release and spread, eventually enhancing the antitumoral activity of oncolytic adenoviruses. Our results indicate that verapamil substantially enhanced the release of adenovirus from a variety of cell types resulting in an improved cell-to-cell spread and cytotoxicity. Furthermore, the combination of the systemic administration of an oncolytic adenovirus (ICOVIR-5) with verapamil *in vivo* greatly improved its antitumoral activity in two different tumor xenograft models without affecting the selectivity of this virus. Overall, our findings indicate that verapamil provides a new, safe, and versatile way to improve the antitumoral potency of oncolytic adenoviruses in the clinical setting.

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INTRODUCTION

Conditionally replicative adenoviruses hold promise for the treatment of cancer.[1] Their selective replication in tumor cells and consequent lysis and progeny release allows the amplification of the virus and lateral spread to neighboring tumor cells. However, certain limitations encountered by adenovirus during systemic administration and in the tumor make the achievement of systemic antitumoral efficacy challenging. Adenovirus is quickly eliminated from the bloodstream following systemic administration,[2] and once in the tumor, oncolytic adenoviruses face physical barriers imposed by the tumor stroma and the recruitment of an antiviral immune response, which may hinder the spread of the antitumor activity.[3] In this particular environment, the improvement of the rate of adenovirus spread is critical to allow the progression of the oncolytic effect.[4]

Strategies to increase systemic oncolytic adenovirus therapy by improving their spread include the expression of proteases,[5] which disrupt connective tissue, or fusogenic proteins.[6] However, the insertion of transgenes into the adenovirus genome is limited by their size and requires transgene compatibility with the adenovirus replication cycle. Alternatively, specific point mutations or deletions in E1B-19K,[7] overexpression of the adenovirus death protein (ADP),[8] or c-truncating mutations in the i-leader protein[9,10] have been described to improve the cell-to-cell spread *in vitro* and enhance its therapeutic potential. Despite the advantages of these approaches, the insertion of these modifications still requires the genetic manipulation of the adenovirus genome. In addition, several side effects of some spread-enhancing mutations, such as virus yield reduction[11] or partial loss of the E3 immunomodulatory functions,[12] may be undesirable in the context of oncolytic adenoviruses. Therefore, a drug capable of increasing the spread of adenovirus without affecting other viral functions would be an attractive alternative.

Using an *in vivo* bioselection approach, we recently isolated a c-terminal mutation in the E3/19K protein, which enhanced the release of adenovirus from the infected cell and improved its antitumoral efficacy,[13] suggesting that the intratumoral spread of adenovirus is limited by the natural rate of adenovirus release. This process is rather inefficient and it does not take place until late times after infection when ADP accumulates to actively promote progeny release.[14] Although the mechanism of adenovirus cell lysis and progeny release is not well understood, several hypothesis suggest that ADP may function by modifying intracellular calcium pools.[14,15] Moreover, a growing body of evidence suggests that different viruses induce cell death and progeny release by altering intracellular calcium concentration.[16,17] Indeed, the c-terminal E3/19K mutation we had previously identified enhanced virus release by disrupting intracellular calcium homeostasis.[13] Based on these observations, we hypothesized that verapamil, a calcium channel blocker, could improve the rate of virus release and spread, eventually enhancing the antitumoral activity of oncolytic adenoviruses.

This study was designed to determine the effect of verapamil on adenovirus spread *in vitro* and to study the selectivity and antitumoral potency of an oncolytic adenovirus, such as ICOVIR-5 (refs. 18,19), in combination with verapamil *in vivo*. Our results demonstrate that verapamil substantially enhances the release of adenovirus in a variety of cell types, resulting in an improved cell-to-cell spread and cytotoxicity without affecting adenovirus replication or native gene expression. *In vivo*, the selectivity profile of ICOVIR-5 was maintained following treatment with verapamil,

RA and MC contributed equally to this work.

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and the antitumoral potency of this oncolytic virus was greatly enhanced in different tumor models. Overall, our findings indicate that verapamil provides a new, safe, and versatile way to improve the antitumoral potency of oncolytic adenoviruses in the clinical setting.

RESULTS

Verapamil enhances virus release and cytotoxicity of adenovirus type 5 *in vitro*

Due to the importance of Ca^{2+} modulation during virus-induced cell death and progeny release,[17,20] and in order to evaluate whether verapamil, a calcium blocking agent, was able to increase the release of adenovirus serotype 5 (Ad5), we assessed the effect of verapamil on the kinetics of virus release and production in lung adenocarcinoma cell line A549. Despite the total yield was the same both in the presence and the absence of verapamil, there was a 30-fold increase in Ad5 release at 40 hours postinfection (p.i.) when combined with verapamil (**Figure 1a**). Addition of verapamil also resulted in an accelerated rate of Ad5 release from human carcinoma–associated fibroblasts CAF1, SkMel-28 melanoma, and NP-9 pancreatic tumor cell lines (**Figure 1b**). The early release improved the cell-to-cell spread of Ad5 as demonstrated by the large plaque size in A549 monolayers. In the presence of verapamil, the plaques of Ad5 appeared earlier and were bigger than the control plaques (**Figure 1c**). In addition, the combination with verapamil rendered Ad5 more cytotoxic because the amount of Ad5 required to cause a reduction of 50% in cell viability (IC_{50}) was 4 times, 3 times, and up to 100 times lower in SkMel-28, NP-9, and A549 cells, respectively, when combined with verapamil (**Figure 1d**).

Verapamil does not alter viral gene expression and enhances the release regardless of ADP levels

In order to study the effects of verapamil on the viral cycle of Ad5, we analyzed adenovirus early and late protein expression in the presence of verapamil. There were no differences in the pattern of E1A, E3/19K, or late L4, and fiber protein expression when combining Ad5 with verapamil, proving that verapamil had no effect on adenovirus protein expression (**Figure 2a**).

Although the exact mechanism that triggers adenovirus release is unknown, ADP plays a major role in this process because ADP mutant viruses display a defect in virus release without affecting the total viral production.[14] In order to evaluate the ADP dependence of the phenotype of Ad5 in the presence of verapamil, we analyzed

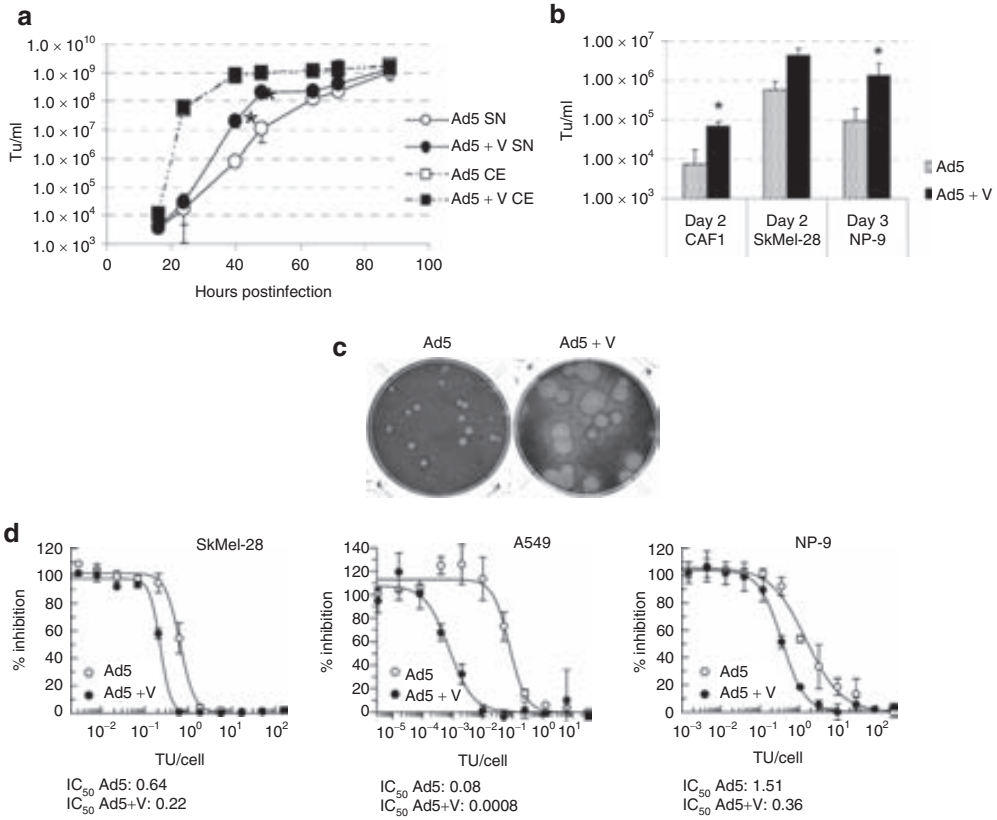

Figure 1 Verapamil enhances the release, spread, and cytotoxicity of Ad5 *in vitro*. (**a**) Viral production and release kinetics of Ad5 combined with 40 µmol/l verapamil in A549 cells. Viral content of the total (CE) and extracellular (SN) fractions were analyzed at the indicated time points. Mean values (n = 3) ± SD are plotted.*Significant ($P = 8.1 \times 10^{-5}$ and $P = 0.03$ at 48 and 64 hours postinfection, respectively) compared to SN of Ad5. (**b**) Viral release of Ad5 in combination with verapamil in CAF1, SkMel-28, and NP-9 cells. The time point at which the biggest difference in virus release was observed is shown. *Significant ($P = 0.015$ for CAF1 and $P = 0.04$ for NP-9) compared to the release of Ad5. (**c**) Plaque size of Ad5 in the presence of verapamil (30 µmol/l final concentration) in A549 cells at day 7 postinfection. (**d**) Comparative cytotoxicity of Ad5 ± verapamil in SkMel-28, A549, and NP-9 tumor cell lines. IC_{50} values (TU/cell of Ad5 required to cause a reduction of 50% in cell viability) for each condition are shown. TU, transducing units.

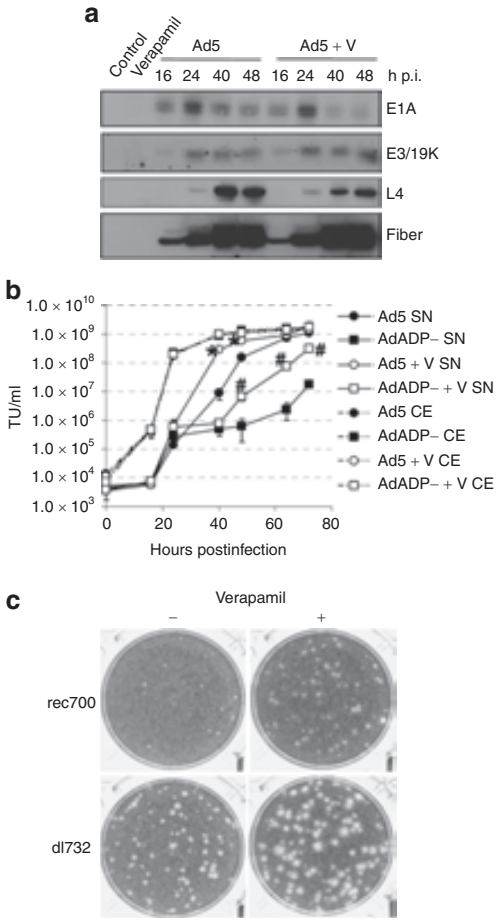

Figure 2 Effect of verapamil on viral protein expression and dependence on ADP. (**a**) Verapamil treatment does not modify adenovirus early and late protein expression pattern. A549 cells were infected with Ad5 with or without verapamil in the extracellular medium, and expression of E1A, E3/19K, L4, and fiber proteins was analyzed at 16, 24, 40, and 48 hours postinfection. (**b**) Viral release kinetics of Ad5 and AdADP⁻ in A549 cells treated with verapamil. Supernatant viral content was quantified at the indicated time points. Mean values (n = 3) ± SD are plotted. *Significant (P = 0.005 and P = 0.001 at 40 and 48 hours p.i., respectively) compared to the release of Ad5 and #significant (P = 0.03, P = 0.003, and P = 0.02 at 48, 64, and 72 hours p.i., respectively) compared to the release of AdADP⁻. (**c**) Comparative plaque size of rec700 and *dl*732 in the presence and absence of verapamil in A549 cells at day 6 p.i. ADP, adenovirus death protein; p.i., postinfection.

the virus release of AdADP⁻ (a virus that expresses a truncated form of the ADP protein that partially retains certain functions of the native form but is defective in promoting cell lysis), alone or combined with verapamil. As expected, AdADP⁻ displayed an impaired release compared to Ad5 due to the deletion of residues near the NH2 terminus of the protein that have been suggested to be important for its transport and stability[21] (**Figure 2b**). Interestingly, incubation of AdADP⁻-infected cells with verapamil also enhanced the release of this defective mutant (**Figure 2b**) as well as its plaque size (data not shown). Although the increase in the release of AdADP⁻ in combination with verapamil was delayed compared to Ad5 (64 hours p.i. for AdADP⁻ versus 40 hours p.i. for Ad5), the presence of the calcium blocking agent increased the release of both viruses to the same extent (~30-fold) (**Figure 2b**). This suggests that verapamil triggers a new pathway that results in the improved release of adenovirus and does not require the cell lysis–promoting function of ADP.

ADP overexpression has also been described to improve the spread of adenovirus and render it more cytotoxic.[15] Because the effect of verapamil on adenovirus release was independent of ADP, we combined a mutant with ADP overexpression, *dl*732, with verapamil in order to evaluate whether it could further enhance the release of this virus. The addition of verapamil in a plaque assay in A549 cells resulted in an even greater plaque size of this mutant that demonstrated that the effects of ADP overexpression and verapamil on adenovirus release were additive (**Figure 2c**).

The early release of Ad5 depends on the calcium channel blocking activity of verapamil

Verapamil is a calcium channel blocker that belongs to the family of phenylalkylamines which as well as the two other types of calcium channel blockers (dihydropyridines and benzothiazepines) inhibits the influx of calcium through passive "slow" channels.[22] In order to study whether the calcium blocking activity of verapamil was causing the fast rate of virus release, we assessed extracellular Ad5 levels in the presence of different calcium channel blocking agents: amlodipine (dihydropyridine) and diltiazem (benzothiazepine). Similar to the effect observed with verapamil, Ad5 displayed a 15-fold increase in virus release at 40 hours p.i. and a large plaque size when combined with both amlodipine and diltiazem in A549 cells (**Figure 3a,b**). Viral release and production in the absence of calcium in the extracellular medium provided further evidence concerning the dependence on the calcium blocking activity of this phenotype. Extracellular calcium deprivation improved the release of Ad5 to a degree similar to that obtained with verapamil (up to 120-fold increase at 48 hours p.i.) without affecting the total viral production (**Figure 3c**) indicating, again, that the calcium blocking activity of verapamil is triggering the observed release enhancement.

Cell death mechanisms triggered by verapamil that may result in enhanced release

The importance of intracellular calcium in the regulation of apoptosis[23] and the fact that apoptosis activation can confer a large plaque size to Ad5 similar to that conferred by verapamil[24] lead us to evaluate apoptosis activation in the presence of the drug. As **Figure 4a** displays, the levels of PARP cleavage, indicative of apoptosis activation, during infection with Ad5 alone or in the presence of verapamil were similar. This demonstrates that verapamil was not enhancing apoptosis and that the verapamil-induced large plaque size of Ad5 was not apoptosis-mediated.

The calcium channel blocking activity of verapamil has recently been reported to enhance autophagic vesicle formation,[25] and prominent autophagy induction at late stages of adenovirus infection has led to speculation that autophagy induction during adenovirus infection may facilitate adenovirus release.[26] In order to study whether the enhanced release in the presence of verapamil associates to a more pronounced induction of autophagy in combination with this calcium channel blocking agent, we performed a western blot anti-LC3 in A549 cells infected with Ad5 alone or in combination with different calcium channel blocking

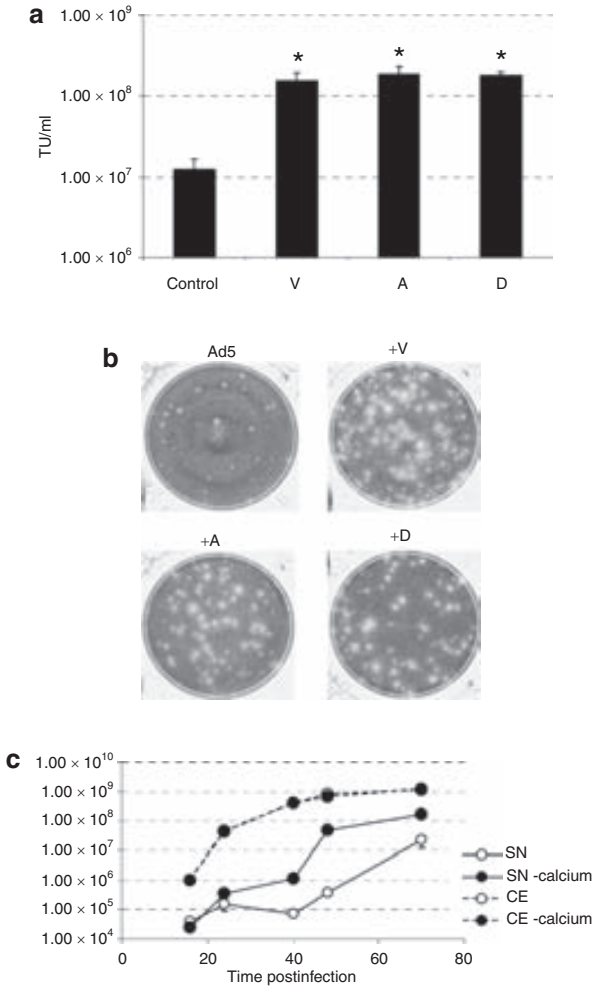

Figure 3 Dependence of the enhanced release phenotype on the calcium blocking activity of verapamil. (**a**) Ad5 release at 40 hours postinfection in the presence of calcium channel blockers. Mean values (n = 3) ± SD are plotted.*Significant compared to the release of Ad5 (P = 0.025, P = 0.023, and P = 0.003 for verapamil, amlodipine, and diltiazem, respectively). (**b**) Plaque size of Ad5 in the presence of calcium channel blockers: verapamil (V), amlodipine (A), and diltiazem (D) at 30 µmol/l. Pictures of representative plaques at 7 days postinfection (p.i.) are shown. (**c**) Virus production and release kinetics in the presence or absence of calcium in the extracellular medium. Mean values (n = 3) ± SD are plotted.

agents. At a time point at which there was a marked increase in adenovirus release (40 hours p.i.), verapamil substantially enhanced the ratio LC3-II/LC3-I both in control A549 cells and in Ad5-infected cells (**Figure 4b**). Indeed, all the calcium channel blockers tested were able to increase the ratio LC3-II/LC3-I (**Figure 4b**). This established an association between increased autophagosome formation or accumulation, and the enhanced progeny release triggered by different calcium channel blocking agents.

The synergistic antitumoral effect of verapamil and adenovirus differs from the effect of other drugs

To further study the association of autophagy and verapamil, we tested virus release in the presence of increasing concentrations of rapamycin. Previously, several drugs capable of inducing autophagy, such as rapamycin, its analogue RAD001 (everolimus) or temozolomide, have been found to improve the antitumoral effect of a telomerase-selective oncolytic adenovirus by inducing autophagy.[27] Although rapamycin was able to induce LC3-I to LC3-II cleavage, which is indicative of autophagy induction, combination of adenovirus with rapamycin displayed wild-type levels of extracellular virus (**Figure 4c**) and small plaque size (data not shown). This indicated that the ability of verapamil to enhance adenovirus release was autophagy-independent. In addition, verapamil-induced large plaque size was not observed in the presence of other drugs that have previously demonstrated synergistic antitumoral effect with oncolytic adenoviruses,[28,29] such as cisplatin, docetaxel, RAD001, and temozolomide (**Figure 4d**). This indicated that the early release observed with verapamil was unique for the combination with the calcium channel blocker.

Verapamil enhances the cytotoxicity of ICOVIR-5 *in vitro* and maintains the selectivity of oncolytic adenovirus ICOVIR-5 *in vivo*

To evaluate whether verapamil was also able to increase the cytotoxicity of tumor-selective oncolytic adenoviruses, we tested its combination with ICOVIR-5. Previously, we have demonstrated that ICOVIR-5 displays a safe toxicity profile after systemic virus administration based on E2F-1 promoter–regulated E1AΔ24 expression.[18,19] As expected, verapamil increased ICOVIR-5 plaque size (data not shown) and enhanced its cytotoxicity *in vitro*. The IC_{50} value of ICOVIR-5 in A549 and NP-9 cells was 0.69 and 4.96 transducing units (TU)/cell, respectively, whereas in the presence of verapamil, the IC_{50} of ICOVIR-5 was reduced to 0.067 and 3.2.

We also evaluated the effects of verapamil on the selectivity of ICOVIR-5 in an immunocompetent model *in vivo*. The toxicity of a single intravenous dose of ICOVIR-5 alone or combined with daily intraperitoneal administration of 20 mg/kg verapamil (a dose chosen based on studies of the combination of verapamil with chemotherapy *in vivo*[30]) was assessed in Balb/C immunocompetent mice and compared to the toxicity of a nonselective control (AdwtRGD). Mice treated with 5×10^{10} viral particles (vp) or 1×10^{11} vp of ICOVIR-5 combined with daily verapamil administration showed a similar body weight variation as the groups treated with ICOVIR-5 alone at day 5 postinjection (**Figure 5a**). Anti-E1A immunostaining of frozen liver sections from mice treated with 5×10^{10} vp of AdwtRGD alone or in combination with verapamil displayed the same levels of E1A expression (**Figure 5b**), whereas ICOVIR-5 alone or combined with verapamil both efficiently abrogated E1A expression compared to AdwtRGD (**Figure 5b**). Furthermore, transaminase levels in AdwtRGD- or ICOVIR-5-treated mice were not affected by daily verapamil administration (**Figure 5c**), and verapamil administration was still able to prevent the reduction in platelet count and lymphopenia in ICOVIR-5-treated mice compared to the AdwtRGD-injected groups (**Figure 5d**). Overall, these data indicated that daily verapamil administration did not increase the toxicity of AdwtRGD and maintained the selectivity profile of ICOVIR-5 regardless of the dose.

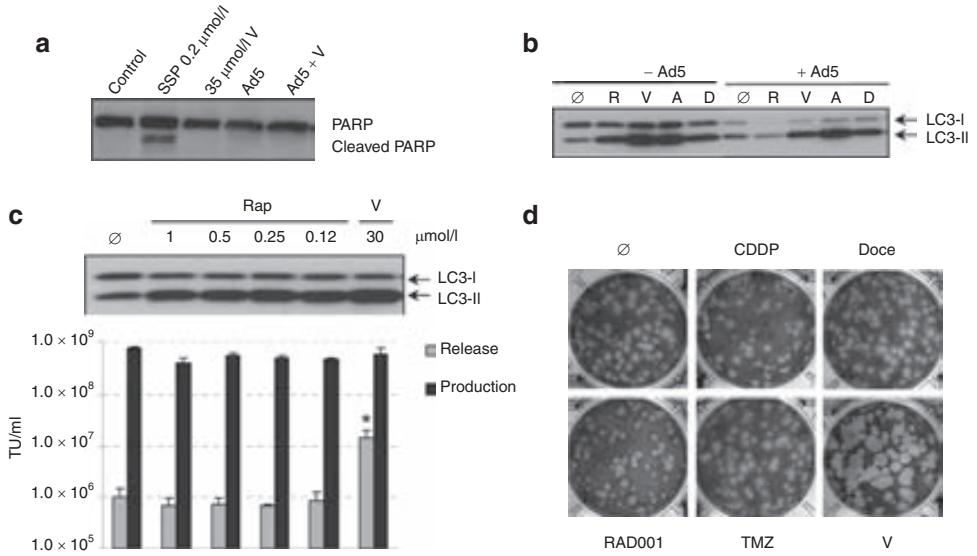

Figure 4 Potential death mode triggered by verapamil and comparison of the effect of verapamil with that of other drugs that synergize with adenovirus. (**a**) Apoptosis activation status in Ad5-infected A549 cells in the presence or absence of verapamil. PARP cleavage was detected by western blot at 40 hours postinfection. Cells incubated with Staurosporine (SSP, 0.2 µmol/l) are used as positive control of apoptosis induction. (**b**) Anti-LC3 western blot in A549 cells in the presence of calcium channel blockers. A549 cells were infected with Ad5 and incubated with normal medium or medium containing rapamycin (positive control of autophagy induction), verapamil (V), amlodipine (A), or diltiazem (D). (**c**) Virus release in the presence of rapamycin. A549 cells were incubated with increasing concentrations of rapamycin or verapamil, and LC3 expression was assessed by western blot 40 hours p.i. At the same conditions, the extracellular and total virus produced were quantified at 40 hours p.i. Mean values (n = 3) ± SD are plotted. *Significant compared to the release of Ad5 (P = 0.04). (**d**) Plaque size of Ad5 in the presence of 30 µmol/l verapamil (V) or 5 µmol/l cisplatin (CDDP), 200 pg/ml docetaxel (Doce), 10 nmol/l RAD001, or 10 µmol/l temozolomide (TMZ) (concentration that gave 10% growth inhibition). Cells were stained 7 days postinfection and pictures of representative plaques are shown. p.i., postinfection.

Verapamil improves the antitumoral efficacy of ICOVIR-5 *in vivo*

Once we had demonstrated the improved cytotoxicity *in vitro* and safety of ICOVIR-5 combined with verapamil, we sought to determine whether it conferred an advantage in antitumoral efficacy *in vivo* compared to ICOVIR-5 alone. A single injection of phosphate-buffered saline (PBS) or 5×10^{10} vp of ICOVIR-5 was injected systemically into mice bearing subcutaneous A549 (lung) or SkMel-28 (melanoma) tumor xenograft models. Starting at day 1 postinjection, half of the mice in each group received daily intraperitoneal injections of 20 mg/kg of verapamil. As **Figure 6a** displays ICOVIR-5 combined with verapamil was more efficient at delaying the growth of A549 subcutaneous tumors *in vivo*. Moreover, four tumors treated with ICOVIR-5 and verapamil completely regressed and maintained the regression status by 4 months after treatment (time of killing), indicating that tumor cells had been completely eradicated. This enhanced antitumoral effect correlated with a more diffuse distribution observed by anti-adenovirus immunostaining on tumor sections at day 13 postinjection (**Figure 6b**), indicating an improved intratumoral spread of ICOVIR-5 in the presence of verapamil. Similarly, whereas only a discrete effect on tumor growth was observed in the ICOVIR-5-treated SkMel-28 subcutaneous tumor xenografts at this dose, the combination with verapamil showed a marked control of tumor growth in this model (sixfold reduction in tumor growth compared to PBS) (**Figure 6c,d**). The enhanced therapeutic effect of the combination of ICOVIR-5 with verapamil in two different tumor xenograft models confirms the benefits of the use of this drug to improve the therapeutic potential of oncolytic adenoviruses.

DISCUSSION

The clinical use of oncolytic adenoviruses revealed a good toxicological and safety profile but also pointed out the need of an improved oncolytic potency of the candidate viruses. Improvement of intratumoral spread of oncolytic adenoviruses can enhance the therapeutic potential of oncolytic adenoviruses, but this step is limited by the natural rate of adenovirus.[13,15] To date, the poor understanding of the mechanism of adenovirus release has restricted the rational approaches to improve progeny release to ADP overexpression[8] or to insertion of transgenes that induce early cell death.[31] Although the contribution of calcium modulation to adenovirus release has not yet been studied, we recently reported that a c-truncation in the E3/19K glycoprotein could enhance adenovirus release by disrupting intracellular calcium homeostasis.[13] Additionally, the modification of the intracellular calcium pools is a widespread mechanism used by viruses to induce cell lysis and progeny release.[16,17] Calcium is important in apoptosis regulation[23] and several hypotheses suggest that ADP, required to induce cell lysis and progeny release, may act as a calcium channel.[14,15] Based on these observations, we hypothesized that calcium channel blocker verapamil could improve the rate of adenovirus release and spread, eventually enhancing the therapeutic activity of oncolytic adenoviruses.

Our results demonstrate that, indeed, the combination of Ad5 with verapamil *in vitro* substantially enhanced the release of adenovirus from the infected cell without affecting virus production. The calcium channel blocker improved the rate of adenovirus release from a variety of cell types including lung, pancreas, and melanoma adenocarcinomas and cancer-associated fibroblasts, and this

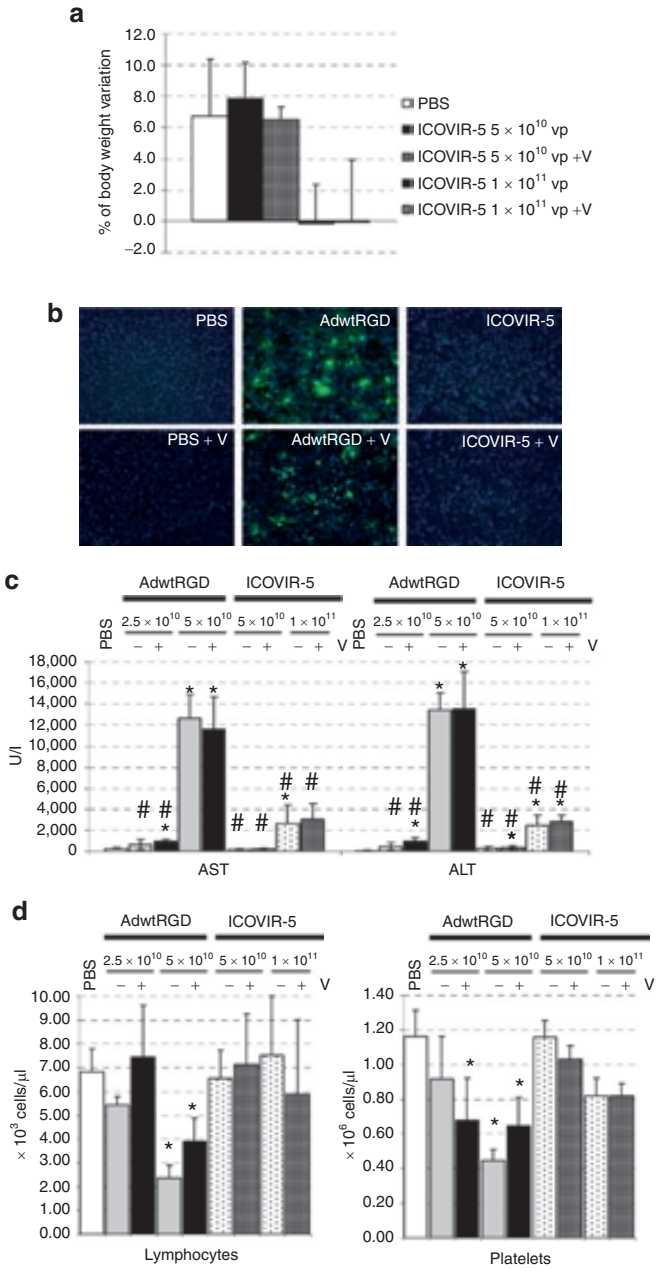

Figure 5 The selectivity of ICOVIR-5 is maintained in the presence of verapamil in an immunocompetent model *in vivo*. (**a**) Percent of body weight variation after systemic administration of PBS or ICOVIR-5 alone or combined with daily i.p. injection of 20 mg/kg of verapamil. (**b**) Liver E1A expression analyzed by immunohistochemistry of representative frozen liver sections of mice treated with PBS or 5 × 10^{10} vp of AdwtRGD, or ICOVIR-5 + daily verapamil (V) at day 5 postinfection (AdwtRGD groups were killed at day 3 due to toxicity). (**c**) Mean values of AST (aspartate animotransferase) and ALT (alanine aminotransferase) in serum and (**d**) lymphocyte and platelet concentrations in peripheral blood at day 5 postinjection (day 3 postinjection for the groups injected with AdwtRGD at 5 × 10^{10} vp) of the doses indicated. Mean values of five animals per group ± SD are plotted. *Significant ($P < 0.05$) compared to PBS and #significant compared to AdwtRGD. PBS, phosphate-buffered saline.

resulted in an improved cell-to-cell spread and cytotoxicity. The benefits of the use of verapamil to improve the spread of oncolytic adenoviruses were confirmed with a highly selective candidate, such as ICOVIR-5. Combination of ICOVIR-5 with verapamil enhanced its cytotoxicity *in vitro*, and most importantly, greatly improved the antitumoral activity of this oncolytic adenovirus in two different human tumor xenograft models *in vivo*. Despite the enhanced cell killing and antitumoral effect *in vivo*, combination of systemic ICOVIR-5 administration with verapamil in an immune competent model strictly maintained the E2F promoter–driven E1AΔ24 selectivity of the virus. Thus, by specifically acting on adenovirus release, a late event in the replication cycle, verapamil preserves the selectivity of conditionally replicative adenoviruses regardless of the regulatory elements used.

Such increase in the antitumoral effect without the requirement of genetic modifications, coupled with the safety of the combination with verapamil make this drug an attractive alternative to improve the efficacy of oncolytic adenoviruses. Whereas other genetic-based methods used to enhance the spread of adenoviruses may compromise some of the E3 immunomodulatory functions[8,32] or reduce virus production by inducing early cell death,[11] verapamil was able to enhance the spread and cytotoxicity without impairing virus production or modifying the expression of adenovirus proteins. The applicability of the combination with verapamil is further augmented by its ability to rescue the impaired progeny release of an ADP-defective adenovirus. The E3 region, considered unnecessary for adenovirus replication *in vitro*, was universally deleted from Ad5 gene therapy constructs until recent efforts to reduce the immune response.[33] Consequently, some oncolytic adenoviruses constructed in an ADP-defective background, which exhibit an impaired spread, could benefit from the combination with verapamil. The calcium channel blocker was also able to further enhance the *in vitro* spread of an ADP-overexpressing mutant (*dl*732). Oncolytic adenoviruses that overexpress ADP display an enhanced antitumoral activity in preclinical models[8,34] and are currently proposed as clinical candidates. Although the additive effect of verapamil and ADP overexpression has not yet been confirmed *in vivo*, our results suggest that the intratumoral spread of these viruses could still be further increased by verapamil. Furthermore, the fast rate of virus release and enhanced intratumoral spread observed in combination with verapamil is also desirable with oncolytic adenoviruses expressing pro-drug converting enzymes, fusogenic proteins, extracellular matrix metalloproteases, or other transgenes to improve the extent of their antitumoral effect.

Combination therapy of oncolytic adenoviruses with other drugs has already shown promising results. Administration of ONYX-015 together with cisplatin and 5-FU led to improved antitumor response in a phase II clinical trial.[35] Doxorubicin and paclitaxel have also shown synergistic antitumor effects when combined with oncolytic adenoviruses *in vivo*,[28,36] and autophagy-inducing agents such as temozolomide or RAD001 also enhance their therapeutic potential.[27,37] The discovery that verapamil can be used to enhance the therapeutic activity of adenoviruses by an independent mechanism is particularly noteworthy, as it is safer than other chemotherapeutic agents used for this purpose. Verapamil has been used for decades in patients to treat arrhythmias and hypertension with minimal side effects. Other properties associated with verapamil make combination of this drug with oncolytic adenoviruses even more appealing. Verapamil is

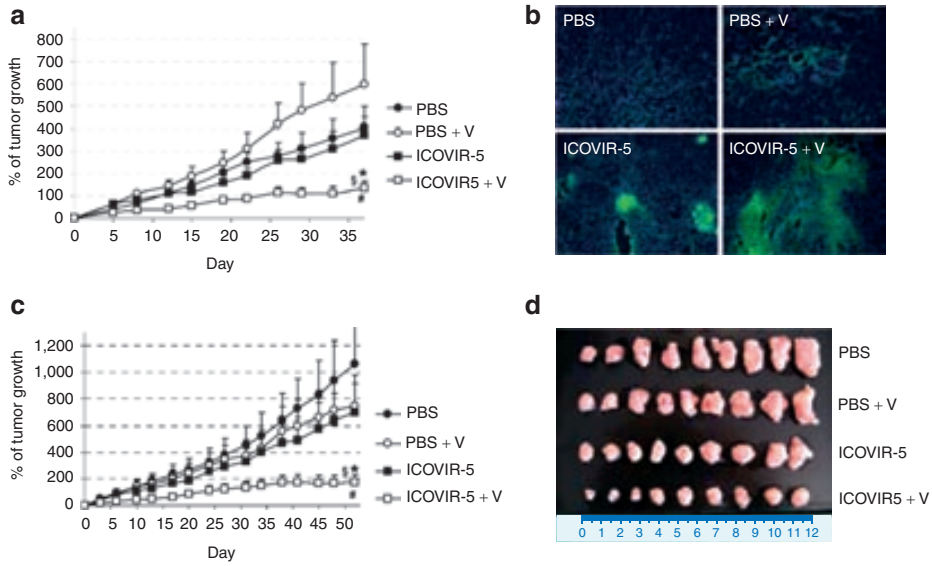

Figure 6 Verapamil enhances the antitumoral activity of ICOVIR-5 *in vivo*. (**a**) Nude mice with A549 tumor xenografts were treated with PBS, PBS combined with daily 20 mg/kg verapamil i.p. injection, or a single dose of 5×10^{10} vp of ICOVIR-5 alone or combined with verapamil. Percent of tumor growth ± SEM is plotted. *Significant ($P = 0.0019$) compared to PBS; §Significant ($P = 0.016$) compared to PBS + V; and $^{\#}$Significant ($P = 0.019$) compared to ICOVIR-5. (**b**) Antiadenovirus immunostaining of frozen A549 tumor sections treated with PBS, PBS + verapamil, ICOVIR-5, or ICOVIR-5 + daily verapamil at day 13 after virus administration. (**c**) Nude mice with SkMel-28 tumor xenografts were treated with PBS, PBS combined with daily 20 mg/kg verapamil i.p. injection, or a single dose of 5×10^{10} vp of ICOVIR-5 alone or combined with verapamil. Percent of tumor growth ± SEM is plotted. *Significant ($P = 0.0009$) compared to PBS; §Significant ($P = 0.0015$) compared to PBS +V; and $^{\#}$Significant ($P = 0.017$) compared to ICOVIR-5. (**d**) Comparative size of SkMel-28 tumor xenografts treated with PBS, PBS + verapamil, ICOVIR-5, or ICOVIR-5 + verapamil at day 52 after virus injection. PBS, phosphate-buffered saline.

able to enhance the cytotoxic effect of certain chemotherapies[38] and reverse multidrug resistance by competitively inhibiting drug transport through P-glycoprotein.[39–42] The positive effect of verapamil on chemotherapy and on the intratumoral adenovirus spread, separately, could further improve the synergistic antiproliferative response observed when combining chemotherapy with oncolytic adenoviruses and makes a multimodal therapy including chemotherapy, an oncolytic adenovirus, and verapamil an interesting option. Additionally, several reports describe that the calcium blocking activity of verapamil also inhibits T-cell activation.[43,44] This activity of verapamil may further enhance the oncolytic effect in the presence of an immune system, where the virus is more exposed to an antiviral immune response.

The exact mechanism of how verapamil promotes adenovirus release remains unknown. The low levels of PARP cleavage indicate that the enhanced release phenotype and spread observed were independent of apoptosis. Moreover, verapamil also enhances the release of an ADP-defective mutant (AdADP$^-$), a mutant that is unable to promote cell lysis. Although this virus still expresses a smaller form of the protein that only partially retains its plaque-development function, the extent of the effect of verapamil on its release is very similar to that observed in Ad5, which suggests that mechanism induced by verapamil is independent of ADP expression. Therefore, the calcium channel blocker verapamil triggers a new, not previously described, pathway that promotes the release of adenovirus from the infected cell. Other calcium channel blocking agents (phenylalkylamines, dihydropyridines, and benzothiazepines) and calcium deprivation had a similar effect on adenovirus release, suggesting that the calcium channel blocking activity could be responsible for the enhanced release observed. Interestingly, Williams and collaborators have recently reported that the calcium blocking activity of verapamil induces autophagy in certain cell types,[25] and autophagy has been proposed as a new pathway induced by adenovirus that leads to cell death[45,46] and progeny release.[26] We found that the ability of calcium channel blockers to enhance Ad5 release correlated with their ability to enhance autophagic vesicle formation or accumulation. However, other autophagy-inducing agents and other drugs that have demonstrated synergies with oncolytic adenoviruses do not reproduce the enhanced release observed with verapamil, which makes the effect of this drug unique. Because different autophagic pathways are currently being characterized, further studies are required to understand the role of autophagy in the process of virus release in verapamil-treated cells.

Our data demonstrate that inhibition of calcium influx can be used as a new rational approach to induce adenovirus release and improve the spread and antitumoral potency of oncolytic adenoviruses. Verapamil and other calcium channel blockers are able to enhance the release, spread, and cytotoxicity of Ad5 *in vitro*, and combination of verapamil with oncolytic adenovirus ICOVIR-5 *in vivo* has demonstrated enhanced antitumor activity and safety. The versatility, broad applicability, and good tolerability of verapamil make the combination of this drug and oncolytic adenoviruses very promising and, as such, they deserve further clinical validation.

MATERIALS AND METHODS

Cell lines, virus, and reagents. Human HEK 293, A549, and SkMel-28 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). NP-9 and CAF1 (human carcinoma–associated fibroblasts) cell lines were established in our laboratory.[13,47] All cell lines were routinely

tested by mycoplasma presence and authenticated by morphology and growth curve analysis. To obtain >80% infection, A549, SkMel-28, NP-9, and CAF1 cells were infected with 25, 35, 30, and 45 TU/cell, respectively. For extracellular calcium deprivation, a calcium-free medium containing 200 mg/l $MgSO_4$, 400 mg/l KCl, 6,400 mg/l NaCl, 3,700 mg/l $NaHCO_3$, 141 mg/l $NaH_2PO_42H_20$, 4,500 mg/l D-glucose (Sigma-Aldrich, St Louis, MO), MEM amino acid, MEM vitamin, MEM nonessential amino acids, and L-glutamine solutions (Invitrogen, Paisley, UK), and 5% FBS were prepared. This medium was supplemented with 1.8 mmol/l $CaCl_2$ when used as a complete medium. Verapamil, amlodipine, and diltiazem were purchased from Abbott Laboratories (Abbott Park, IL), Laboratorios Almirall (Barcelona, Spain), and Pfizer (New York, NY), respectively. RAD001 (everolimus) was supplied by Novartis (Basel, Switzerland), and rapamycin, cisplatin, docetaxel (Taxotere), and temozolomide (Temodal) were purchased from Calbiochem (Darmstadt, Germany), Sigma-Aldrich, Aventis Pharma (Dagenham, UK), and Schering-Plough (Madrid, Spain), respectively.

Human Ad5 was obtained from ATCC. Adenovirus mutant *dl*732, which overexpresses ADP,[48] and its wild-type counterpart, rec700, were kindly provided by WSM Wold (St Louis University, St Louis, MO). To construct an ADP-defective mutant ($AdADP^-$), a T was introduced at position 7 of the ADP coding sequence in pAd5CAU[13] to generate a STOP codon at position 3 of amino acid sequence. Resulting plasmid ($pAdADP^-$) was Pac*I*-digested and transfected into 293 cells. $AdADP^-$ virus expresses a truncated form of ADP from Met[41] in the native protein sequence and lacks the entire luminal sequence of ADP (data not shown). ICOVIR-5 (Ad-DM-E2F-K-Δ24RGD) has been previously described.[18] All the viruses were propagated in A549 cells, and TU were quantified using an antihexon staining–based method in 293 cells.[49]

Virus production and release kinetics. Preliminary experiments were carried out to evaluate the effect of increasing doses of calcium channel blockers on Ad5 release and cell viability. For each cell type, the concentration of verapamil that gave the lowest toxicity and highest viral release was chosen (40 μmol/l verapamil for A549, NP-9, and CAF1 cells; 25 μmol/l for SkMel-28 cells; 30 μmol/l amlodipine or 30 μmol/l diltiazem for A549 cells) to carry out the virus production and release kinetics experiments. Briefly, A549, SkMel-28, NP-9, and CAF1 cells were seeded in 24-well plates and infected to allow >80% infection. Two hours after infection, cells were washed twice and incubated with fresh medium or medium containing verapamil, other calcium channel blockers, or rapamycin. The virus released into the supernatant and the total virus produced (cell + media suspension) were collected in triplicate at different time points after infection and quantified using an antihexon staining–based method. The supernatant was centrifuged at 5,000 rpm before titration of the extracellular virus in order to eliminate detached cells and debris.

Plaque assay. A549 monolayers were seeded in six-well plates and infected with serial dilutions of Ad5, rec700, or dl732. Four hours after infection, the medium was removed and cells were washed twice with PBS. A 1:1 (DMEM 10% FBS: 1% agarose) solution was added to the cells and, once the agarose overlay had solidified, another layer was added of fresh DMEM 5% FBS alone or containing verapamil (30 μmol/l final concentration), other calcium channel blockers (amlodipine 30 μmol/l and diltiazem 30 μmol/l) or other drugs (5 μmol/l cisplatin, 200 pg/ml docetaxel, 10 nmol/l RAD001, and 10 μmol/l temozolomide; concentration that gave 10% growth inhibition). The plaque assay was stained at the day indicated by incubation with 0.5 mg/ml thiazolyl blue tetrazolium bromide during 3 hours at 37 °C and 5% CO_2. Pictures of representative plaques were taken.

In vitro *cytotoxicity assay.* 2×10^4 (SkMel-28), 3×10^4 (A549), or 1×10^4 (NP-9) cells were seeded in 96-well plates in the absence or presence of 20 μmol/l (SkMel-28 cells) or 35 μmol/l (A549, NP-9) of verapamil and infected with serial dilutions starting at 150, 85, and 260 TU/cell, respectively. At day 6 postinfection, plates were processed as previously described.[13] IC_{50} value was calculated from dose–response curves by standard nonlinear regression (GraFit; Erithacus Software, Horley, UK) using an adapted Hill equation.

Western blot analysis. A549 cells (1.5×10^6 cells/well of six-well plate) were infected with Ad5 to allow >80% infection. Two hours after infection, cells were washed twice and incubated in fresh media or media containing calcium channel blockers. At the indicated time points postinfection, cell extracts were obtained with Iso-Hi-pH buffer.[13] Protein samples (20 μg/lane) were separated electrophoretically on SDS-PAGE and transferred to membranes. Blots were probed with primary antibody anti-Ad2 E1A (clone 13 S-5; Santa Cruz Biotechnology, Santa Cruz, CA), anti-E3/19K [Tw1.3 (ref. 50) kindly provided by Jonathan W Yewdell, NIAID/NIH, Bethesda, MD], anti-L4/100K (clone 7/199 kindly provided by WC Russell, St Andrews University, St Andrews, UK), antiadenovirus fiber A-4 mAb (clone 4D2; Fitzgerald Industries International, Concord, MA), antihuman PARP [poly-(ADP ribose) polymerase] (clone #551024; Becton Dickinson, Erembodegem, Belgium) or anti-LC3 (Novus Biologicals, Littleton, CO).

Measurement of autophagy. For measurement of autophagy, cells were seeded in six-well plates, infected with 25 TU/cell of Ad5, and incubated with fresh medium or medium containing verapamil (40 μmol/l), amlodipine (30 μmol/l), diltiazem (30 μmol/l), or increasing concentrations of rapamycin (positive control). Forty hours postinfection, protein cell extracts were collected and LC3-II/LC3-I ratio was calculated after densitometry of the corresponding bands detected by western blot with anti-LC3 antibody (Novus Biologicals).

In vivo *toxicity assay in immune competent mice.* All animal studies were carried out in the facility of IDIBELL (AAALAC Unit 1,155) after approval by IDIBELL's Ethical Committee of Animal Experimentation. Six-week-old immunocompetent Balb/C male mice were injected intravenously by the tail vein with PBS, 2.5×10^{10} vp, or 5×10^{10} vp of AdwtRGD, or 5×10^{10} vp or 1×10^{11} vp of ICOVIR-5 in a final volume of 200 μl ($n = 10$ animals per group). Starting at day 1 postinjection, 20 mg/kg of verapamil was daily injected intraperitoneally into five animals from each experimental group. Animals were monitored for signs of morbidity and body weight recorded. At day 5 after virus administration, animals were killed and blood was drawn by intracardiac puncture. Complete clinical biochemistry and hematology assessment were performed at the Clinical Biochemistry or Hematology Service of the Veterinary Faculty at the Autonomous University of Barcelona. Mice livers were collected and fixed in 4% formaldehyde (for paraffin embedding and hematoxylin/eosin staining) or frozen in OCT for anti-E1A immunofluorescence staining, as previously described.[49]

In vivo *antitumoral efficacy studies.* Subcutaneous A549 or SkMel-28 tumor xenografts were established by injection of 1×10^7 cells into the flanks of 6-week-old Balb/C *nu/nu* mice. Once the mean tumor volume reached 100 mm^3, mice were tail vein-injected with PBS or 5×10^{10} vp of ICOVIR-5 ($n = 24$) in a final volume of 200 μl. Starting at day 1 after virus administration, half of the mice from each group ($n = 12$) were injected with 20 mg/kg of verapamil intraperitoneally daily until the end of the experiment. Tumor size and mouse body weight were recorded at 3- to 4-day intervals. Tumor volume and tumor growth were calculated as previously described.[18] Tumor samples were OCT-included to assess virus replication by antiadenovirus immunostaining of A549 tumor xenografts at day 13 postinjection, as previously described.[13]

Statistical analysis. Data from *in vitro* studies were tested for significance by means of the Student's *t*-test. The *t*-test was also used for comparing the toxicity and tumor progression in mice in the different treatment groups. All *P* values are two-tailed. *P* value <0.05 was considered to be statistically significant in Student's *t*-test.

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Hyaluronidase expression by an oncolytic adenovirus enhances its intratumoral spread and suppresses tumor growth.

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Abstract

Successful virotherapy requires efficient virus spread within tumors. We tested whether the expression of hyaluronidase, an enzyme which dissociates the extracellular matrix, could enhance the intratumoral distribution of an oncolytic adenovirus and improve its therapeutic activity. As a proof of concept, we demonstrated that intratumoral coadministration of hyaluronidase in mice bearing tumor xenografts improves the antitumor activity of an oncolytic adenovirus. Next, we constructed a replication-competent adenovirus expressing a soluble form of the human sperm hyaluronidase (PH20) under the control of the major late promoter (AdwtRGD-PH20). Intratumoral treatment of human melanoma xenografts with AdwtRGD-PH20 resulted in degradation of hyaluronan, enhanced viral distribution and induced tumor regression in all treated tumors. Finally, the PH20 cDNA was inserted in an oncolytic adenovirus which selectivelly kills pRb pathway-defective tumor cells. The antitumoral activity of the novel oncolytic adenovirus expressing PH20 (ICOVIR-17) was compared to that of the parental virus ICOVIR-15. ICOVIR-17 showed more antitumor efficacy following intratumoral and systemic administration in mice with pre-stablished tumors, along with an improved spread of the virus within the tumor. Importantly, a single intravenous dose of ICOVIR-17 induced tumor regression in 60% of treated tumors. These results indicate that ICOVIR-17 is a promising candidate for clinical testing.

Introduction

Oncolytic adenoviruses are promising agents for the treatment of cancer. These viruses are designed to selectively target, replicate in and kill cancer cells, while sparing normal cells. The release of virus progeny from lysed tumor cells results in an exponential increase of the virus inoculum, which can spread laterally throughout the tumor mass and potentially induce tumor regression. Early clinical trials with oncolytic adenoviruses established the safety of this therapy 1, even after i.v. administration 2, 3. However, only limited responses were noted, and treatment with first generation of oncolytic adenoviruses did not significantly alter the course of the disease.

One of the main limitations which contributes to these suboptimal results is the inability of oncolytic adenoviruses to effectively spread in the solid tumors. Oncolytic adenoviruses, along with most novel targeted therapeutics, face tremendous transport barriers in the tumor interstitium, due in part to their relatively large size (90 nm), much larger than chemotherapeutic drugs. Also, solid tumor presents unique characteristics that hinder the transport of macromolecules 4. Among them, the presence of high amounts of extracellular matrix (ECM) and high interstitial fluid pressure (IFP) in tumor interstitium are the main source of physical resistance to drug transport.

One attractive method for improving viral spread through the tumor mass is to degrade extracellular matrix (ECM) with proteolytic enzymes. In this regard, pretreatment of tumors with trypsin or collagenase has been shown to enhance distribution of viral vectors after intratumoral administration 5, 6. Also, replication-competent adenoviruses expressing relaxin, a peptide hormone that modulates collagen levels, have been constructed and results showed that ECM degradation can enhance viral spread and prolong the survival of tumor-bearing mice 7, 8.

Hyaluronan (HA) is an important structural element of the ECM. HA is a high molecular weight linear glycosaminoglycan consisting of repeating disaccharide units. Concentrations of HA are elevated in several cancers, and often HA levels correlate with invasive and metastatic behavior 9. Degradation of HA with hyaluronidase has been shown to enhance the action of various chemotherapeutic agents in patients 10, 11. Hyaluronidase is assumed to facilitate penetration

and to decrease IFP 12, permitting anticancer agents to reach malignant cells. Moreover, it has been proposed that hyaluronidase may itself have intrinsic anti-cancer activity 13.

Interestingly, a recent report indicated that intratumoral coadministration of hyaluronidase and replication-competent adenoviruses can enhance viral distribution and improve virus potency *in vivo* 14. In the present study, we confirmed these results by administering a single dose of hyaluronidase and an oncolytic adenovirus in a melanoma model which expresses high levels of HA. Although successful results were obtained, this strategy is limited to intratumoral administration. As many patients have metastasic disease, intravenous administration would improve the utility of an oncolytic virotherapeutic approach for cancer. Therefore, we sought to determine whether expression of hyaluronidase by an oncolytic adenovirus, instead of coadministration, could enhance viral spread throughout the tumor after systemic administration.

As an initial step, we constructed a replication-competent adenovirus expressing PH20 hyaluronidase under the control of the major late promoter (MLP). PH20 is a GPI-anchored hyaluronidase found in mammalian sperm 15. Human PH20 hyaluronidase was chosen for its ability to degrade HA at neutral pH (pH 5 to 8), in contrast to other human hyaluronidases (as HYAL1 and HYAL2) which are active only at acid pH (pH 3 to 4). In order to obtain a soluble hyaluronidase, we removed the GPI membrane attachment motif of PH20. Expression of such a soluble PH20 enhanced virus spread and improved therapeutic outcome when compared to the parental virus, resulting in tumor regression in all treated tumors. We next constructed an oncolytic adenovirus expressing PH20 hyaluronidase (ICOVIR17). The insertion of eight E2F binding sites and one Sp1 binding site in the E1a endogenous promoter render ICOVIR17 selective for replication in pRb pathway-defective tumor cells. HA degradation enhanced the intratumoral spread of ICOVIR17 and greatly improved its antitumor efficacy without increasing its toxicity.

Results

HA expression in tumor cell lines. In order to choose a suitable model for *in vivo* experiments, we first measured hyaluronan expression levels in supernatants of various tumor cell lines using an ELISA-like assay. As shown in Fig. 1c SkMel-28 melanoma cells and PC3 prostate carcinoma cells produced the highest amounts of HA. Next, we analyzed HA production in human xenograft tumors (Fig. 1d) by histochemical staining. Quantification of the percentage of stained areas indicated that both SKMel-28 and NP9 tumors expressed high amounts of HA (18.4 ± 6.6 % and 14.2 ± 9.8 %, respectively) around tumor cells. By contrast, NP18 tumors expressed 6-fold less HA than SKMel-28 cells (2,7 ± 0.1%; $p = 0.028$), and this HA was mainly expressed within the tumor stroma. SkMel-28 melanoma cells, which produced high amounts of HA both *in vitro* and *in vivo*, were selected for *in vivo* experiments.

Intratumoral administration of hyaluronidase enhances the efficacy of an oncolytic adenovirus *in vivo*. To investigate whether the degradation of the hyaluronan present in the ECM of solid tumors can enhance virus spread we used an oncolytic adenovirus, ICOVIR5 16, which replication is restricted to cancer cells with a disrupted Rb pathway (Fig. 1a). Established s.c. SkMel-28 melanoma tumor xenografts (average size of 170 m^3) were treated with a single intratumoral injection of PBS, testicular bovine hyaluronidase (100 U/tumor), ICOVIR5 (1 x 10^9 vp/tumor) or a combination of hyaluronidase and ICOVIR5 (Fig. 2a). A significant amount of tumor growth inhibition was detectable in tumors treated with the combination of virus and hyaluronidase compared with tumors treated with PBS ($p \leq 0.01$) or hyaluronidase alone ($p \leq 0.01$). Importantly, hyaluronidase administration increased the antitumor efficacy of ICOVIR5 compared with the virus administered in monotherapy. Although differences in tumor volume

between these groups do not reach statistical significance, they do when percentage of tumor growth is analyzed ($p \leq 0.05$ at days 11 and 14 post-administration).

To assess whether the enhanced efficacy observed in animals treated with the combination was related to a decrease in HA levels in the tumor mass, as well as an improved distribution profile of ICOVIR5, immunohistochemical analysis was done on tumors obtained at 14 days after virus treatment (Fig. 2b-c). SkMel28 tumors treated with ICOVIR5 and hyaluronidase showed lower levels of expression of HA and more diffuse adenoviral capsid protein expression (a marker of virus replication) compared with tumors treated with ICOVIR5 alone. Control tumors injected with PBS presented high content of HA but no adenovirus positive cells (data not shown).

Expression of PH20 hyaluronidase enhances the efficacy of a replication-competent adenovirus *in vivo*. Next, we constructed AdwtRGD-PH20 (Fig. 1a), a replication-competent adenovirus that expresses a soluble form of the human testicular hyaluronidase (PH20) lacking the GPI membrane attachment motif (Fig. 1b). To restrict hyaluronidase expression to the later phases of the viral life cycle, PH20 cDNA was inserted in the AdwtRGD genome (Fig. 1a) downstream of the fiber gene, under the control of the MLP. To confirm that AdwtRGD-PH20 expressed a functional soluble hyaluronidase, A549 cells were infected with AdwtRGD and AdwtRGD-PH20. After 48 hours, supernatants were collected and analyzed for hyaluronidase activity. While no hyaluronidase activity was detected in supernatants of A549 cells infected with AdwtRGD, supernatants of AdwtRGD-PH20 infected cells were able to digest high molecular weight hyaluronan to oligosaccharides of different sizes (data not shown). In vitro characterization of AdwtRGD-PH20 in a panel of tumor cell lines showed similar levels of viral replication and cell cytotoxicity compared with the control virus, AdwtRGD (data not shown).

To analyze whether expression of the human PH20 hyaluronidase by a replication-competent adenovirus enhances the antitumor effect *in vivo*, mice bearing SkMel-28 tumors were administered with a single dose of AdwtRGD or AdwtRGD-PH20 (1×10^9 vp/tumor). In this experiment, tumors treated with AdwtRGD showed a pattern of tumor growth similar to that of PBS-treated tumors (Fig. 3a). In contrast, treatment with AdwtRGD-PH20 showed a significant reduction in tumor growth compared with PBS ($p \leq 0.005$) and AdwtRGD ($p \leq 0.025$). At day 27 after administration 100% of tumors treated with AdwtRGD-PH20 showed partial regressions (10-70 % of tumor reduction), while no tumor regressions were observed in mice treated with PBS or AdwtRGD. The enhanced antitumor activity displayed by the hyaluronidase expressing adenovirus, AdwtRGD-PH20, correlated with a decrease in tumor HA levels and wider areas of virus replication compared with tumors treated with AdwtRGD, as showed by immunohistochemical analysis (Fig. 3b).

Generation and characterization of ICOVIR17, an oncolytic adenovirus expressing PH20 hyaluronidase. On the basis of the observations described above, we constructed an oncolytic adenovirus expressing PH20 hyaluronidase. Insertion of the PH20 cDNA into ICOVIR5, in absence of compensatory deletions, would result in a net increase in virion DNA of 2.5 Kb, exceeding the accepted packaging size limit of 1.8 kb. To address this problem we used a new oncolytic adenovirus, ICOVIR15, which displays similar selectivity and potency to that of ICOVIR5 (Rojas et al. unpublished results) but with a shorter genome. In ICOVIR15 selectivity was achieved by the insertion of eight E2F binding sites and one Sp1 binding site in the E1a endogenous promoter (Fig 1a). Insertion of the PH20 expression cassette from AdwtRGD-PH20 in ICOVIR15, downstream of the fiber gene, resulted in the vector ICOVIR17 (Fig 1a). The genome of ICOVIR17 is ~1.7 kb larger than Adwt genome.

Analysis of hyaluronidase activity in the supernatants of tumor cells infected with ICOVIR15 and ICOVIR17 indicated that ICOVIR17 expresses a soluble hyaluronidase which is capable of digesting high molecular weight hyaluronan. As expected, no hyaluronidase activity was detected in supernatants of tumor cells infected with ICOVIR15 (Fig. 4A).

To analyze the impact of incorporating the PH20 cDNA on viral replication, we compared the replication kinetics of ICOVIR15 and ICOVIR17 in SkMel-28 cells. As shown in figure 4B, both viruses reached approximately the same viral titer at all time points, indicating

similar viral replication. Next, we examined the ability of ICOVIR17 to kill tumor cells when compared with ICOVIR15. IC_{50} values obtained in SkMel-28 and PC3 were 0.5 and 0.065 for ICOVIR15 and 0.33 and 0.099 for ICOVIR17, respectively. These results indicate that *in vitro*, where virus spread is not impaired, expression of PH20 hyaluronidase does not increase cytotoxicity of a conditionally replicative adenovirus.

ICOVIR17 displays potent antitumor activity after intratumoral administration *in vivo.* We next analyzed the antitumor activity of ICOVIR17 in established melanoma tumors after intratumoral administration. Mice bearing SkMel-28 tumors (average size of 100 mm^3) were treated with a single intratumoral injection of PBS, ICOVIR15 or ICOVIR17 (1×10^9 vp/tumor). Treatment with both viruses resulted in considerable inhibition of tumor growth when compared with untreated tumors. These effects were observed throughout the duration of the study (Fig. 5a). At day 42 after administration, ICOVIR15 and ICOVIR17 induced a reduction of tumor growth of 60% ($p \leq 0.02$) and 95% ($p \leq 0.002$) respectively, compared to the control group. At this time point, animals were sacrificed and the weight of the tumors was measured. PBS and ICOVIR15 treated-tumors reached an average weight of 0.552 ± 0.4 g and 0.342 ± 0.18 g, respectively. Tumors treated with the PH20-expressing adenovirus, ICOVIR17, were significantly smaller (0.199 ± 0.11 g) when compared with tumors treated with PBS ($p = 0.022$) or with the control virus, ICOVIR15 ($p = 0.05$), indicating that PH20 expression can significantly enhance the antitumor activity of an oncolytic adenovirus after intratumoral administration.

ICOVIR17 displays potent antitumor activity after systemic administration *in vivo.* The efficacy of ICOVIR17 via intratumoral administration led us to evaluate the ability of ICOVIR17 to control tumor growth when administered i.v. Mice bearing SkMel-28 melanoma tumor xenografts (average size of 100 mm^3) were treated with a single dose of PBS, ICOVIR15 or ICOVIR17 at 5×10^{10} vp/mouse administered via the tail vein. In this tumor model, which expresses high levels of HA, tumors treated with ICOVIR15 showed no therapeutic response and all tumors continued to grow in a similar pattern to those treated with PBS (Fig. 5b). In contrast, a single intravenous injection of ICOVIR17 had an immediate effect on tumor growth that lasted throughout the study. Within one week of virus administration, the tumors treated with ICOVIR17 showed a significantly reduction on tumor growth compared with PBS ($p=0.0012$) and ICOVIR15 ($p=0.0057$) treated mice. At the end of the study (day 49 post-treatment) the PH20-expressing adenovirus, ICOVIR17, displayed a significantly greater antitumor effect than ICOVIR15 ($p=0.000008$), with 60% of ICOVIR17 treated tumors showing regression between 20% to 40% of their pretreatment size.

To confirm that the antitumor activity shown by the PH20-expressing adenoviruses was not restricted to a single cell line, we performed an additional study in human pancreatic tumor xenografts. Mice carrying NP18 tumors (average size of 60 mm^3) were treated with a single i.v. dose of PBS, ICOVIR15 or ICOVIR17 at 5×10^{10} vp/mouse (Fig. 5c). By day 30 after treatment, animals were sacrificed due to the large size of PBS-treated tumors. In these tumors, which express lower levels of HA compared with SkMel-28 tumors (Fig. 1d), ICOVIR15 induced a significant tumor growth inhibition when compared with PBS treated mice. Importantly, ICOVIR17 showed a drastically greater reduction in tumor progression that was significant compared with PBS and ICOVIR15 groups from early after the start of the treatment through the end of the study.

Finally, immunofluorescence detection of virus capsid proteins was performed in SkMel-28 and NP18 tumors harvested at the end of the study, and percentage of stained areas was quantified (Fig 5d-e). All tumors treated with ICOVIR15 and ICOVIR17 showed areas of virus replication that were not present in untreated tumors. ICOVIR15-treated tumors showed compact islands of infected cells that represented 6% to 8% of the total viable tissue. Importantly, tumors treated with the PH20-expressing adenovirus, ICOVIR17, showed a more diffuse pattern of adenovirus replication (about 13% of viable tissue was positive for virus capsid protein expression), which was significant compared to ICOVIR15-treated tumors in both tumor models.

ICOVIR17 displays similar toxicity to ICOVIR15 after systemic administration in hamsters. Our *in vivo* studies showed that hyaluronidase expression enhanced the intratumoral spread of ICOVIR17 and greatly improved its antitumor efficacy. An important question was therefore whether this also resulted in increased toxicity. Because mice are poorly permissive for human adenoviruses, we performed this safety study in Syrian hamsters, which are immune competent and permissive for Ad5 replication. Hamsters were injected with PBS or with a previously determined maximum tolerable dose of $4x10^{11}$ vp of ICOVIR15 or ICOVIR17 via the cephalic vein. Weight loss, liver enzymes and hematological profile were determined at day 5 after viral injection. Both ICOVIR15 and ICOVIR17 viruses slightly reduced body weight and caused a modest increase in AST and ALT levels (Fig 6a-b). Also both viruses induced a modest but significant decrease in platelets and a significant increase in neutrophils and basophils compared with the PBS-treated hamsters (Fig 6c-d). Importantly, no significant differences in body weight loss, serum levels of liver enzymes or hematological profile were observed between mice treated with ICOVIR15 or ICOVIR17, indicating that PH20 expression did not significantly increase toxicity caused by an oncolytic adenovirus.

In addition, histological analysis was performed on liver samples obtained at day 5 after systemic administration. Microscopic examination of liver sections from animals treated with ICOVIR15 and ICOVIR17 showed some areas of infiltration with marginal Councilman bodies in the more superficial areas of the liver. No apparent differences were noted between liver sections treated with ICOVIR15 or ICOVIR17.

Discussion

A major obstacle to the successful application of oncolytic adenovirus in cancer therapy is their inability to effectively spread in the solid tumors. In the present study, we attempted to enhance the intratumoral distribution of an oncolytic adenovirus by arming it with a soluble form of the human sperm hyaluronidase, PH20. By using this strategy, we have demonstrated that PH20 expression by an oncolytic adenovirus degrades the extracellular matrix, improves viral spread through the tumor mass and enhances the overall antitumor efficacy without increasing its toxicity. We have also demonstrated, for the first time, that an oncolytic adenovirus armed with hyaluronidase displays great antitumor efficacy after systemic administration.

As a proof of principle, we demonstrated that a single dose of hyaluronidase in combination with an oncolytic adenovirus is enough to improve adenovirus distribution within the tumor mass and enhance its antitumor activity. This data, confirm previous results suggesting that ECM degradation enhances viral spread and is therefore greatly advantageous for the virotherapy of cancer 7, 8, 14. However, this approach presents some limitations. The first limitation relies on the fact that the effect of hyaluronidase on the ECM modulation is transient and reversible within 48 hours. Thus, the enhancement of the antitumoral activity observed with the combination therapy is probably due to an improvement in the spread of viruses produced early after the start of the treatment. Therefore, one might expect that maintained modulation of the ECM would further enhance adenoviral spread through the tumor. One possibility, which has already been used by Ganesh et al, is to inject hyaluronidase one every other day. By using this method, Ganesh et al. demonstrated that combination treatment of an oncolytic adenovirus and human recombinant PH20 significantly increases the antitumor activity and survival of tumor-bearing animals in two different models. However, although these strategies gave encouraging results, the utility of hyaluronidase coinjection is limited to intratumoral administration into localized tumors.

Our goal in this study was to develop an oncolytic adenovirus with improved spread capabilities for the treatment of disseminated disease. We speculated that hyaluronidase

expression by replication-competent adenoviruses should allow maintained ECM degradation around areas of viral replication, enhancing therefore virus spread within the tumor. To this end, two viruses were generated expressing a soluble version of PH20 hyaluronidase under the control of the major late promoter: AdwtRGD-PH20, a replication-competent adenovirus; and ICOVIR17, a replication-selective adenovirus. *In vitro* characterization of the new viruses showed that expression of a soluble hyaluronidase lacking the GPI attachment motive was feasible, and did not impair virus replication. *In vivo*, both PH20-expressing viruses displayed greater antitumor efficacy compared with their non-armed counterparts in the tumor models tested. Importantly, PH20 expression increased oncolytic activity of ICOVIR17 after i.v. administration in two different tumor models compared to its counterpart, ICOVIR15. In all the experiments, this enhanced oncolytic activity was achieved after a single administration of the virus, and the tumor burden at the time of treatment (2 or 3 weeks after tumor cell implantation) was extensive. It is important to note that although 60% of tumors showed partial regressions and all tumors showed active ICOVIR17 replication areas, complete tumor responses were rarely achieved. One possible explanation to the low rate of complete tumor regressions is that tumor stroma of murine origin precludes a complete intra-tumoral spread, as mouse cells are not permissive for human adenovirus replication. However, fibrotic tissue may not be such an important issue for viral spread in the clinical setting as preliminary results indicate that ICOVIR15 efficiently replicates in Carcinoma Associated Fibroblast (CAF) of human origin (Guedan, et al. unpublished results).

Other studies have sought to improve adenovirus spread by degrading matrix components. For example, two different groups constructed oncolytic adenoviruses armed with relaxin, a peptide hormone that downregulates expression of collagen and upregulates expression of MMPs. Consistent with our results, ECM degradation by relaxin-expressing oncolytic viruses increased virus spread and improved antitumor efficacy 7, 8 supporting the idea that ECM components inhibit viral spread following administration of adenoviruses. One important issue that may be considered is whether expression of these proteolytic enzymes can increase the toxicity of the oncolytic virotherapy. In this regard, Kim et al demonstrated that injections of subcutaneous tumors with a relaxin-expressing virus yielded no increase in metastases, although their in vitro results showed that relaxin expression could increase cell invasion, which raises potential safety concerns [8]. In our work, we sought to prevent uncontrolled expression of hyaluronidase in normal cells by expressing PH20 late in the adenoviral infection cycle, under the control of the MLP. Late PH20 expression by an oncolytic adenovirus should be restricted to tumor cells, thus maintaining the safety of the original virus in normal cells. In the toxicology studies performed in hamsters, we have not detected significant differences in body weight loss, serum levels of liver enzymes or hematological profile following i.v. injection of ICOVIR17 compared to ICOVIR15, indicating that PH20 expression does not seem to affect the safety profile of a conventional oncolytic adenovirus. Importantly, the safety and tolerability of hyaluronidase has been analyzed in clinical trials, showing that hyaluronidase seemingly has no toxicity towards normal tissues or other adverse effects 10, 17, 18. In particular, hyaluronidase administration in preclinical studies and clinical trials has not been related to an increase of metastasis 13, 14, 19-21. In this regard, Bookbinder et al. demonstrated that the size of channels created in the interstitial matrix after depletion of hyaluronan by PH20 hyaluronidase allowed the diffusion of particles up to 200 nm in diameter 22. Therefore, adenovirus particles, but not tumor cells, would be allowed to penetrate through these channels.

Hyaluronidase has shown promise as an adjuvant in local and systemic cancer therapy. The mechanism by which hyaluronidase enhances therapeutic effect of chemotherapeutics is not well known, but degradation of the ECM is assumed to improve the penetration of the drug into the tumor tissue. However, it has also been suggested that hyaluronidase may itself have intrinsic anti-cancer activity. In this regard, Shuster et al. reported that hyaluronidase administration in SCID mice bearing human breast tumors caused rapid reduction in tumor size, and they suggest that hyaluronidase treatment of tumor cells appears to have induced an

irreversible change in cell cycle kinetics 13. Recently, Lokeshwar et al. stably transfected prostate cancer cell lines to generate moderate, high and low hyaluronidase producers 23. They demonstrated that when hyaluronidase is expressed at high doses it can act as a tumor suppressor by inducing apoptosis. In this report, we show that either hyaluronidase coadministration or hyaluronidase expression by an oncolytic adenovirus enhances virus distribution within the tumor, resulting in a widespread viral replication and improved therapeutic outcome. In contrast, when PH20-expressing viruses were analyzed *in vitro*, where viral spread is not impaired, all viruses showed similar cytotoxicity in all conditions tested. Therefore, our results suggest that the main mechanism by which hyaluronidase increases oncolytic potency *in vivo* is probably related to the ECM degradation and IFP reduction. However, we cannot rule out the possibility that other mechanisms, such as apoptosis induction in cancer cells, could be contributing to the enhanced potency of PH20-expressing adenoviruses.

Taken together, our results provide conclusive evidence that ECM disruption by hyaluronidase greatly enhances the distribution of oncolytic adenovirus within the tumor mass, which results in a more potent antitumor effect in all tumor models tested. Also, we show that ICOVIR17, an oncolytic adenovirus expressing PH20, has strong antitumor activity after both intratumoral and systemic administration, resulting in tumor regression. Taken into account that oncolytic adenoviruses have shown promising results in combination with chemotherapy 24, and that hyaluronidase administration improves antitumor effects of various chemotherapeutics, we believe that combination of ICOVIR17 with chemotherapy may present synergistic effects. We believe that ICOVIR17 is highly promising for clinical development for the treatment of disseminated cancers, and it might be also combined with other therapies, such as chemotherapy.

Materials and Methods

Cell lines. HEK293, A549 lung adenocarcinoma cells, SkMel-28 melanoma cells, PC3 prostate carcinoma cells, BxPC3 pancreatic tumor cells and HTC116 colon cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). BZR cells were derived from Beas-2B normal lung cells and were also obtained from the ATCC. NP9 and NP18 pancreatic tumor cells were established in our laboratory 25. All cells lines were routinely tested by mycoplasma presence and authenticated by morphology and growth curve analysis. All cell lines were grown in DMEM supplemented with 5% fetal bovine serum (FBS).

Hyaluronan determination. To determine the hyaluronan content in the supernatants of various cell lines, cell cultures were grown to 60% confluence in DMEM medium containing 5% FBS. Medium was removed and cells were incubated in serum free medium. After 24h, medium was collected and digested overnight with 0,1mg/ml of pronase. Hyaluronan content in samples was measured using an ELISA assay as previously described 26.

Recombinant adenoviruses. A schematic diagram outlining the structure of the viruses used in this study is shown in Fig. 1A. AdwtRGD and ICOVIR5 have been described previously 16. ICOVIR15 is a conditionally replicative adenovirus in which expression of the adenovirus E1A-Δ24 gene is regulated by a modified endogenous E1A promoter, which contains eight E2F-1 binding sites and one Sp1 binding site. To generate PH20-expressing adenoviruses, a shuttle plasmid, pNKFiberPH20, was constructed as described below. PH20 exons II, III and IV were amplified by PCR using A549 cellular DNA as the template DNA. The reverse primer used to amplify exon IV was designed such that the nucleotide sequence encoding for the GPI membrane attachment motif was excluded. The PCR products corresponding to exons II, III and IV were mixed and reamplified with specific primers engineered to amplify the complete PH20 cDNA. The forward primer contained the acceptor splicing sequence of the virus protein IIIa and the kozak sequence, and the reverse primer contained a polyadenylation signal for PH20.

Both primers contained MfeI restriction sites. The PCR product, containing the PH20 cassette, was digested with MfeI and ligated to the pNKFiberRGD plasmid 27 to generate pNKFiberPH20. The NotI-KpnI fragment of pNKFiberPH20 and the partially SwaI digested pVK50cau (a pVK50 28-derived plasmid, containing the yeast replication elements and a selectable marker to allow the homologous recombination in yeast) were recombined by homologous recombination, performed in yeast. This recombination event resulted in generation of the pAdwtRGD-PH20. pICOVIR17 was obtained using a similar strategy; pICOVIR15 was partially digested with SpeI and recombined with the SpeI-PacI fragment of pAdwtRGD-PH20. AdwtRGD-PH20 and ICOVIR17 were generated in HEK293 and plaque purified in A549 cells. All viruses were amplified in A549 cells and purified on CsCl gradients according to standard techniques.

Production assays. A549 and SkMel-28 cells were infected with 25 TU/cell of each virus to allow for 80% to 100% infection. Four hours later, infection medium was removed; cells were washed twice with PBS and incubated with fresh medium. At indicated time points, cells and medium (CE) were harvested and subjected to three rounds of freeze-thaw lysis. Viral titers of CE were determined in triplicate according to an anti-hexon staining-based method in HEK293 cells 29.

Assay for hyaluronidase activity. A549 cells were infected with 25 TU/cell of each virus to allow for 80% to 100% infection. After 24 hours, infection medium was removed and cells were incubated with serum free medium. 24 hours later, medium was collected and concentrated by filtration using Amicon Ultra-4 columns (Milipore, Billerica, USA). The hyaluronidase activity of concentrated supernatants was analyzed as previously described 30. Briefly, supernatant samples containing soluble hyaluronidase were mixed with a hyaluronan (Sigma. St Louis, MO) solution in phosphate buffer (pH 6.0) containing 0.1 M NaCl and 0.05% BSA, and samples were incubated overnight at 37ºC. Hyaluronan fragment sizes after digestion with samples were analyzed by polyacrylamide gel electrophoresis (PAGE) and visualized using a combined alcian blue and silver staining protocol.

In vitro cytotoxicity assay. Cytotoxicity assay was performed by seeding 10,000 SkMel-28 and PC3 cells in 96-well plates in DMEM with 5% FBS. Cells were infected with serial dilutions starting with 180 TU/cell. At day 5 post-infection (PC3) or 8 post-infection (SkMel-28), plates were washed with PBS and stained for total protein content (bicinchoninic acid assay, Pierce Biotechnology) and absorbance was quantified. The TU per cell required to produce 50% growth inhibition (IC_{50} value) was estimated from dose-response curves by standard nonlinear regression (GraFit; Erithacus Software), using an adapted Hill equation.

***In vivo* efficacy studies.** Animal studies were performed at the IDIBELL facility (AAALAC unit 1155) and approved by the IDIBELL's Ethical Committee for Animal Experimentation. For in vivo coadministration studies, subcutaneous SkMel-28 melanoma tumors were established by injection of 5×10^6 cells into the flanks of 6-week-old male Balb/C nu/nu mice. Once tumors reached 170 mm^3 mice were randomized (n = 6) and tumors were treated with a single intratumoral injection of 25 µl of PBS, bovine hyaluronidase (100 U/tumor), ICOVIR5 (1×10^9 vp/tumor) or a combination of ICOVIR5 (1×10^9 vp/tumor) and PH20 hyaluronidase (100 U/tumor).

To evaluate the intratumoral efficacy of viruses expressing the PH20 hyaluronidase, mice bearing established SkMel-28 tumors (100-150 mm^3) received a single intratumoral injection of PBS or 1×10^9 viral particles of each virus. To evaluate systemic efficacy, subcutaneous SkMel-28 melanoma or NP18 pancreatic tumors were established by injection of 5×10^6 cells into the flanks of 6-week-old male Balb/C nu/nu mice. Once tumors reached the desired mean tumor volume (100 mm^3 for SkMel-28 tumors, and 60 mm^3 for NP18 tumors), mice were randomly distributed into treatment groups (n = 10 tumors per group) and treated with a single intravenous injection of PBS or 5×10^{10} vp of ICOVIR15 or ICOVIR17 given via tail vein.

In all animal experiments, tumor progression and morbidity status were monitored three times weekly. Tumor volume and the percentage of tumor growth were calculated as previously described 16. The two-tailed Student's t test was used to compare the differences in tumor volume and tumor progression in mice in each cohort.

At desired time points, animals from each group were euthanized and tumors were collected and cut into halves. One half was frozen in OCT and the other half was fixed in 4% formaldehyde for 24 hours and embedded in paraffin.

***In vivo* toxicity studies.** Female 5-week-old immune competent Syrian (golden) hamsters (*Mesocricetus auratus*) were used. A single dose of 4 x 10^{11} vp of ICOVIR15 and ICOVIR17 was administered intravenously into the cephalic vein on day 0 in a volume of 300 µl of PBS. The control group was injected with an equal volume of PBS. Body weights were measured at days 0, 3 and 5 after administration. At these times, animals were examined for clinical signs of toxicity. On day 5, Hamsters were sacrificed and whole blood and serum samples were collected through cardiac puncture. Clinical biochemistry of transaminase levels and hematological determinations were performed by the Clinial Biochemistry and Hematological Services of the Veterinary Faculty at the Autonomous University of Barcelona. Hamster livers were collected and portions were fixed in 4% formaldehyde for 24 hours or frozen in O.C.T.

Immunohistochemistry. Adenovirus late protein immunofluorescence was performed on O.C.T.-embedded tumor sections, as previously described 16, 29. Images of sections were obtained on an Olympus BX60 fluorescence microscope. To quantify adenovirus positive replication areas, five random fields in viable tissue zones for each tumor were captured at ×100 magnification. Quantification of percentage of stained areas was done using ImageJ software. For immunohistochemical analysis, paraffin-embedded sections of SKMel-28 tumors were treated with an anti-adenovirus antibody (Ab6982. Abcam; Cambrige, UK). Immunohistochemical staining was performed with EnVision (DAKO; Hamburg, Germany), according to the manufacturer's instructions.

Histochemical staining of hyaluronan. Paraffin-embedded blocks were cut into 4-µm thick sections. Sections were deparaffinized and endogenous peroxidase activity was blocked by incubation for 30 minutes in 0,3 % H_2O_2 in methanol. After re-hydratation, sections were blocked for 30 minutes with 10% Normal Goat Serum diluted in PBS. For HA staining, the slides were incubated with 5 µg/ml of a bionylated hyaluronic acid binding protein (HABP-b. Seikagaku; Japan) overnight at 4ºC. The specificity of HA staining was tested by pretreating some samples with 20 U/ml of bovine testes hyaluronidase (Sigma; St Louis, MO) at 37°C for 1h, prior to the addition of the HABP-b. After incubation with HABP-b, the slides were washed in PBS and treated with avidin-biotin-peroxidase kit (ABC KIT PK-4000. Vectastain; Burlingame, CA). After washings, sections were developed with DAB (DAKO Laboratories; Glostrup, Denmark), and counterstained with hematoxylin. To quantify HA positive areas, five random fields in viable tissue zones for each tumor (n=4) were captured at ×400 magnification. Quantification of percentage of stained areas was done using ImageJ software.

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Figure 1. **(a)** Schematic representation of viruses used in this study. All viruses contain the RGD peptide in the HI loop of the fiber knob protein and all oncolytic adenoviruses contain a deletion of 24 bp within the E1a region (E1a-Δ24). ICOVIR5 is an oncolytic adenovirus in which the endogenous E1A promoter has been replaced by the human E2F-1 promoter insulated with the DM-1. AdwtRGD and AdwtRGD-PH20 are RGD-modified wild-type adenoviruses. AdwtRGD-PH20 also contains a PH20 cassette inserted downstream of the fiber gene, consisting of a splice acceptor (SA) and a kozak sequence (k) in front of the PH20 cDNA and a polyadenylation sequence (pA). ICOVIR15 and ICOVIR17 are novel oncolytic adenoviruses that contain an E1a promoter modified by the insertion of eight E2F binding sites and one Sp1 binding site at the nucleotide site 415 of the Ad genome. ICOVIR17 also contains the PH20 cassette after the fiber gene. DM, myotonic dystrophy locus insulator; MLP, major late promoter. **(b)** Amino acid sequence of the C-terminal human PH-20 protein. The hydrophobic region responsible for signal attachment of a GPI anchor is underlined, and the cleavage site is represented in boldface type. The viruses used in this study express a soluble hyaluronidase lacking the cleavage site and the GPI membrane attachment motif. **(c)** Measurement of HA levels in the supernatants of various tumor cell lines by an Elisa-like assay. Mean ± SD is plotted. **(d)** Evaluation of HA levels within SKMel-28, NP9 and NP18 tumors by histochemical analisis using the biotinylated hyaluronan-binding protein (HABP-b).

Figure 2. **Coadministration of testicular bovine hyaluronidase enhances spread and antitumor activity of ICOVIR5. (a)** SkMel-28 s.c. tumor xenografts were treated intratumorally with PBS, testicular bovine hyaluronidase (PH20), ICOVIR5, or a combination of PH20 hyaluronidase and ICOVIR5. Tumor volume + SEM is plotted (n=10). #, significant ($p \leq 0.01$) compared with tumors treated with PBS; *, significant ($p \leq 0.01$) compared with tumors treated with PH20 hyaluronidase. **(b-c)** Analysis of extracellular matrix and adenovirus distribution in melanoma tumors treated with ICOVIR5 or ICOVIR5 and hyaluronidase obtained 14 days after administration. **(b)** Deparaffinized tumor sections were stained for HA using the HABP-b and counterstained with hematoxylin; Upper panel, magnification 40x. Lower panels, magnification 200x. **(c)** Upper panels, adenovirus immunodetection was performed in frozen sections with an anti-adenovirus antibody and counterstained with 4',6'-diaminidino-2-phenylindole (DAPI); 40x magnification. Lower panels, adenovirus immunodetection was performed in deparaffinized tumor sections with an anti-adenovirus antibody and counterstained with hematoxylin; 40x magnification.

Figure 3. **Expression of human testicular hyaluronidase enhances spread and antitumor activity of a replication-competent adenovirus. (a)** SkMel-28 s.c. tumor xenografts were treated intratumorally with PBS, AdwtRGD or AdwtRGD-PH20. Tumor volume + SEM is plotted (n=10). #, significant ($p \leq 0.005$) compared with tumors treated with PBS from day 10 to day 27; *, significant ($p \leq 0.025$) compared with tumors treated with AdwtRGD from day 7 to day 27. **(b)** Immunohistochemistry of melanoma tumors treated with AdwtRGD or AdwtRGD-PH20. Upper panel, tumor sections from tumors obtained 14 days after administration were stained for HA using the HABP-b; 400x magnification. Lower panels, adenovirus immunodetection was performed in frozen sections from tumors obtained at day 27 post-administration with an anti-adenovirus antibody and counterstained with DAPI; 100x magnification.

Figure 4. ***In vitro*** **characterization of ICOVIR17**. **(a)** PAGE-stained patterns of HA samples digested with supernatants of A549 cells obtained 48 h after infection with ICOVIR15 and ICOVIR17. Electrophoresis shows that ICOVIR17 expresses a soluble protein that mediates the depolymerization of hyaluronan. **(b)** SkMel-28 cells were infected with ICOVIR15 or ICOVIR17 at high MOI. At indicated time points, cell extracts were harvested and titrated by α-hexon staining. Mean ± SD is plotted. TU, transduction units.

Figure 5. **Expression of human PH20 enhances the antitumor activity of an oncolytic adenovirus. (a)** SkMel-28 s.c. tumor xenografts were treated intratumorally with PBS, ICOVIR15 or ICOVIR17. Tumor volume + SEM is plotted (n=10). #, significant ($p \leq 0.05$) compared with PBS-treated tumors from day 29 to day 42; *, significant ($p \leq 0.05$) compared with PBS-treated tumors from day 23 to day 42. **(b)**, SkMel-28 s.c. tumor xenografts were treated i.v. with a single dose of PBS, ICOVIR15 or ICOVIR17. Tumor volume + SEM is plotted (n=7-10). #, significant ($p \leq 0.03$) compared with tumors treated with PBS from day 20 to day 48; *, significant ($p \leq 0.05$) compared with tumors treated with ICOVIR15 from day 14 to day 48. **(c)** NP18 s.c. tumor xenografts were treated i.v. with a single dose of PBS, ICOVIR15 or ICOVIR17. Tumor volume + SEM is plotted (n=10). #, significant ($p \leq 0.02$) compared with PBS-treated tumors from day 14 to day 30; &, significant ($p \leq 0.05$) compared with PBS-treated tumors from day 14 to day 30; *, significant ($p \leq 0.02$) compared with ICOVIR15-treated tumors from day 12 to day 30. **(d)** SkMel-28 and NP18 tumors treated with ICOVIR15 or ICOVIR17 were obtained at the endpoint of each experiment (b-c) and were frozen in OCT. Adenovirus immunodetection was performed in frozen sections with an anti-adenovirus antibody and counterstained with DAPI; Representative sections of ICOVIR15 and ICOVIR17-treated tumors are shown. 40x magnification. **(e)** To quantify stained areas (which are indicative of virus replication), five random fields in viable tissue zones for each tumor (n=7-10) were captured at ×100 magnification. Quantification of percentage of stained areas was done using ImageJ software. *, significant ($p \leq 0.01$) compared with ICOVIR15-treated tumors

Figure 6. **Toxicity profile after systemic administration of ICOVIR17 in Hamsters.**
Syrian hamsters were injected intravenously with vehicle or with 4×10^{11} vp of ICOVIR15 or ICOVIR17. After 5 days of virus administration, animals were weighted and sacrificed and blood and liver samples were collected **(a)** Percentage weight gain of individual animals. The average values for **(b)** serum transaminases, **(c)** platelet and **(d)** leucocytes concentration in Hamster peripheral blood at day 5 after virus administration. Mean values + SE of 4-5 hamsters/group are plotted. #, significant ($p \leq 0.05$) compared with PBS-treated animals. **(e)** Livers of hamsters treated with PBS, ICOVIR15 or ICOVIR17 were excised at day 5 after virus administration. Paraffin sections of the livers were prepared and each section was stained with H&E. x400 magnification.

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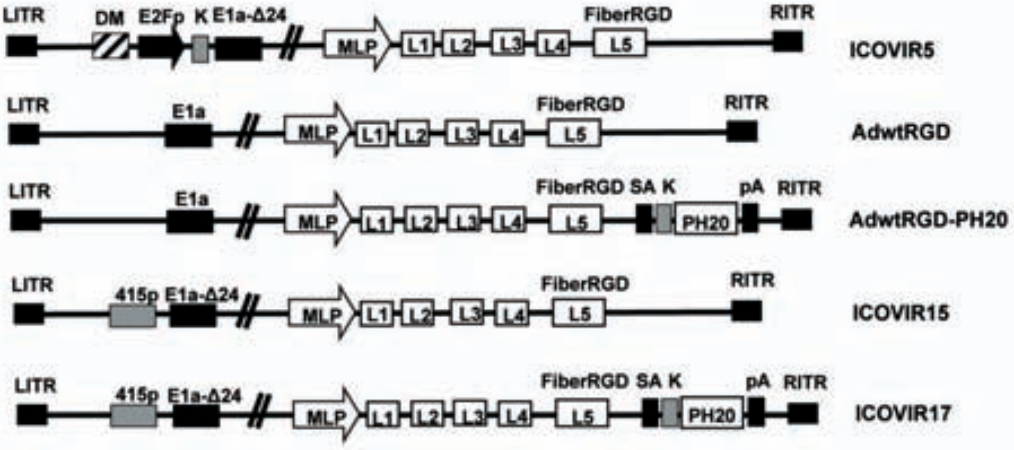

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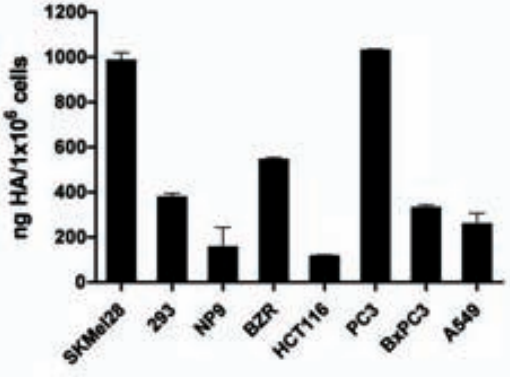

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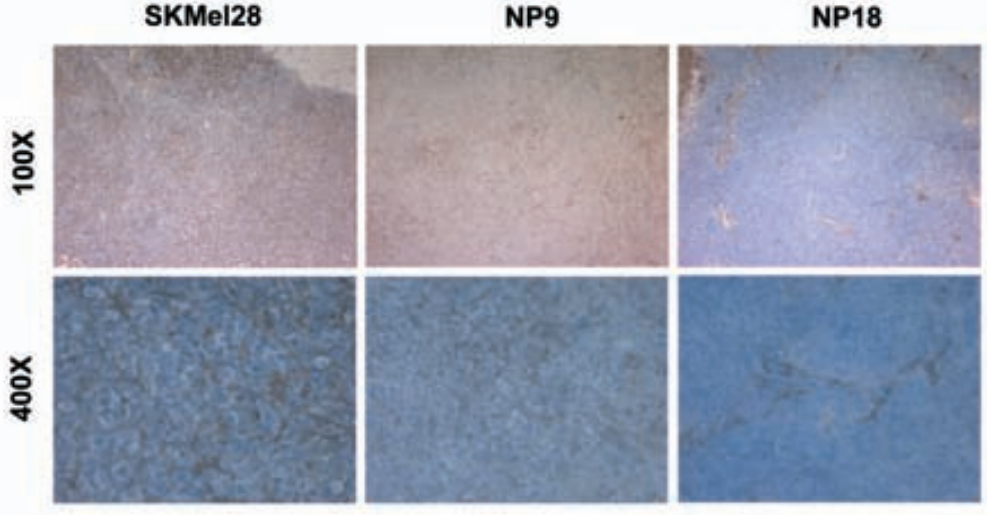

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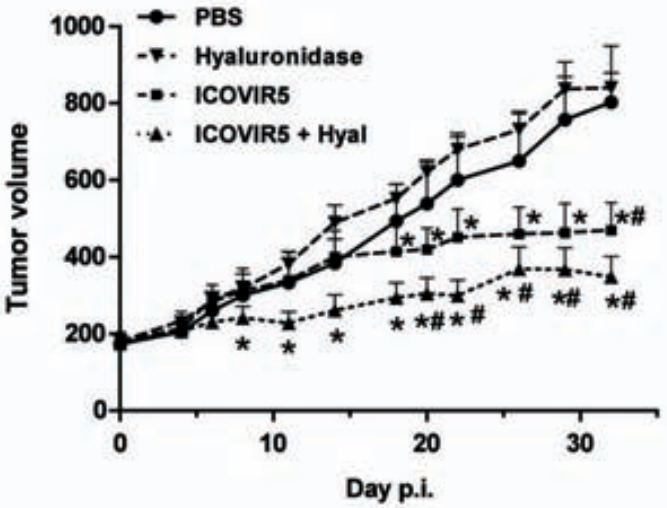
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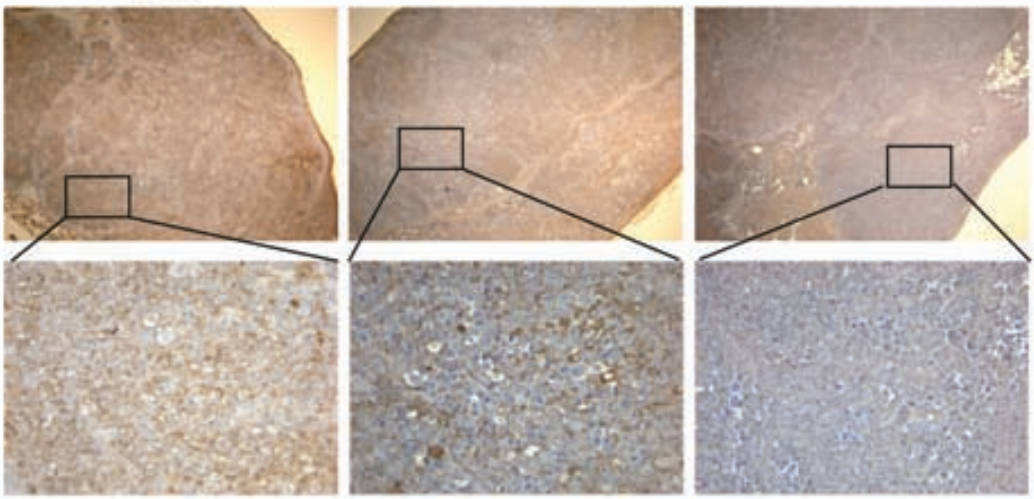
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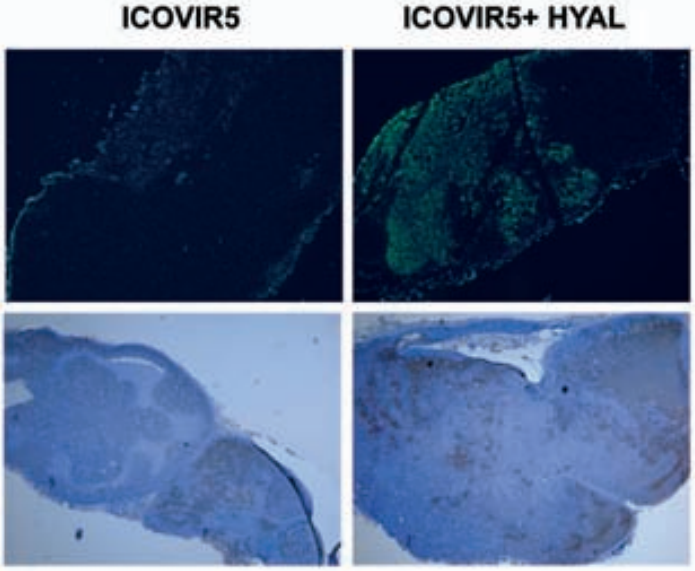
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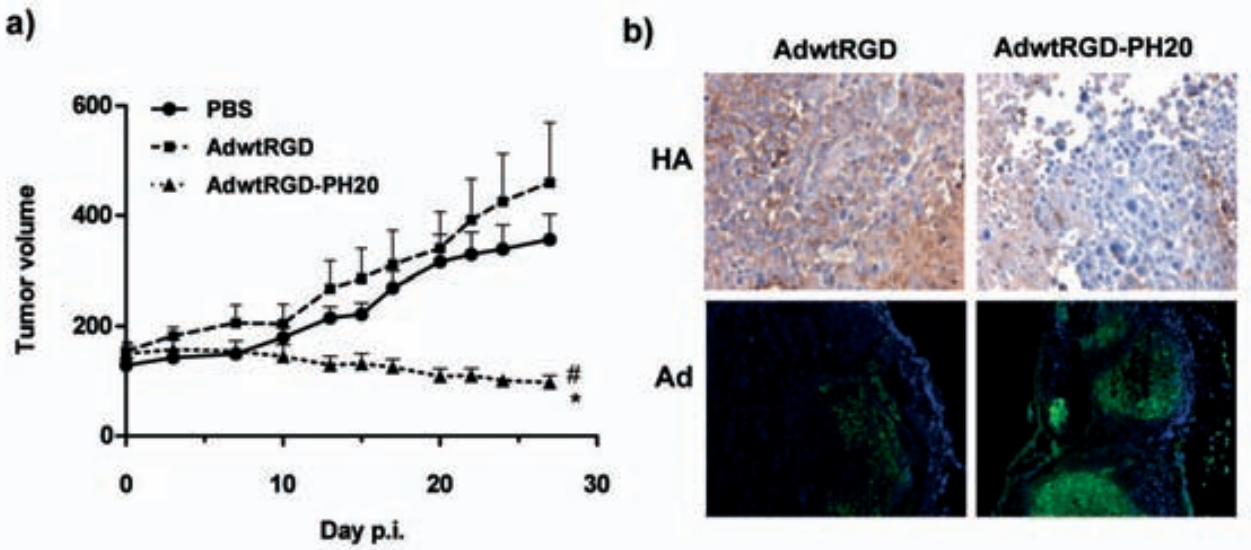
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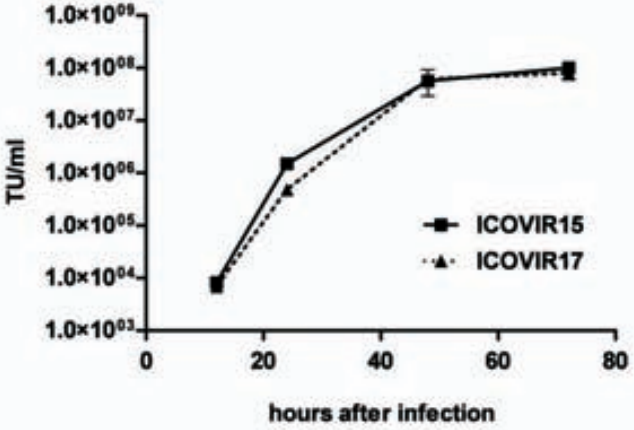
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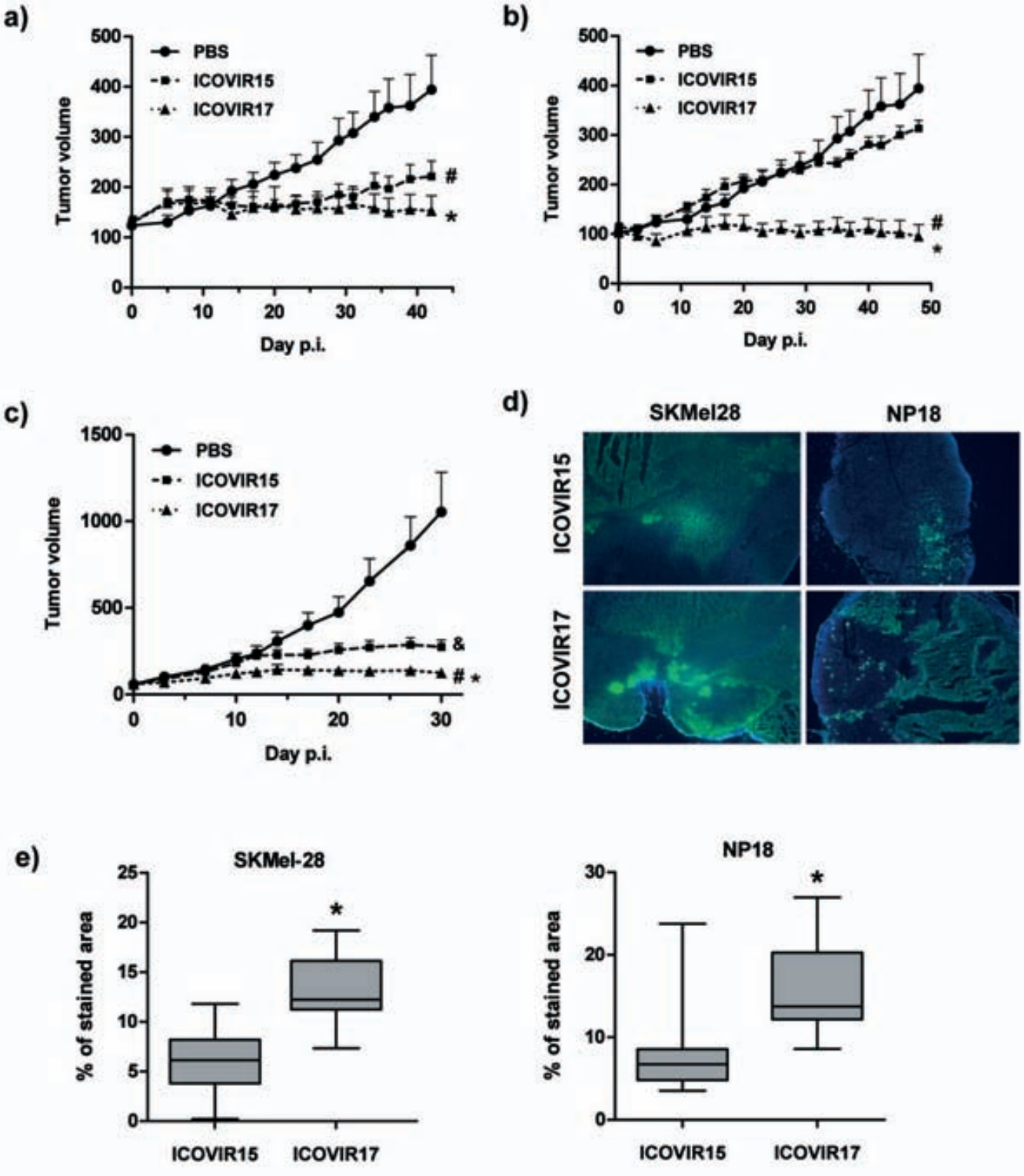
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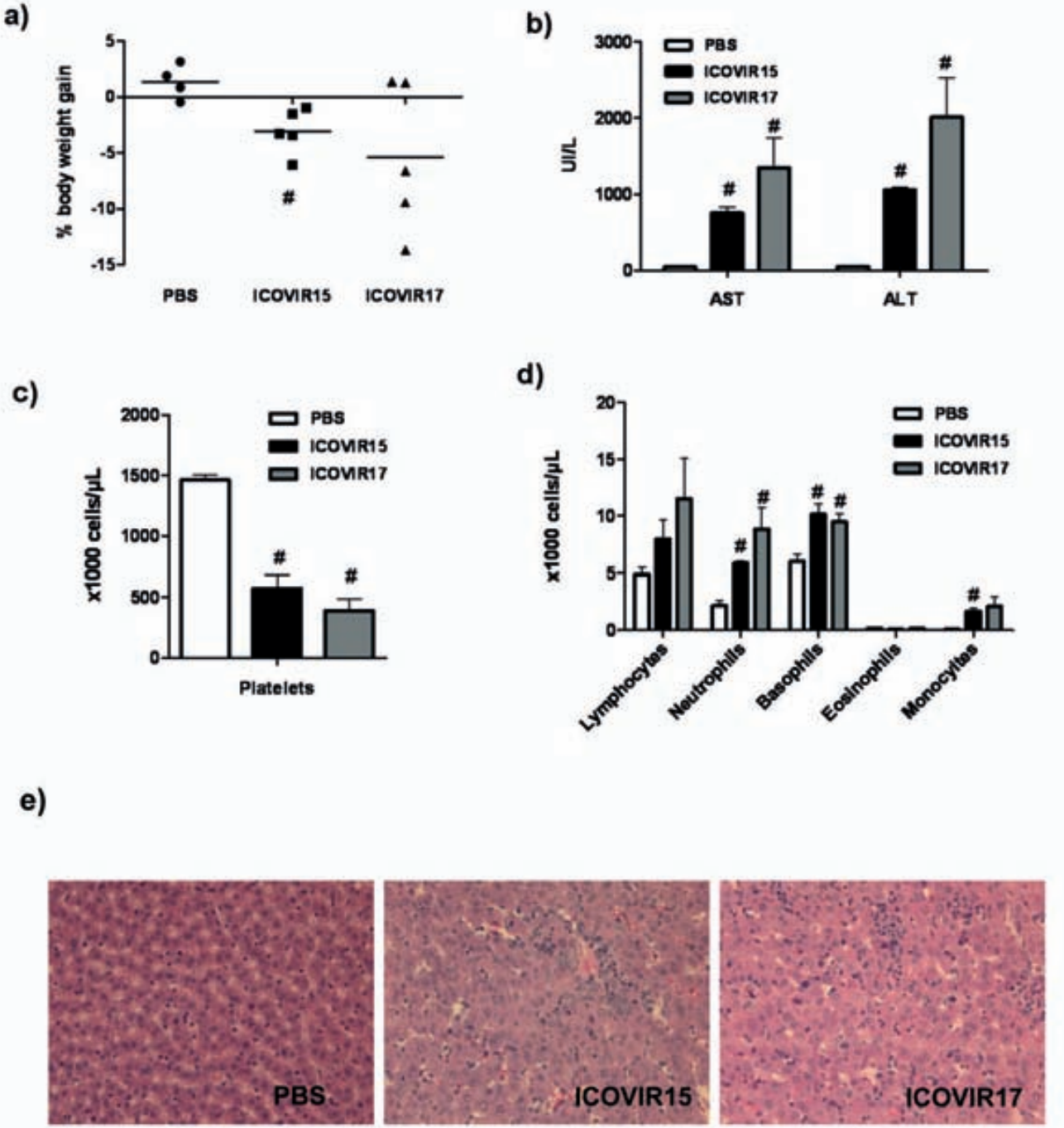
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Article enviat a Molecular Therapy

Minimal RB-responsive E1A promoter modification to attain potency, selectivity and transgene-arming capacity in oncolytic adenoviruses

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RUNNING TITLE: Improving systemic efficacy of RB-responsive CRADs

KEYWORDS: oncolytic adenovirus; transgene expression; E1A; E2F-binding sites; selectivity; antitumor efficacy

ABSTRACT

Oncolytic adenoviruses are promising anticancer agents due to their ability to self-amplify at the tumor mass. However, tumor stroma imposes barriers difficult to overcome by these agents. Transgene expression is a valuable strategy to counteract these limitations and to enhance anti-tumor activity. For this purpose, the genetic backbone in which the transgene is inserted should be optimized to render transgene expression compatible with the adenovirus replication cycle and to keep genome size within the encapsidation size limit. In order to design a potent and selective oncolytic adenovirus that keeps intact all the viral functions with minimal increase in genome size, we inserted palindromic E2F-binding sites into the endogenous E1A promoter. The insertion of these sites controlling E1A-Δ24 results in a low systemic toxicity profile in mice. Importantly, the E2F-binding sites also increased the cytotoxicity and the systemic anti-tumor activity relative to wild-type adenovirus in all cancer models tested. The low toxicity and the increased potency results in improved anti-tumor efficacy after systemic injection and increased survival of mice carrying tumors. Furthermore, the constrained genome size of this backbone allows an efficient and potent expression of transgenes, indicating that this virus holds promise for overcoming the limitations of oncolytic adenoviral therapy.

INTRODUCTION

Despite great advances in the treatment of cancer, it remains one of the leading causes of mortality world-wide. Hence, research on novel cancer therapies with a high therapeutic index limited to malignant tissues is crucial. Among new treatments proposed to target cancer, oncolytic adenoviruses are a promising and appealing therapy due to their ability to self-amplify selectively at the tumor site [1]. Several oncolytic adenoviruses have already been tested in clinical trials involving a variety of tumors and routes of administration. Clinical data revealed a good toxicological and safety profile, but some potentially concerning adverse effects were observed after administration at high doses [2]. With regard to efficacy, most responses detected were transient and the treatment was not able to alter significantly the course of the disease. Overall, this data indicates out a critical need for improved oncolytic potency to result in sustained therapeutic responses in humans.

For an efficient treatment of tumors at an advanced stage, systemic delivery is required. However, the virus encounters some limitations when injected systemically. First of all, it is quickly eliminated from the bloodstream by the liver [3] or inactivated by binding to blood cells, neutralizing antibodies or complement [4], and only a minimal proportion of the injected dose reaches the tumor. Once in the tumor, the stroma and the antiviral immune response limit the spread of the virus throughout the tumor [5]. The expression of a therapeutic transgene from the adenoviral backbone is a rational and efficient approach to circumvent these limitations. Armed replicating adenoviruses are a combination of virotherapy and gene therapy strategies in which the input of transgene dose is amplified by replication of the virus and, above all, gene transfer can amplify the antitumor activity of virotherapy. In this regard, several transgenes have been inserted into oncolytic adenoviruses in order to increase cytotoxicity, to stimulate immune responses or to digest the connective tissue to facilitate intratumoral spread [6]. However, encapsidation size of adenoviruses is limited to 105% of the wild-type genome and larger genomes result in genetic instability and packaging problems [7]. E3 genes have been deleted to create space for transgenes, but E3 has important immune-inhibitory functions that may facilitate virus spread in immunocompetent hosts [8]. Thus, further research is needed to optimize the transgene expression machinery and the adenoviral backbone in which the transgene is inserted in order to minimize genome size and make transgene-expression compatible with both selective and potent replication.

Taking into consideration the concerning adverse effects observed in clinical trials after systemic injection of oncolytic adenoviruses [2], it is important to restrict virus gene expression to tumor cells to ensure virus safety. In mice, E1A expression in hepatocytes is enough to cause transaminitis and severe liver injury [9], and this toxicity is not prevented by deletions in other

viral genes that confer selectivity. Modification of E1A transcriptional control is a useful approach to avoid this toxicity. Tissue-specific promoters have been tested in this regard to treat certain types of cancer, such as the PSA promoter to target prostate cancer [10] or the uPAR promoter for pancreatic tumors [11]. However, promoters with a broader tropism are more appealing due to their applicability to different tumor types. Those active in tumors and repressed in normal tissues, such as E2F-1 [12], HIF [13] or hTERT [14], are an ideal option. Nevertheless, some losses of potency with respect to wild-type transcription control were reported when these promoters were placed to control E1A, especially when tested in a wide-range of cancer models.

In a recent work [15], we demonstrated that the incorporation of E2F-responsive palindromes in an insulated E2F-1 promoter controlling E1A-Δ24 resulted in increased oncolytic potency with a low systemic toxicity profile. The E2F-responsive palindromes boosted a positive feedback loop turned on in cancer cells involving E1A and E4-6/7. However, the combination of genetic elements present in the resulting oncolytic adenovirus (ICOVIR-7) raised its genomic size close to the 105% packaging limit and this hindered the incorporation of transgenes to this highly tumor-selective backbone. In the present study, we developed a novel oncolytic adenovirus (ICOVIR-15) that achieved selective and potent replication in tumor cells with a genomic size that only exceeds the native Ad5 size by 151 base pairs. Four palindromic E2F binding sites and one Sp-1 binding site were inserted in the endogenous E1A promoter to redirect E1A-Δ24 transcription towards pRB deregulation. As a consequence, viral replication was enhanced compared to the wild-type virus in all cancer models tested and potent anti-tumor activity was achieved when injected systemically in tumor models *in vivo*. Toxicity in mice of the wild-type virus was considerably reduced by this modification and allowed safe systemic administration at high doses. Importantly, the reduced genome size of ICOVIR-15 improves its suitability for transgene expression and this virus offers considerable promise for cancer gene-virotherapy.

RESULTS

E1A promoter modification

The transcription of the E1A gene in wild-type Ad5 is regulated by a variety of cellular and viral protein factors [16]. The transcriptional control region of E1A extends from the left terminus of the virus genome to the E1A cap site and contains the replication origin of Ad DNA, the inverted terminal repeat, the packaging elements, the transcriptional enhancer elements for the E1A gene and other viral early genes, and the promoter proximal elements of the E1A gene (Figure 1). ICOVIR-15 is a novel AdΔ24-RGD-derivative oncolytic adenovirus which incorporates four E2F-responsive palindromes downstream of the packaging signal to redirect E1A transcription towards deregulation of the retinoblastoma (Rb/p16) pathway. These palindromic binding sites are responsible for the potency and selectivity of the E2F-1 promoter and play a dual role controlling transcription in response to E2F status [17]. Furthermore, one Sp-1 binding site was also inserted as both transcription factors interact to cooperatively activate transcription [18]. Figure 1 represents schematically the binding sites inserted into the E1A promoter and their different role in tumor or normal cells. Moreover, genome sizes of Adwt-RGD and ICOVIR-15 are also indicated. Importantly, this modification preserves the original structure of the adenovirus genome and the function of the E1A enhancer region, which may contribute to an efficient selective replication in the cancer cell without affecting the antitumor potency.

The palindromic E2F-binding sites in ICOVIR-15 enhance the antitumor activity of Adwt-RGD in vitro *and* in vivo

Due to deregulation of the retinoblastoma (Rb/p16) pathway, E2F is ubiquitously released from the E2F-Rb complexes in tumor cells [19]. The binding of free E2F to E2F-palindromic sites in the E1A promoter of ICOVIR-15 should enhance the transcription of the E1A gene. Higher levels of E1A proteins should enhance the transactivation of the other

adenoviral early proteins, including E4-6/7. E4-6/7 protein forms a complex with two E2F transcription factors and induces the cooperative and stable binding of this complex to the palindromic E2F-binding sites structure present in the E2 promoter [20] and in the E1A promoter of ICOVIR-15, activating a positive feedback that should culminate in higher production of virus progeny and lysis of the tumor cell. To confirm this hypothesis, a panel of tumor cell lines was evaluated for the effects of ICOVIR-15 infection. As shown in Figure 2a, E1A protein levels after infection with ICOVIR-15 were higher than those after infection with the parental virus Adwt-RGD in all cancer cell lines tested when analyzed by western blot and quantified by densitometry. E1A protein levels from ICOVIR-15 were 1.8 times, 3.4 times and 4 times higher in A549, Sk-mel28 and NP-9, respectively, compared to Adwt-RGD levels.

To determine whether this increase in E1A amounts has a positive impact on the anti-tumoral potency, virus progeny production and cytotoxicity assays were performed. Virus progeny production was increased significantly for ICOVIR-15 in two out of four cancer cell lines tested, showing a trend for improvement in the other two (Figure 2b). When cytotoxicity was analyzed at days 5-8, the improvement became more evident since more than one cycle of replication occurred. The E2F-binding sites rendered ICOVIR-15 more cytotoxic since the amount of virus required to reduce the cell culture viability by 50% (IC_{50}) was 3.5 times, 2.5 times, 2.5 times and 1.8 times lower in Sk-mel28, NP-9, Saos-2 and NP-18 cells, respectively, compared to the parental virus Adwt-RGD (Figure 2c).

Once we had demonstrated the improved oncolytic potency of ICOVIR-15 *in vitro*, we tested whether this benefit is maintained in a tumor xenograft model. As disseminated disease is the most relevant clinical situation, systemic injection was used. Mice bearing Sk-mel28 (melanoma) tumors were injected with a single intravenous dose of phosphate-buffered saline (PBS), Adwt-RGD or ICOVIR-15. The virus dose was $2.5x10^{10}$ viral particles per mouse, which is the maximum tolerated dose for systemic injection of wild-type adenoviruses in mice [12]. ICOVIR-15 was more efficient at delaying the growth of Sk-mel28 subcutaneous tumors *in vivo* (Figure 2d). At day 37, when untreated mice had to be killed due to uncontrolled tumor growth, tumor size of Adwt-RGD treated mice was 1.5-fold larger than in those treated with ICOVIR-15 ($P<0.025$). Tumor size in PBS-treated mice was 1.8-fold larger than in the ICOVIR-15 group ($P<0.005$).

In vivo *systemic toxicity after ICOVIR-15 administration in Balb/C immunocompetent mice*

Binding of pRb-E2F-HDAC complexes to E2F sites inserted into the E1A promoter should restrict transcription and diminish E1A-mediated toxicity in normal cells, thus allowing systemic administration for the treatment of disseminated neoplasias. The toxicity associated to a single intravenous dose of ICOVIR-15 or controls was assessed in Balb/C immunocompetent mice. Viruses were injected at a dose of $5x10^{10}$ viral particles per mouse and body weight was monitored daily. Adwt-RGD-injected mice were sacrificed at day 4 after injection due to high decrease in body weight and high morbidity. In contrast, ICOVIR-15-injected group only suffered a 1% reduction in the body weight (not significant) at day 3 post-administration and underwent a rapid recovery to PBS levels (Figure 3a).

After systemic injection of adenoviruses, a large proportion of the oncolytic agent ends up in the liver. As a consequence, transaminase elevation has been reported in clinical trials after high-dose injection [2]. When transaminase enzymes (AST and ALT) were analyzed in Adwt-RGD-treated mice at day 4 after injection, up to a 55-fold elevation of both AST and ALT was detected compared with non-treated animals (Figure 3b). On the other hand, only a slight, transient transaminase elevation was detected in the ICOVIR-15-injected group. At day 4 post-administration, a sevenfold elevation in AST (not significant) and an 11-fold in ALT ($P<0.05$) were detected, but these elevations resolved by day 12. Furthermore, although a serious thrombocytopenia was observed after Adwt-RGD injection (fivefold reduction, $P<0.02$), platelet depletion mediated by ICOVIR-15 was only 1.5-fold at day 4 and absent at day 12 (Figure 3c).

In addition, E1A expression was evaluated in murine liver sections by immunostaining as it is the main cause of toxicity in mice [9]. Strong E1A staining was detected throughout livers from Adwt-RGD-injected mice (Figure 4a). On the contrary, little E1A was detected in livers from mice treated with ICOVIR-15 and this correlates with a histological analysis of liver

sections (Figure 4b). While evident symptoms of degenerative cirrhosis (macrosteatosis, presence of Councilman bodies and large necrotic areas) were detected in livers from the Adwt-RGD group, livers from ICOVIR-15-treated mice displayed an almost normal phenotype, with only marginal Councilman bodies in the superficial areas. These data indicate that the E2F-responsive palindromes inserted to control E1A expression are actively repressing transcription in normal cells. Once we confirmed that the dose of $5x10^{10}$ viral particles of ICOVIR-15 per mouse was well-tolerated, we evaluated the systemic anti-tumoral activity of this novel oncolytic agent at this dose in several tumor xenograft models.

ICOVIR-15 exhibits potent anti-tumor efficacy and prolongs mouse survival after systemic administration

A549 (lung), NP-9 and NP-18 (pancreatic adenocarcinoma), PC-3 (prostate) and Sk-mel28 (melanoma) were selected as subcutaneous models in order to represent a range of tumor types. Mice carrying tumors were injected with a single intravenous dose of ICOVIR-15 at $5x10^{10}$ viral particles per mouse or PBS. When untreated animals displayed uncontrolled tumor growth, mice were sacrificed. At sacrifice, ICOVIR-15 treatment induced a reduction of more than 3.7-fold ($P<0.0012$), threefold ($P<0.012$), 2.3-fold ($P<0.00007$), 2.8-fold ($P<0.0001$) and 1.7-fold ($P<0.03$) in the tumor size of NP-18, A459, Sk-mel28, NP-9 and PC-3 subcutaneous tumors, respectively (Figure 6a). Importantly, efficient tumor growth reduction was achieved in all subcutaneous tumor models tested. In addition, ICOVIR-15 treatment was able to significantly increase survival in all subcutaneous tumor models tested (Figure 6b).

The reduced genome size of ICOVIR-15 allows potent transgene expression without affecting viral kinetics

Late phase expression of transgenes which promote viral spread or have cytotoxic effects may improve the ability of oncolytic adenovirus to eradicate tumors [1]. It is preferable to restrict transgene expression to the late phase of the virus replication cycle in order to minimize any antagonism between transgene activity and virus replication [21]. However, the ability of Ad5 to carry exogenous DNA is limited by its encapsidation capacity, which is approximately 38 kb [7]. To determine if the ICOVIR-15 backbone could be compatible with late transgene expression, we cloned the NfsA nitroreductase gene downstream of the fiber (Figure 6a). The NfsA gene included the 3VDE splicing acceptor (IIIa virus infection-dependent splicing enhancer) and a polyA sequence in order to promote its expression during the late phase of the viral life cycle.

To establish whether the insertion of NfsA alters virus replication, we compared virus progeny production and release of ICOVIR-15 and ICOVIR-15-NfsA. A549 cells were infected at high MOI and the amount of virus produced and released was determined at different time points. As shown in Figure 6b, the production and release kinetics of both viruses were similar at every time point, indicating that the transgene is not reducing the oncolytic potency of ICOVIR-15. Furthermore, the timing of nitroreductase expression was evaluated by Western-blot analysis to confirm late phase expression. As shown in Figure 6c, NfsA was undetectable 24 hours after infection, its expression was slightly detected at 48 hours, and high amounts of transgene accumulated 72 hours post-infection.

In summary, these results indicate that the new E2F binding sites introduced into the E1A promoter in ICOVIR-15 are able to increase the antitumor potency of the wild-type adenovirus while drastically reducing toxicity after systemic injection. Moreover, its constrained genome size permits the insertion of transgenes without affecting the oncolytic potency.

DISCUSSION

Oncolytic adenoviruses have proven efficacious and relatively safe in the clinics [2]. However, clinical response rates are suboptimal and further research is needed to improve the antitumor potency of these agents. One of the main limitations for efficacy is the inability of oncolytic adenoviruses to spread sufficiently within solid tumors [5]. Some strategies aiming to increase adenovirus spread include mutations in E1B-19K [22], overexpression of the adenovirus death protein (ADP) [23], or c-terminal truncating mutations in the i-leader protein [24]. However, several side-effects such as virus yield reduction [25] or partial loss of the E3 immunomodulatory functions [26] have been reported with these approaches. An alternative strategy aiming to increase virus spread within the tumor mass is the expression of transgenes from the adenovirus backbone, such as proteases to disrupt the extracellular matrix [5] or enzymes that convert prodrugs to diffusible cytotoxins that provide bystander killing of surrounding cells [27]. However, the insertion of transgenes into the adenovirus genome is limited by the encapsidation size and requires transgene compatibility with the adenovirus replication cycle.

For an effective therapy, systemic delivery of the oncolytic agent is preferred in the clinical setting as patients may have inaccessible tumors or tumors that are already metastatic at the time of detection. Our group has previously described ICOVIR-7, an oncolytic adenovirus showing antitumor efficacy in a wide range of subcutaneous tumor models after systemic administration, with a low toxicity profile [15]. ICOVIR-7 controls the transcription of the *kozak*-E1A-Δ24 protein via an improved E2F-1 promoter insulated with the myotonic dystrophy locus insulator. It also contains an RGD-modified fiber to increase virus infectivity. Due to the benefits that the expression of certain transgenes may grant in terms of oncolytic potency, we considered arming this virus. However, the combination of all the genetic elements present in ICOVIR-7 raised its genome size to 37053 bp, close to the 105% packaging limit [7], and when we attempted to introduce transgenes into this backbone, it resulted in genome instability and packaging problems (data not shown). As a measure to gain DNA insertion capacity, we deleted ORF 1 and 2 or ORF 1, 2 and 3 of the adenovirus E4 region. Although no important functions in replication have been associated with these proteins [28], the resulting viruses showed significant loss of potency and replication defects in cancer cells compared with their controls (unpublished data). Thus, we sought a novel modification that restricted a potent E1A transcription to cancer cells with only minimal increase in genome size, in order to retain the potential for efficient delivery and expression of additional transgenes.

Deregulation of the retinoblastoma (Rb/p16) pathway is a hallmark of tumor cells [29]. As a consequence of Rb pathway defects, E2F transcription factors are released, allowing activation of promoters containing E2F sites. Furthermore, the same E2F-binding sites can mediate silencing of these promoters in quiescent cells due to the formation of a complex involving E2F-pRb complexes and histone deacetylases [30]. An oncolytic adenovirus which controls E1A transcription by E2F sites may acquire potent, tumor-selective replication in a wide range of cancer cells. Importantly, a palindromic E2F-binding site pattern was described to perform an important role in adenovirus life cycle: the E4-6/7 protein forms a complex with two E2F transcription factors and induces the cooperative and stable binding of E2F to this palindromic pattern to activate transcription [20]. This pattern is present in the E2F-1 cellular promoter [17] as S-phase induction is a requisite for the adenoviral life cycle. Moreover, this pattern is also present in the adenovirus type 5 E2A promoter to boost its transcription in response to E1A and E4 activation [31]. The presence of this pattern in the adenoviral genome suggests an optimal structure in terms of DNA length to confer efficient E2F-responsiveness. Thus, we decided to construct a novel oncolytic adenovirus which contained palindromic E2F-binding sites in the E1A promoter. ICOVIR-15 incorporates eight new E2F-binding sites organized as four imperfect palindromes downstream of the packaging signal (Figure 1). Downstream of these palindromic sites, we also inserted one Sp-1-binding site, as Sp-1 was reported to play an important role in the E2F-controlled promoters by interacting and cooperating to activate transcription [18]. Contrary to an insulated exogenous promoter, this modification preserves the function of the element II in the E1A enhancer, which was previously reported to activate transcription of all of the early promoters in *cis* [32]. Moreover, as a measure to prevent an auto-activation loop by E2F

in case of promoter leakage, the pRb-binding site of E1A was deleted (Δ24 deletion) [33]. An RGD motif was also inserted at the HI-loop of the fiber to increase virus infectivity to cancer cells [34].

Taking into consideration that the mouse is not permissive for human adenovirus replication, the main toxicity in mice is associated with E1A expression [9]. The palindromic E2F-binding sites present in ICOVIR-15 may repress E1A transcription in quiescent cells due to binding of E2F-pRb-histone deacetylase complexes, which strengthen the association of nucleosomes with DNA [30]. The systemic administration of wild-type human adenovirus at a dose of $5x10^{10}$ viral particles per mouse caused an increase in transaminases levels, degeneration of liver tissue, hematological alterations and severe weight loss (Figures 3 and 4). In fact, this dose represents more than the LD_{50} value for adenoviruses controlling E1A under the wild-type promoter [12]. In contrast, the injection of ICOVIR-15 at the same dose only caused slight alterations in ALT levels and in platelet concentration at day 4 (Figure 3). This toxicity was transient and a complete recovery was achieved 12 days after the treatment. Thus, we can conclude that the insertion of palindromic E2F sites drastically reduces the toxicity following systemic administration of wild-type human adenovirus to mice.

Furthermore, our results demonstrate that the novel palindromic E2F-binding sites enhance the anti-tumor activity compared to wild-type human adenovirus. In contrast to some reports in which reduced E1A expression had little effect on replication [35, 36], an increased level of E1A led to an improved viral yield, and this resulted in improved cytotoxicity in a variety of cell types including melanoma, osteosarcoma and pancreatic adenocarcinomas (Figure 2a, b and c). Importantly, oncolytic potency improved in all tumor cell lines tested, in contrast to our previously described viruses where the presence of an insulated E1A promoter may limit the capacity of E1A enhancers to activate the other viral proteins [12, 15]. The benefit of ICOVIR-15 in terms of oncolytic potency was confirmed *in vivo*, where a single intravenous injection significantly increased the tumor growth inhibition relative to Adwt-RGD (Figure 2d). This experiment was performed at the maximum tolerated dose for Adwt-RGD by systemic injection. As ICOVIR-15 has lower toxicity than this non-selective virus and allows systemic injection at higher doses, the anti-tumor activity of a dose of $5x10^{10}$ viral particles per mouse was tested in a wide range of subcutaneous xenograft tumors, including melanoma, prostate, lung and pancreatic adenocarcinoma. A substantial benefit in the control of tumor growth and a significant increase in survival were observed compared to non-treated animals (Figure 5), suggesting an important anti-tumor activity of ICOVIR-15 when injected systemically.

As previously reported [37], a histology analysis of tumors after adenovirus administration revealed that the presence of tumor stroma limits the complete intratumoral spread of the oncolytic agent (data not shown). This was especially noteworthy in the PC-3 xenograft model, which expresses high amounts of matrix components [38]. The expression of transgenes which contribute to disruption of the stromal barriers, such as proteolytic enzymes to degrade the matrix components [39] or prodrug converting enzymes to kill fibroblastics [27], may be used to enhance the viral spread within the tumor. Importantly, ICOVIR-15 has a similar genome size to wild-type adenovirus (only 150 base pairs more), which may facilitate the incorporation of transgenes. In order to evaluate whether the insertion of a transgene has negative effects on ICOVIR-15 replication, we constructed an armed version which incorporates an expression cassette of the transgene NfsA. NfsA is the major *Escherichia coli* nitroreductase and it can activate a variety of nitroaromatic prodrugs for cancer gene therapy [40]. The replication and release of ICOVIR-15-NfsA were indistinguishable from those of its unarmed control (Figure 6b), indicating the suitability of ICOVIR-15 for delivering additional transgenes. To maintain tumor-selective expression of the transgene, we inserted additional splicing signals within the major late transcription unit. This strategy confers E1A-dependent transcription and achieves efficient, regulated transgene expression with a small DNA sequence. The 3VDE sequence (IIIa virus infection-dependent splicing enhancer) from the adenoviral IIIa protein is a useful splicing signal for late transgene expression because it is subject to a strict regulation during virus infection [41, 42]. As shown in Figure 6c, the expression of NfsA driven by the 3VDE sequence in ICOVIR-15-NfsA was strictly restricted to the late phase of the viral cycle.

In addition to transgenes which enhance cytotoxicity or adenoviral distribution within the tumor, other trangenes may enhance the antitumor activity of ICOVIR-15. The expression of transgenes that modulate the tumor microenvironment, such as murine endostatin to inhibit the process of angiogenesis or TIMP3 to inhibit the action of matrix metalloproteinases, may improve the oncolysis by hindering tumor development [43, 44]. Furthermore, the expression of fusogenic proteins, such as GALV protein, may also enhance anti-tumor activity by syncytium formation [41]. On the other hand, a different strategy is the expression of factors which recruit immune cells to the site of infection and induce their proliferation and activation. This strategy has the potential to destroy not only the primary tumor, but also remote metastases. Cytokines, such as GM-CSF or MCP-3, or interleukins, such as IL-4 or IL-24, have been used previously to arm oncolytic adenoviruses [6]. All these strategies would confer different characteristics to ICOVIR-15 and warrant testing as candidates for clinical trials.

In summary, our results indicate that ICOVIR-15 displays an appealing efficacy to toxicity ratio. On one hand, the inserted palindromic E2F-binding sites reduce the toxicity caused by systemic injection of adenoviruses. On the other hand, the same binding sites enhance the oncolytic potency even when compared to the non-selective wild-type adenovirus, which is particularly noteworthy due to the requirements of enhanced potency in the clinics. Importantly, the broad spectrum and the small genome size of this novel oncolytic adenovirus represents an optimal backbone to insert transgenes without deleting viral functions, which may help to overcome the barriers imposed by the complex architecture of tumors.

MATERIALS AND METHODS

Cell culture

HEK293 (human embryonic kidney cells), A549 (human lung adenocarcinoma), SkMel-28 (melanoma), PC-3 (prostate adenocarcinoma) and Saos-2 (osteosarcoma) cell lines were obtained from the American Type Culture Collection (Manassas, VA). NP-9 and NP-18 (pancreatic adenocarcinomas) cell lines were established in our laboratory [45]. Isrec-01 (colon cancer cell line) was a kind gift from Dr. R. Iggo (Institut Bergonié, Bordeaux, France). All tumor cell lines, excluding Isrec-01, were maintained in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37ºC, 5% CO_2. Isrec-01 cells' DMEM was supplemented with 10% fetal bovine serum.

Viruses

Adwt-RGD and AdTL-RGD have been previously described [46, 47]. Adwt-RGD was propagated in A549 cells and the replication-deficient AdTL-RGD and Ad-CMV-NfsA were propagated in HEK293 cells. ICOVIR-15 was created by inserting four E2F-binding site hairpins and one Sp-1-binding site following nucleotid 415 in the E1a promoter of AdΔ24-RGD [46]. To achieve this, a unique *BsiWI* site was created by site-directed mutagenesis in the adenoviral shuttle vector pEndK/SpeI. An Sp1 site was introduced using this plasmid digested with *BsiWI* and the annealed oligonucleotides Sp1F (5'-GTACGTCGACCACAAACCCCGCCCAGCGTCTTGTCATTGGCGTCGACGCT-3') and Sp1R (5'-GTACAGCGTCGACGCCAATGACAAGACGCTGGGCGGGGTTTGTGGTCGAC-3'). E2F hairpins were introduced using the annealed oligonucleotides E2FF2 (5'-GTACGTCGGCGGCTCGTGGCTCTTTCGCGGCAAAAAGGATTTGGCGCGTAAAAGTG GTTCGAA-3') and E2FR2 (5'-GTACTTCGAACCACTTTTACGCGCCAAATCCTTTTTGCCGCGAAAGAGCCACGAGCC GCCGAC-3') to create pEndKBsi415Sp1E2F2. For homologous recombination in yeast, the yeast replication elements and a selectable marker (CAU fragment [48]) were cloned into this vector. The pEndKBsi415Sp1E2F2CAU linearized with *KpnI* was recombined with AdΔ24-RGD genomic DNA in *Saccharomyces cerevisiae* YPH857 to construct pICOVIR-15. ICOVIR-15 was obtained by transfection into HEK293 cells of the large *PacI* fragment of pICOVIR-15. ICOVIR-15-NfsA was constructed taking advantage of an *SpeI* site present in pAdwt-GALV-CAU, a replication competent adenovirus previously described by us [41]. By homologous

recombination in yeast, the E2F and Sp1 sites, as well as the Δ24 mutation, were introduced. To clone the NfsA gene downstream of the fiber protein, plasmid pPS1374J1 was created by amplifying the NfsA from the pSV035 plasmid [40] using oligonucleotides 5'-CGTCAATTGTACTAAGCGGTGATGTTTCTGATCAGCCACCATGACGCCAACCATTG-3' and 5'-CAGCAATTGAAAAATAAAGTTTATTAGCGCGTCGCCCAACCCTG-3' and cloning the *MfeI* digested PCR product into the *MfeI* site of pNK-FiberRGD [41, 49]. The pICOVIR-15-NfsA plasmid, containing the complete ICOVIR-15-NfsA genome, was created by homologous recombination in yeast between pPS1374J1, digested with *KpnI* and *NotI*, and the pAdwt-GALV-CAU with E2F and Sp1 sites, digested with *SpeI*. ICOVIR-15-NfsA was obtained by transfection in HEK293 cells of the *PacI* fragment of pICOVIR-15-NfsA. Viruses were plaque-purified, amplified in A549 cells and purified using a CsCl gradient. Viral genomes were verified by restriction analysis and by sequencing E1A promoter, E1A-Δ24, RGD-modified fiber and nitroreductase NfsA using oligonucleotides oligo22 (5'-AAGTGTGATGTTGCAAGTGT-3'), Ad670F (5'-ATCTTCCACCTCCTAGCCAT-3'), FiberUp (5'-CAAACGCTGTTGGATTTATG-3') and FiberDown2 (5'-GGCTATACTACTGAATGAA-3').

Protein expression analysis

Cell cultures seeded in 24-well plates were infected at a multiplicity of infection that allowed at least 80% infectivity (MOI of 20 for Sk-mel28, NP-9 and PC-3, MOI of 10 for A549 cells). Whole-cell protein extracts were prepared at indicated time after infection by incubation in lysis buffer (400 mM NaCl, 1 mM EDTA, 5 mM NaF, 10% glycerol, 1mM sodium orthovanadate, 0.5% Nonidet NP-40 and a mixture of protease inhibitors (Sigma, St. Louis, MO) in 10 mM Tris-HCl pH 7.4) for 1 hour at 4ºC. Clarified samples (15 μg/lane) were separated by a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare, Arlington Heights, IL). For E1A protein, detection was performed by immunoblotting membranes using a polyclonal anti-E1A primary antibody (Rabbit, Clone 13S-5) (Sta. Cruz Biotechnology, Santa Cruz, CA) and a polyclonal anti-rabbit conjugated with HRP (Goat, DakoCytomation). E1A bands from Western-blot were quantified using BIO-RAD GS-800 densitometer and normalized using unspecific bands detected by the anti-E1A primary antibody. For NfsA detection, membranes were immunoblotted using a sheep anti-NfsA serum obtained from Alta Bioscience (Birmingham, UK), using purified NfsA protein kindly provided by Dr. E. Hyde and Dr. D. Jarrom (School of Biosciences, University of Birmingham, UK), and peroxidase-conjugated donkey anti-sheep secondary andibody (Sigma). A mouse monoclonal anti-β tubulin antibody and a peroxidase-conjugated anti-mouse antibody (Goat, Sigma) were used for immunoblotting of β-tubulin as a loading control.

Virus release and production assays

Cell cultures (~$2x10^5$ cells seeded in 24-well plates) were infected at an MOI that allowed at least 80% infectivity (MOI of 20 for Sk-mel28, NP-9 and Isrec-01, MOI of 10 for A549 cells). Four hours after infection, cultures were washed thrice with PBS and incubated in fresh virus-free medium. At indicated time points after infection, a small fraction of the supernatant (SN) was collected and the cells and the medium were harvested and frozen-thawed three times to obtain the cell extract (CE). Viral titers were determined by an anti-hexon staining-based method [50].

In vitro *cytotoxicity assays*

Cytotoxity assay was performed by seeding 20000 NP-18 cells, 15000 Saos-2 or NP-9 cells, or 10000 Sk-mel28 per well in 96-well plates in DMEM with 5% FBS. Cells were infected with serial dilutions starting with 800 TU/cell for NP-9 cells, 150 TU/cell for NP-18 or Saos-2 cells, or 100 TU/cell for Sk-mel28 cells. At day 5 post-infection, plates were washed with PBS and stained for total protein content (bicinchoninic acid assay (BCA), Pierce Biotechnology, Rockford, IL). Absorbance was quantified and the TU per cell required to produce 50% inhibition (IC50 value) was estimated from dose-response curves by standard nonlinear regression (GraFit; Erithacus Software, Horley, UK), using an adapted Hill equation.

In vivo *toxicity study*

Mice for toxicology and efficacy studies were maintained in the facility of the Institut de Recerca Oncològica-IDIBELL (Barcelona), AAALAC unit 1155. All animal studies have been approved by the Institut d'Investigacio Biomedica de Bellvitge Ethical Committee for Animal Experimentation. $5x10^{10}$ purified viral particles were injected intravenously into the tail vein in 6-week-old immunocompetent Balb/C male mice in a volume of 10 ml/kg in PBS (n = 5). Daily observations for body weight, morbidity and moribundity were performed. At day 4 or 12 post-injection, mice were sacrificed and different samples were collected. Blood samples were collected by intracardiac puncture and clinical biochemical and hematological determinations were performed by the Clinical Biochemistry and Hematological Services of the Veterinary Faculty at the Autonomous University of Barcelona. The significance of differences in biochemical and hematological rates between treatment groups were assessed by a two-tailed Student's unpaired *t*-test. Mice livers were resected and portions were fixed in 4% formaldehyde for 24h at room temperature (for paraffin embedding and further hematoxylin/eosin staining) or frozen in OCT (Sakura Finetek, Zoeterwoude, The Netherlands). E1A-immunodetection was performed by incubating OCT-embedded liver sections with a primary polyclonal antibody anti-adenovirus-2 E1A (clone 13 S-5, Santa Cruz Biotechnology) and an AlexaFluor® 488-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, OR). Slides were counterstained with 4',6-diamino-2-phenylindole and visualized under a fluorescent microscope (Olympus BX51; Olympus Optical Company, Hamburg, Germany).

In vivo *antitumoral efficacy*

Subcutaneous A549, SkMel-28, NP-9, NP-18 or PC-3 carcinoma tumors were established by injection of 1 x 10^7 cells into the flanks of 6-week-old male Balb/C *nu/nu* mice. When tumors reached 100 mm^3 (experimental day 0), mice were randomized (n=10-16 per group) and were injected by a single intravenous injection of PBS, $2.5x10^{10}$ viral particles of Adwt-RGD or ICOVIR-15, or $5x10^{10}$ viral particles of ICOVIR-15 in a volume of 10 ml/kg in PBS via the tail vein. Tumor size and mice status were monitored thrice per week. Tumor volume was defined by the equation $V(mm^3)= \pi/6 \times W^2 \times L$, where W and L are the width and the length of the tumor, respectively. Data are expressed as relative tumor size to the beginning of the therapy, which was set as 100%. The significance of differences in relative tumor size between treatment groups was assessed by a two-tailed Student's unpaired *t*-test. For Kaplan-Meier survival curves, end point was established at $\geq$500 mm^3. The survival curves obtained were compared for the different treatments. Animals whose tumor size never achieved the threshold were included as right censored information. A log-rank test was used to determine the statistical significance of the differences in time-to-event.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

FIGURE 1. Schematic diagram of E1A transcriptional control. ICOVIR-15 incorporates eight extra E2F-responsive sites organized in four palindromes. Furthermore, one extra Sp-1 binding-site was also inserted. Genetic modifications in ICOVIR-15 increase genome size by only 151 bp. In cancer cells, the E2F transcription factor is released from E2F-Rb complexes, due to pRb pathway deregulation. When ICOVIR-15 infects a tumor cell, free E2F binds to the E2F-palindrome sites present in the modified E1A promoter and activates E1A-Δ24 transcription. The Sp-1 transcription factor cooperates with E2F to activate transcription. E1A proteins activate the transcription of the other early adenoviral proteins, including E4-6/7, which forms a complex with free E2F that binds to the palindromic E2F sites inserted in ICOVIR-15 promoter to further activate E1A transcription. The same palindromic E2F motif is also present in the E2 promoter, resulting in an efficient loop of autoactivation and lysis of cancer cells. The RGD-modified fiber increases virus infectivity. In normal, quiescent cells, functional pRb forms an inhibitory complex with the E2F transcription factor. After infection of normal cells, binding of E2F-pRb complexes to the E2F-responsive sites inserted in ICOVIR-15 docks histone deacetylases to the E1A promoter and prevents E1A-Δ24 transcription. Whereas the wt E1A protein can release free E2F from the pRb-E2F complexes, the Δ24-deletion in E1A prevents this, thereby preventing an auto-activation loop driven by E2F in the event of leaky E1A-Δ24 expression. Adwt-RGD, wild-type adenovirus with RGD-modified fiber; bp, base pairs; ITR, inverted terminal repeat; HDAC, histone deacetylase; RB, retinoblastoma protein; II, enhancer element II.

FIGURE 2. ICOVIR-15 improves antitumor efficacy compared to Adwt-RGD. (a) The E2F-responsive palindromes enhance the E1A expression driven by the endogenous E1A promoter. Anti-E1A Western blots were performed 24 hours after infecting cells with a dose of each virus that allowed more than 80% transduction (multiplicity of infection (MOI) of 20 for Sk-mel28 and NP-9; MOI of 10 for A549 cells). The relative intensities of bands were measured by densitometry. AdTL-RGD is a replication-deficient control and Adwt-RGD is a wild-type adenovirus with an RGD-modified fiber. (b) Viral production of ICOVIR-15 in tumor cells. Different tumor cell lines were infected as indicated in (a) or with an MOI of 20 for Isrec-01. Virus production was measured 3 days after infection. Viral yield was evaluated in quadruplicate for each cell line, by carrying out two independent experiments. (c) Comparative cytotoxicity of ICOVIR-15. Cells were infected with the indicated viruses at doses ranging from 500 to 0.0001 transduction units (TU)/cell. IC_{50} values (TU/cell required to cause a reduction of 50% in cell culture viability) at day 5-8 after infection are shown. Four different replicates were quantified for each cell line. +SD error bars are plotted. (d) Relative tumor growth after systemic injection of Adwt-RGD or ICOVIR-15. Nude mice harboring subcutaneous xenografts of Sk-mel28 cells (melanoma) were randomized and injected with a single intravenous dose of $2.5x10^{10}$ viral particles per mouse of Adwt-RGD or ICOVIR-15. Phosphate-buffered saline (PBS) administration was used as a control. ICOVIR-15 significantly reduced tumor growth compared to Adwt-RGD and PBS from early days in the experiment. Mean values of 16 tumors/group +SE are depicted. #, significant $P<0.05$ by two-tailed unpaired Student's t-test, compared to Adwt-RGD group. *, significant ($p<0.05$) by two-tailed unpaired Student's t-test, compared to PBS group. TU, transduction units; Adwt-RGD, wild-type adenovirus with RGD-modified fiber.

FIGURE 3. Toxicity profile after systemic injection of ICOVIR-15 in immunocompetent mice. (a) Body weight variation was monitored after intravenous administration of $5x10^{10}$ viral particles per mouse of Adwt-RGD or ICOVIR-15. Phosphate-buffered saline (PBS) administration was used in the control group. Adwt-RGD-injected mice were sacrificed at day 4 due to lethal toxicity, while ICOVIR-15-injected mice presented a similar weight profile than those injected with PBS. At day 4 or 12 after administration, serum transaminases (b) and platelet (c) concentrations were analyzed in peripheral blood. Mean values +SD of 5-10 mice/group are depicted. *, significant ($p<0.05$) by two-tailed unpaired Student's t-test,

compared to Adwt-RGD group. #, significant ($p<0.05$) by two-tailed unpaired Student's t-test, compared to PBS group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; IU/l, International units per litre; Adwt-RGD, wild-type adenovirus with RGD-modified fiber.

FIGURE 4. ICOVIR-15 reduces hepatic E1A expression *in vivo*. Mouse livers were collected at day 4 after intravenous administration of PBS or viruses and E1A expression was assessed by immunohistofluorescence in frozen sections (a). E1A was barely detected in livers from mice injected with ICOVIR-15, while intense detection was observed throughout livers of Adwt-RGD-injected mice. E1A detection correlated with evident signs of hepatitis when analysed by eosin-haematoxylin staining of paraffin-embedded liver sections (b). Pathological changes including macrosteatosis, presence of Councilman bodies and large necrotic areas were present in livers from mice injected with Adwt-RGD, but not in those injected with ICOVIR-15. Adwt-RGD, wild-type adenovirus with RGD-modified fiber.

FIGURE 5. Efficacy and survival after systemic administration of ICOVIR-15. (a) Nude mice bearing subcutaneous xenografts of lung (A549), prostate (PC-3), pancreatic carcinoma (NP-9 and NP-18) or melanoma (Sk-mel 28) were injected with a single intravenous dose of phosphate-buffered saline (PBS) or $5x10^{10}$ viral particles (vp) per mouse of ICOVIR-15. Relative tumor volume (percentages of size at treatment, +SE) of 12-18 tumors/group are plotted. #, significant $p<0.05$ by two-tailed unpaired Student's *t*-test compared with mice injected with PBS. (b) Kaplan-Meier survival curves after administration of a single intravenous dose of PBS or $5x10^{10}$ vp per mouse of ICOVIR-15. The end point was established at a tumor volume of $\geq$500 mm^3. #, significant $p<0.05$ by log-rank test compared with mice injected with PBS.

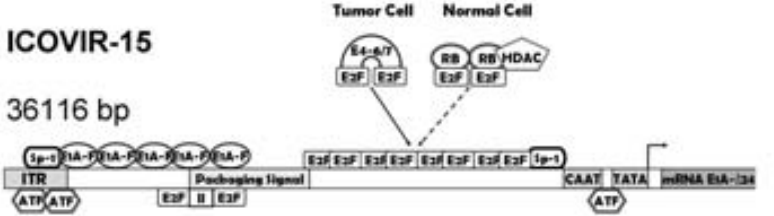
FIGURE 6. ICOVIR-15 is able to efficiently express transgenes. (a) Schematic representation of the genetic components present in ICOVIR-15 and ICOVIR-15-NfsA. To drive NfsA expression in ICOVIR-15-NfsA from the MLP, the Ad5 IIIa protein splice acceptor (3VDE) was inserted in front of the NfsA cDNA, followed by a polyA sequence (pA). These three elements were inserted downstream of the fiber-RGD in the ICOVIR-15 genome. The total lengths of both genomes are depicted in the figure. (b) Virus yield and release after infection with ICOVIR-15 and ICOVIR-15-NfsA. Confluent A549 cells were infected at an MOI of 10. Four hours post-infection, the viral solution was removed and cell cultures were washed thrice with PBS and incubated with 1ml of fresh virus-free medium. Cell extracts and supernatants were harvested 4, 12, 24, 48 and 72 hours after infection and titrated by an anti-hexon staining-based method. Viral yield and release were evaluated in quadruplicate. +SD error bars are plotted. (c) Transgene expression driven by ICOVIR-15-NfsA. Anti-NfsA Western-blots were performed at indicated time points after infection of PC-3 cells at an MOI that allowed high transduction. β-tubulin staining was used as loading control. MLP, major late promoter; 3VDE, IIIa virus infection-dependent splicing enhancer; pA, polyA sequence; TU, transduction units.

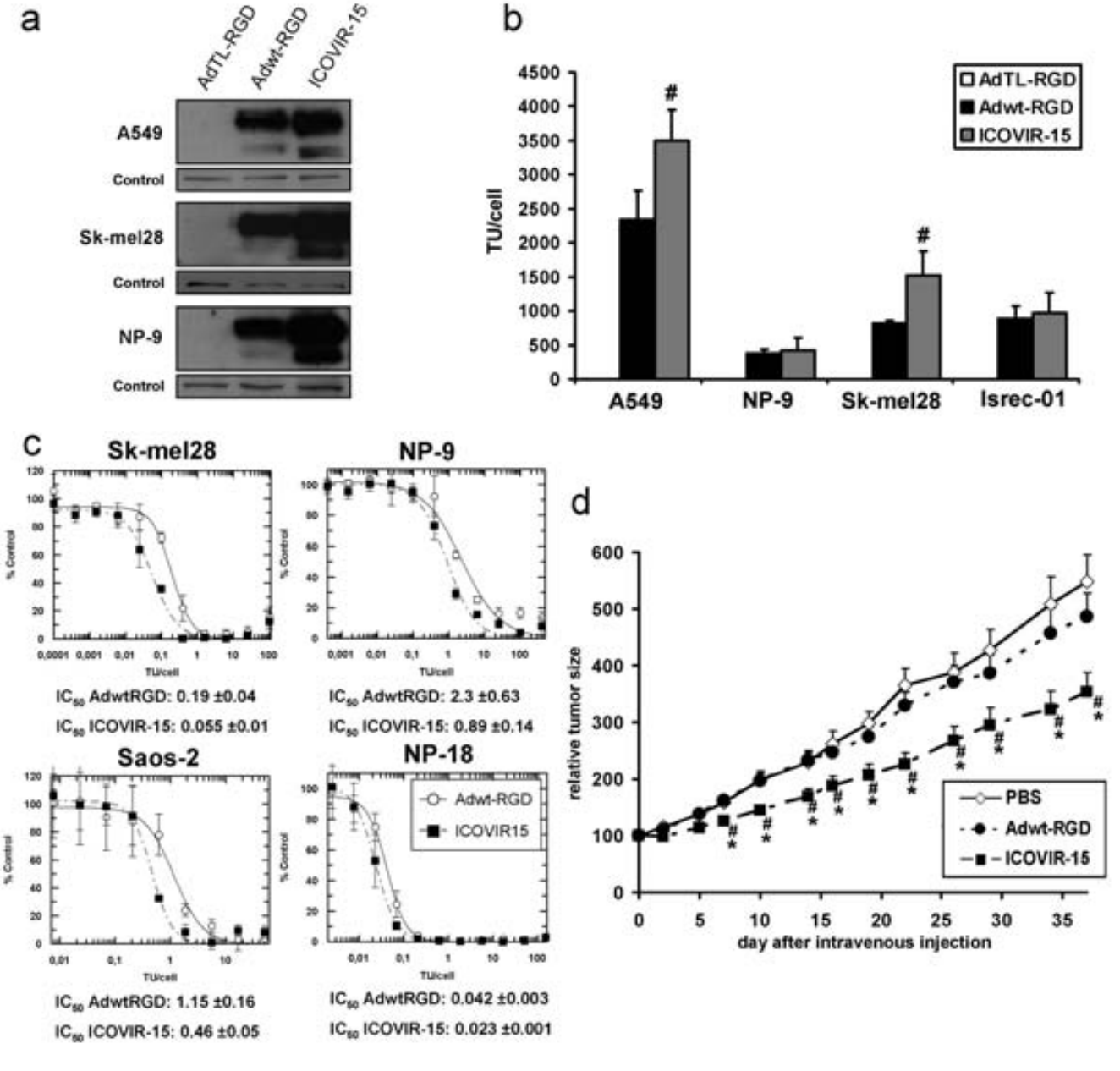
Adwt-RGD

35965 bp

ICOVIR-15

36116 bp

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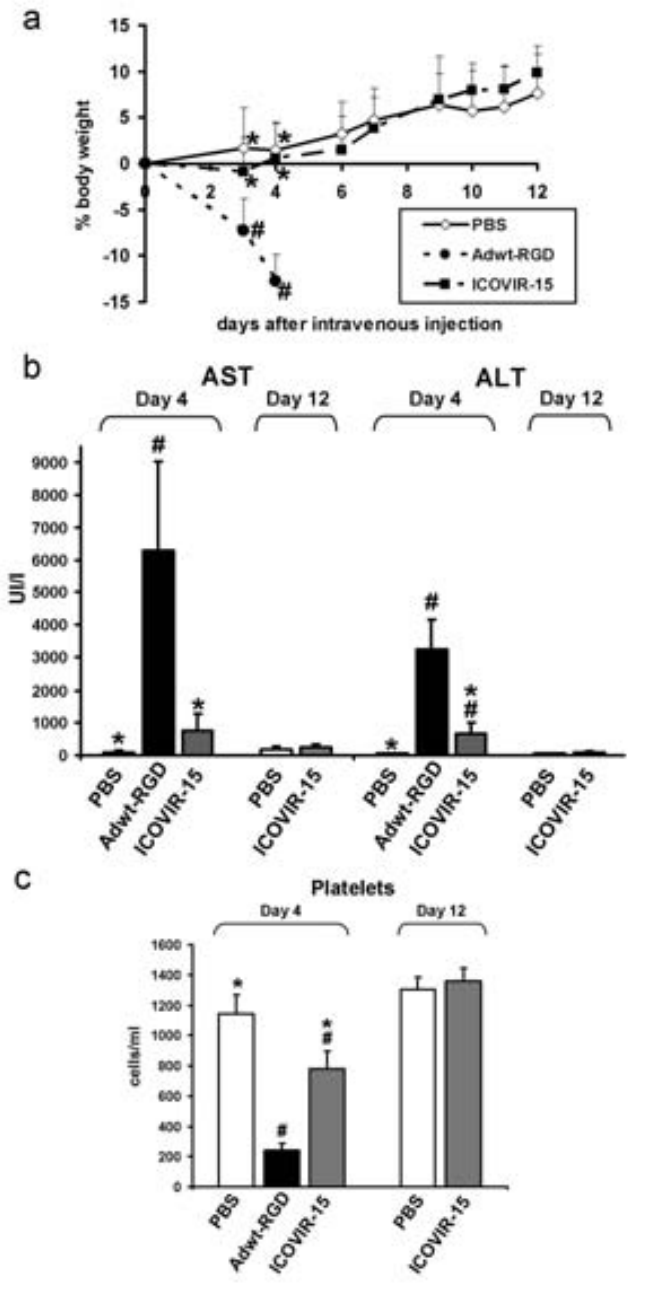
AdTL-RGD
Adwt-RGD
ICOVIR-15
A549
Control
Sk-mel28
Control
NP-9
Control
b
AdTL-RGD
Adwt-RGD
ICOVIR-15
TU/cell
4500
4000
3500
3000
2500
2000
1500
1000
500
0
#
#
A549
NP-9
Sk-mel28
Isrec-01
c
Sk-mel28
% Control
TU/cell
IC50 AdwtRGD: 0.19 ±0.04
IC50 ICOVIR-15: 0.055 ±0.01
NP-9
% Control
TU/cell
IC50 AdwtRGD: 2.3 ±0.63
IC50 ICOVIR-15: 0.89 ±0.14
Saos-2
% Control
TU/cell
IC50 AdwtRGD: 1.15 ±0.16
IC50 ICOVIR-15: 0.46 ±0.05
NP-18
Adwt-RGD
ICOVIR15
% Control
TU/cell
IC50 AdwtRGD: 0.042 ±0.003
IC50 ICOVIR-15: 0.023 ±0.001
d
relative tumor size
600
500
400
300
200
100
0
0
5
10
15
20
25
30
35
day after intravenous injection
PBS
Adwt-RGD
ICOVIR-15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% body weight
days after intravenous injection
PBS
Adwt-RGD
ICOVIR-15
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AST
ALT
Day 4
Day 12
U/l
PBS
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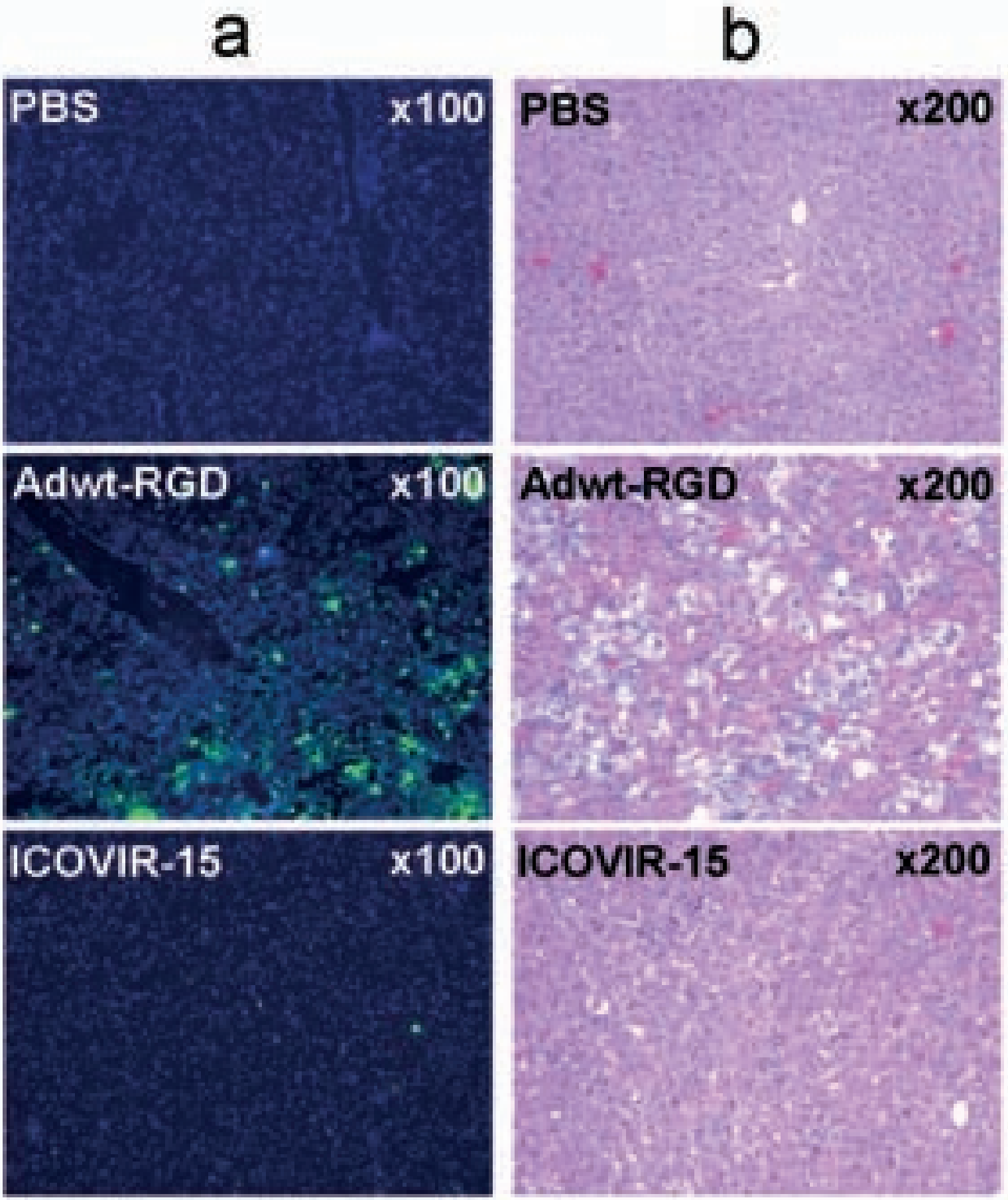
ICOVIR-15
c
Platelets
cells/ml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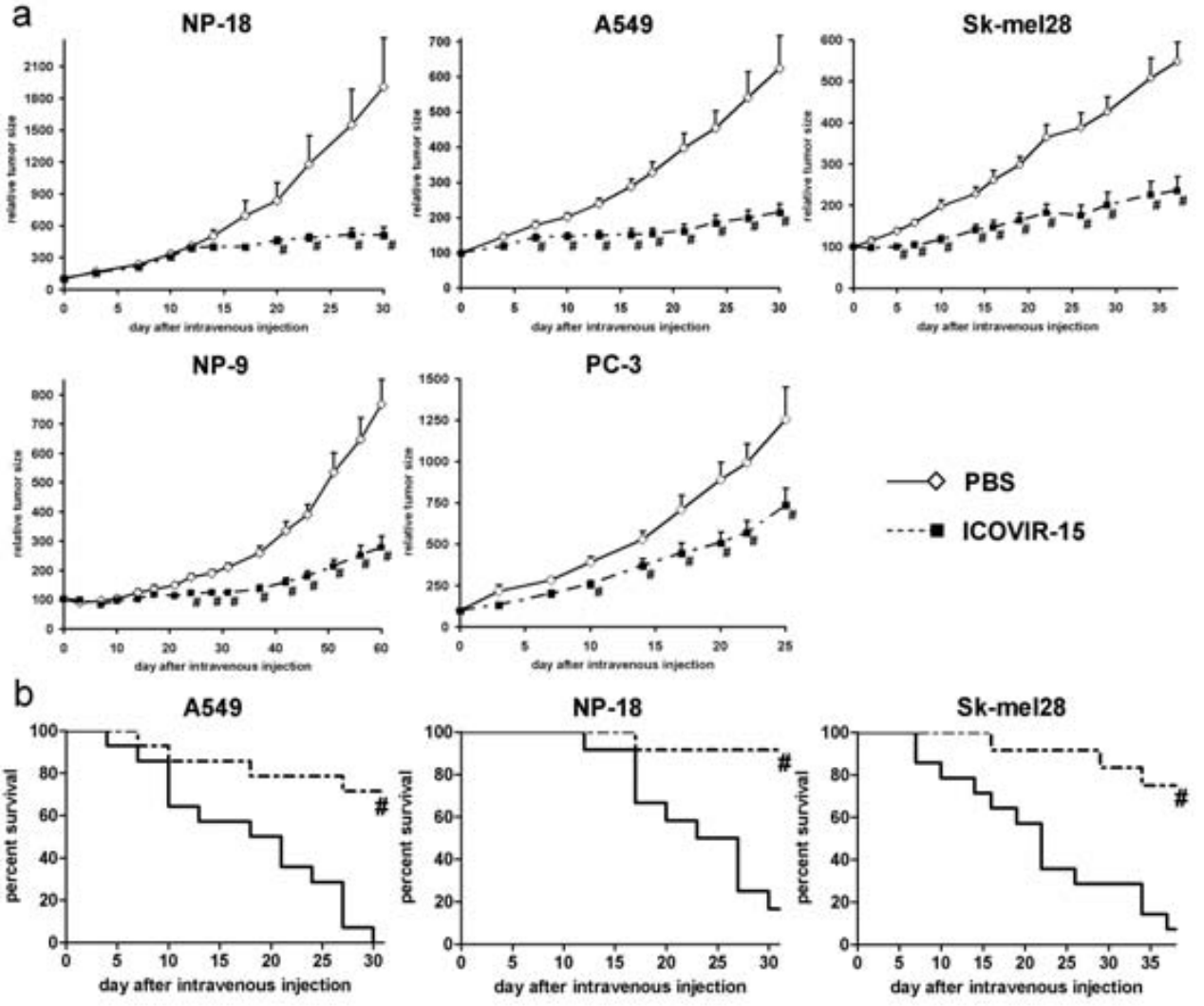
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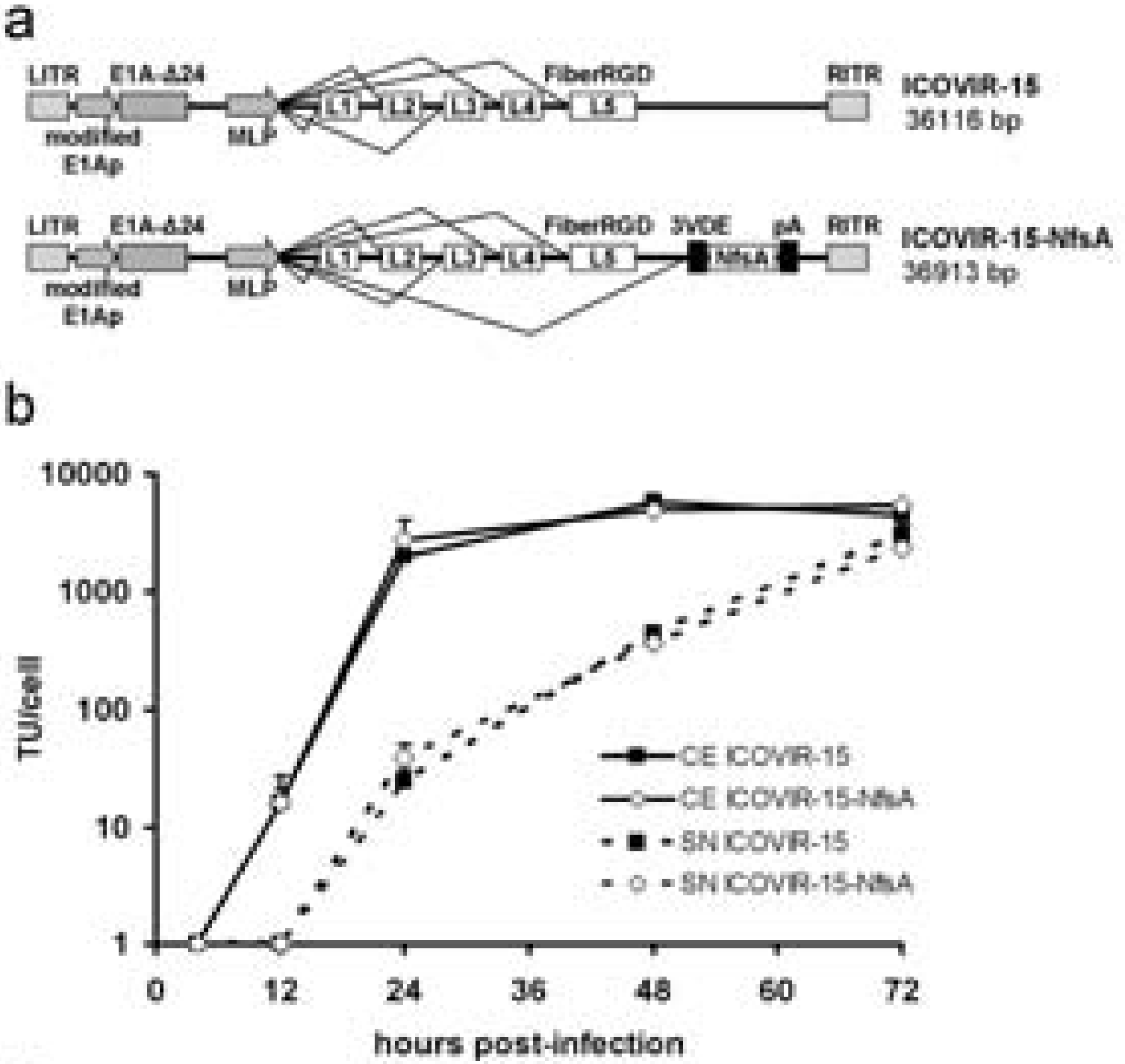
NP-18
A549
Sk-mel28
NP-9
PC-3
relative tumor size
day after intravenous injection
PBS
ICOVIR-15
b
A549
NP-18
Sk-mel28
percent survival
day after intravenous injection
#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L2
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L4
FiberRGD
L5
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36116 bp
LITR
E1A-Δ24
modified E1Ap
MLP
L1
L2
L3
L4
FiberRGD
3VDE
ΔA
NfsA
RITR
ICOVIR-15-NfsA
36913 bp
b
10000
1000
100
10
1
TU/cell
0
12
24
36
48
60
72
hours post-infection
CE ICOVIR-15
CE ICOVIR-15-NfsA
SN ICOVIR-15
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Article enviat a Clinical Cancer Research

Oncolytic adenovirus ICOVIR-7 in patients with advanced and refractory solid tumors

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Abbreviations list:

Ad	Adenovirus
CA	Carbohydrate antigene
CAR	Coxsackie-adenovirus receptor
DM-1	Myotonic dystrophy locus
HU	Hounsfield Unit
IL	Interleukin
NAb	Neutralizing antibody
PSA	Prostate specific antigene
Rb	Retinoblastoma
RGD	Arginine-lysine-aspartic acid
TNF-α	Tumor necrosis factor alpha
VP	Viral particle

Statement of Translational Relevance

Despite recent developments in cancer therapeutics there are still no effective treatments for advanced metastatic solid tumors. Oncolytic adenoviruses provide a novel approach against cancer and they can be successfully combined with conventional therapies improving treatment outcomes. In addition, oncolytic adenoviruses have showed good safety and tolerance in clinical trials which underline their suitability with advanced refractory tumors. However, improvements in technology are still needed in order to enhance efficacy of treatments.

In this experimental study we report first time the use of ICOVIR-7, an oncolytic RGD capsid modified adenovirus with an enhanced tumor specificity via modified E2F-promoter, in 21 cancer patients with advanced metastatic tumors. ICOVIR-7 treatment showed a good safety profile, immunological activity and objective evidence of anti-cancer effect with more than half of patients. These improvements in viral technology and promising clinical results encourage further evaluation in clinical trials.

Abstract

Purpose: In this study we treated 21 cancer patients with a single round of oncolytic adenovirus ICOVIR-7.

Experimental Design: ICOVIR-7 doses ranged from 2 x 10^{10} VP to 1 x 10^{12} VP. All patients had advanced and metastatic solid tumors refractory to standard therapies. ICOVIR-7 features an RGD-4C modification of the fiber HI-loop of serotype 5 adenovirus for enhanced entry into tumor cells. Tumor selectivity is mediated by an insulator, a modified E2F promoter and an Rb binding site deletion, while replication is optimized with E2F binding hairpins and a Kozak sequence.

Results: ICOVIR-7 treatment was well tolerated with mild to moderate fever, fatigue, elevated liver transaminases, chills and hyponatremia. One patient had grade 3 anemia but no other serious side effects were seen. At baseline, 8/20 of evaluable patients had neutralizing antibody titers (NAb) against ICOVIR-7 capsid. Treatment resulted in NAb induction within four weeks in 16/17 patients. No elevations of serum

pro-inflammatory cytokine levels were detected. Viral genomes were detected in the circulation in 18/21 of patients after injection and 7/15 of samples were positive several weeks later suggesting virus replication.

Conclusions: Overall, objective evidence of anti-tumor activity was seen in 9/17 evaluable patients. Clinical benefit in radiological analysis was seen in 5/12 evaluable patients, consisting of one partial response, two minor responses and two cases of stable disease. In addition, 3/3 evaluable patients had a decrease in tumor density. In summary, ICOVIR-7 treatment seems safe, results in anti-cancer activity and is therefore promising for further clinical testing.

Introduction

Oncolytic adenovirus based therapy represents a novel treatment approach for cancer refractory to conventional therapies (1-3). Oncolytic adenoviruses can also be combined with currently available treatment modalities resulting in synergistic effects (4-7). Oncolytic adenoviruses have been safe in dozens of clinical trials where the observed adverse events have usually been flu-like symptoms, fever and pain in injection site (5, 8, 9).

ICOVIR-7 is based on a serotype 5 adenovirus and features a 24 bp deletion in E1A region, which has been placed under control of a tumor specific E2F-1 promoter (10). Both features convey selectivity for cells defective in the Rb-p16 pathway (10-12), which includes most if not all advanced solid tumors (13). The capsid has been modified with a RGD motif in the HI-loop of the fiber knob, for enhanced infectivity of various cancer types (14-18). This modification improves viral transduction to cancer cells poorly expressing CAR. Low CAR expression is known to exist in many cancer types limiting treatment efficacy (19). Improved tumor transduction has enhanced treatment efficacy significantly in preclinical models (15-17). In addition, a DM-insulator has been placed 5' of the promoter for increased specificity and a Kozak sequence leads E1A for optimal expression. These modifications in virus structure increase viral replication in cancer cells and minimize viral replication in normal cells.

In this study we report the safety and efficacy of experimental oncolytic adenovirus ICOVIR-7 in 21 cancer patients (Table 1). According to our knowledge, the use of RGD capsid modified oncolytic adenovirus in humans has not been reported previously. We found that ICOVIR-7 is well tolerated and shows promising anti-cancer activity.

Materials and Methods

Patients

21 patients with solid tumors refractory to available treatment modalities were treated with a single round of oncolytic adenovirus ICOVIR-7. Detailed patients characterization is described in Table 1. All patients had progressive metastatic tumors despite previous oncology treatments. The principles of oncolytic adenovirus treatment including possible side effects were explained verbally and in writing to all patients and they signed written informed consent. Side effects were graded according to CTCAE v3.0 criteria. Treatments were evaluated and approved by the Medicolegal Department of the Finnish Ministry of Social Affairs and Health and The Gene Technology Board. Treatments were performed according Good Clinical Practise and the Helsinki Declaration of World Medical Association.

Treatment protocol

All patients received a single round of ICOVIR-7 at viral doses ranging from 2×10^{10} viral particles (VP) up to 1×10^{12} VP intratumorally (primary tumor and/or any metastasis) in ultrasound guidance. If intratumoral injection was not possible, intravenous injection was performed. In case of intraperitoneal or intrapleural disease, injection was performed intracavitary. Patients were monitored for 24 hours in the hospital and for

four weeks as outpatients. Clinical status and side effects were recorded at each visit. Laboratory data was collected before treatment and at several time points thereafter.

Analysis of efficacy

Tumor assessment by computer tomography (CT) or magnetic resonance imaging (MRI) was performed before treatment and again circa 6 weeks later. Response Evaluation Criteria in solid tumors (RECIST) criteria (20, 21) were applied to overall disease status including injected and non-injected tumors. In addition to the standard criteria, we used minor response (MR, 10-30 % reduction in the size of lesions) as an indicator of cases where biological activity might be present.

Virus

ICOVIR-7 is based on serotype 5 adenovirus (10). The virus capsid has been modified with the RGD-4C motif in the HI loop of the fiber. It features the 24bp deletion in the E1 region conferring cancer cell specificity (22). A tumor specific E2F-1 promoter controls E1A region, which was further modified by additional E2F binding hairpins for enhanced activity. The myotonic dystrophy locus (DM-1) insulator sequence reduces transcriptional leakage from the left ITR and a Kozak sequence ensures optimized transcription. ICOVIR-7 was produced on A549 cells to avoid the risk of recombination with transcomplementing sequences. The viral particle titer of ICOVIR-7 was 1.2 x 10e12 VP/mL and functional titer 2.58 x 10e10 pfu/mL, genome to pfu ratio (VP/pfu) 46.5. Virus stock buffer formulation was 10mM Trizma®base, 75mM NaCl, 5% (w/v) sucrose, 1mM MgCl, 10mM L(+) histidine, 0.5% (v/v) EtOH, 0.02% Tween®, 100µM EDTA. 0.9% (w/v) NaCl solution (B. Braun, Melsungen, Germany) was used as a diluent.

Cytokine analysis

Cytokine analysis was performed with BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Set (Becton Dickinson, Franklin Lakes, NJ, US) according to manufacturer's instructions. FCAP Array (TM) v1.0.1 software was used for data analysis.

Neutralizing antibody titer determination

293 cells were seeded at a density of 1 x 10^4 cells/well on 96-well plates and cultured overnight. Samples were incubated at 56 °C for 90 min to inactivate complement, and 4-fold dilution series (1:1 to 1:16384) was prepared in serum-free DMEM (23). Ad5lucRGD (15) (identical capsid with ICOVIR-7), was mixed with serum dilutions and incubated at room temperature for 30 min. Cells in triplicates were infected with 100 VP/cell, and growth medium with 10% FCS was added 1h later. 24h post-infection, cells were lysed and luciferase activity was measured (Luciferase Assay System, Promega, Madison, WI and TopCount luminometer, PerkinElmer, Waltham, MA). Luciferase readings were plotted relative to gene transfer achieved with Ad5lucRGD alone. The NAb titer was determined as the lowest degree of dilution that blocked gene transfer more than 80%.

Quantitative Real Time PCR for presence ICOVIR-7 in serum

Total DNA from serum was extracted (QIAamp DNA mini kit, Qiagen, Hilden, Germany**).** PCR amplification was based the E1A region targeting flanking the 24-bp deletion (forward primer 5´-TCCGGTTTCTATGCCAAACCT-3´, reverse primer 5´-TCCTCCGGTGATAATGACAAGA-3´ and probe onco 5´^{FAM}-TGATCGATCCACCCAGTGA-3´^{MGBNFQ}). An additional probe was used to test for the presence

of wild-type Ad5 infection (probe wt 5´^{VIC}-TACCTGCCACGAGGCT-3´^{MGBNFQ}). The rt-PCR conditions: 2X LightCycler480 Probes Master Mix (Roche, Mannheim, Germany)**,** 800 nM each forward and reverse primer, 200 nM each probe and 250 ng extracted DNA. **C**ycling conditions: 10 min at 95°C, 50 cycles of 10 s at 95°C, 30 s at 62°C and 20 sec at 72°C and 10 min at 40°C. TaqMan exogenous internal positive control reagents (Applied Biosystems) were used to test for the presence of PCR inhibitors. A regression standard curve was generated using DNA extracted from serial dilutions of Ad5/3-D24-Cox2L (1.0 x 10^8 – 10 vp/ml). The detection and quantification limits for the assay were 500 VP/ml. The presence of ICOVIR-7 virus in PCR positive samples was confirmed by real-time PCR using LightCycler480 SYBR Green I Master mix (Roche, Mannheim, Germany) and specific primers (forward primer 5´-GCGGGAAAACTGAATAAGAGG-3´ and reverse primer 5´- CGGAGCGGTTGTGAACTG-3´).

Results

Treatment of cancer patients with ICOVIR-7 is well tolerated and safe

Treatments were generally well tolerated up to the highest dose used: 1 x 10^{12} VP**.** All patients experienced mild to moderate grade 1 or 2 side effects. Most common detected side effects were fever (18 of 21 patients), fatigue (16 of 21 patients), elevated liver transaminases (13 of 21 patients), anemia (13 of 21 patients), hyponatremia (12 of 21 patients), abdominal pain (11 of 21 patients) and chills (10 of 21 patients). There was no apparent relationship between viral dose and the severity of side effects. Grade 3 post-treatment anaemia was detected with one patient (H111) and no grade 4-5 side effects were observed. All side effects are reported in Table 2.

Treatment effect on pro-inflammatory cytokines

Interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF-α) levels were measured for all patients. All values were compared to pre-treatment reference values. Highest absolute cytokine concentration was measured with patient H41 having pancreatic cancer whose IL-8 value was 1159 pg/ml four days after treatment. However pre-treatment IL-8 value for same patient was already 626 pg/ml indicating only moderate increase (Supplementary Table 1). There was no apparent relationship between measured cytokine levels, patients' clinical status or treatment response. Generally only minor post-treatment elevations in pro-inflammatory cytokine levels could be detected.

Induction of neutralizing antibodies

Neutralizing antibody titers (NAb) against the capsid of ICOVIR-7 could be analyzed for 20 patients (Table 3). Eight patients had titers of higher than 4, which was considered the cutoff for neutralizing activity. In addition, 5 and 3 patients had titers of 1 and 4, respectively, possibly reflecting past infection with adenovirus. Treatment resulted in NAb induction within four weeks in 16/17 patients.

ICOVIR-7 replication

All patients were analyzed by qRT-PCR for the number of viral genomes present in the circulation. Viral genomes were detected in the circulation in 18 of 21 patients (Table 4). Three patients (R85, O48, O92) remained negative at all time points analyzed. The highest viral titer (> 4 mil. VP/ml) was seen at day 4 in a patient with prostate cancer (P44). In 10/15 patients there was more virus detected between days 2-7 than on day 1, suggesting virus replication. Virus could be detected in 7/15 patients for several weeks, which also suggests virus replication since injected virus is typically cleared rapidly (5, 9, 24-26). Three patients (G59,

N106 and N127) received virus only intravenously. Interestingly, all three had virus present in blood on day 1. G59 was negative on two subsequent measurements and N127 had no other data points. However, N106 had circulating virus on day 7, which seems to suggest some virus replication.

Anti-tumor efficacy of ICOVIR-7

Overall, objective evidence of anti-tumor activity was seen in 9/17 evaluable patients. In 5 out of 12 radiologically evaluable patients (Y120, N127, H107, O48 and U157), tumor size measurements suggested benefit from the treatment (Table 4). These consisted of one partial response, two minor responses and two cases of stable disease (all patients were progressing before treatment). In addition, all three evaluable patients had a decrease in tumor density, which has been suggested to indicate antitumor activity with oncolytic viruses (27). 3 patients (R39, P44 and O48) had a decrease or stabilization of tumor markers.

Patient U157 was a 9 year old boy with Wilms tumor, a pediatric kidney malignancy. He had a partial response with a 37 % overall reduction in the sum of tumor diameters. Also, he had complete eradication of some of his tumor lesions (Figure 1). Patients N127 and O48 also showed minor responses (-10 % and -17 % respectively). The RECIST measurements of the tumors of Y120 and H107 resulted in classification of stable disease. Y120 also had a decrease in tumor density.

2/3 patients injected only intravenously could be evaluated for anti-tumor efficacy. One had an increase in CA12-5, while the other had a 10% minor reduction in the tumors. This may suggest some anti-tumor efficacy as her tumor was progressing prior to treatment. After a minimum of 9 months of follow-up (Figure 2), 4 patients were still alive (longest follow-up 371 days).

Discussion

Treatment of metastatic cancer refractory to available treatments requires novel approaches such as oncolytic adenoviruses. The first generation of such viruses has completed clinical testing with good safety data, and although there are some examples of efficacy in nearly all trials, the overall single agent efficacy has been less than satisfying (1-3, 28). However, it is promising that even such prototype viruses seem quite effective when combined to chemotherapy or radiation (4, 6, 7). ICOVIR-7 embodies several improvements over viruses tested previously in patients including RGD modification of the fiber and a deletion of constant region 2 of E1 combined with a tumor specific promoter enhanced by E2F binding hairpins. These modifications may represent an important improvement in the technology and the use of RGD modified viruses in humans has not been reported previously.

No serious side effects were detected in this study, except for one patient with pancreatic cancer who experienced grade 3 anaemia (patient H111). His haemoglobin value decreased from 97 g/l to 68 g/l on the first post-treatment day. This may have been treatment related, as such a rapid decrease is rare despite anaemia being very common in cancer patients especially in the context of pancreatic cancer (29). The haemoglobin value of H111 was 65 g/l five days before treatment and he had received several red blood cell transfusions before and after treatment. At the seventh post-treatment day patient's haemoglobin was 94 g/l and it varied between 80-94 g/l during following four weeks.

Although adenoviruses have been quite safe in the treatment of cancer with more than 15 000 patients reported in the literature (1-7, 28), some safety concerns remain in the context of newer generation viruses. It has been proposed that cytokines could predict harmful inflammation (30, 31). IL-6, IL-8 and TNF-α were selected for analysis due their pro-inflammatory role (32, 33) which can be helpful for analyzing systemic inflammatory response. High levels are associated with systemic inflammatory response syndrome and may induce at worst multi organ failure (30, 31). However, IL-10 is an anti-inflammatory cytokine that is up-regulated in many inflammatory diseases. All patients had elevated cytokine levels prior to treatment which is in line with previous reports on cancer patients with advanced tumors (34-36). Taking into account the detected side effects and cytokine data it seems reasonable to propose that ICOVIR-7 treatment was well

tolerated at the used dose range. The highest dose used here is 6-fold lower than the highest dose of oncolytic adenovirus safely used in humans previously (9).

About one third of patients had pre-existing NAb against Ad5. This suggests that those patients had experienced a recent wild type Ad5 infection. The most likely targets of the pre-existing NAb are hexon, penton and fiber shaft, as those parts of the ICOVIR-7 is identical to wild type Ad5. As suggested by others (7, 26, 37-39), no apparent relationship was seen between NAb titers and the injected viral dose, as NAb increased in nearly all evaluable patients, except O92. No virus replication was seen in O92 either perhaps correlating with lack of NAb induction. However, samples were only available from day 4 and we cannot exclude an increase of NAb at later time points. In the two patients with the highest baseline NAb titers (R85 and O48), there was no virus detected in blood after treatment. However, there seemed to be no correlation between baseline NAb and efficacy. For example, O48 had a minor response despite a baseline NAb titer of 1024 and N127 had a minor response after intravenous injection, despite a baseline NAb titer of 256. These data seem to support the hypothesis that antiviral antibodies can increase the efficacy of treatment, as they can help in clearance of (tumor) cells containing virus. However, this should be confirmed in larger patient materials. It is clear that the patient series reported here is not large enough to conclusively resolve these issues.

Although most patients received the virus intratumorally, there were three patients whose tumors could not be injected. It is tantalizing that there was a minor response in one of these patients. Although there is substantial body of preclinical evidence suggesting that efficacy is possible through the intravenous route (10, 40), initial trials did not seem to support this notion (6, 26, 37, 39). However, in one trial there were prostate specific antibody responses in 3/8 patients treated at the highest dose level (9), perhaps suggesting anti-tumor activity. These findings might be explained in part by the low activity of ONYX-015 in general (7, 26, 37-39), while the virus used in Small et al may have been more active (9). Preclinical data suggests that ICOVIR-7 may be even more active (10).

All patients in this study were heavily pretreated with a mean of 5 previous chemotherapy regimens, in addition to other modalities such as radiotherapy and surgery. Despite this, 9/17 of patients had objective evidence of anti-tumor activity of the virus. The preliminary activity seen here might justify further evaluation of the virus in larger studies which might ultimately lead to randomized trials, which are needed to reliably evaluate the safety and efficacy of any cancer therapeutic. Nevertheless it is interesting to note that the patients who experienced some clinical effect had different tumor types including cholangio cancer, head & neck cancer, pancreatic cancer, prostate cancer, ovarian cancer and Wilms tumor. This is a promising indication that ICOVIR-7 can be utilized successfully for various tumor types. One issue that complicates the analysis of treatment efficacy is the lack of suitable evaluation methods. RECIST criteria were originally developed for chemotherapy agents, may give incorrect results because viral replication might cause local inflammation enlarging tumors and thus leading to false conclusion of progression. The same applies to tumor marker analysis, as cell lysis due to oncolytic replication might misleadingly increase tumor markers temporarily (5). One proposed mechanism for marker surge is activation of the CEA promoter by virus replication (41).

In summary, our data suggests that ICOVIR-7 is safe for treatment of human cancer and that there is promising anti-tumor activity. Further increases in efficacy could be obtained by treating less advanced patients, combining with standard therapy (4, 6, 25, 37), and by treating patients with more than one injection. Also, given the lack of dose limiting toxicity, perhaps higher doses could yield higher efficacy. Randomized trials are needed to ultimately determine the safety and efficacy of the approach.

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Tables

Table 1.

Characteristics of patients treated with oncolytic adenovirus ICOVIR-7.

Table 2.

Treatment related side effects according to CTCAE v3.0 criteria.

Table 3.

Neutralizing antibody titers for patients treated with ICOVIR-7.

Table 4.

Summary of all ICOVIR-7 treated patients in this study according to viral dose. * = alive at the end of follow-up, ‡ = cyclophosphamide 50 mg/ day per os starting 1 week before treatment until 4 weeks after treatment, § = cyclophosphamide intravenously 500 mg on day of virus injection and 25 mg/day per os starting 2 weeks later, † = intravenous treatment only, ↓ = tumor marker decreased, ↑ = tumor marker increased, blanks indicate data not available

Supplementary Tables

Supplementary Table 1.

Cytokine analyze results for patients treated with ICOVIR-7.

Figure Legends

Figure 1.

For patient U157 having Wilms tumor MR-images are shown 14 days before *(1A* and *1C)* and 36 days after *(1B and 1D)* treatment to demonstrate the clinical effect of ICOVIR-7. Total size reduction in all tumor lesions according to RECIST criteria was 37 % scored as partial response. *A* and *B,* tumor lesion at the left side of abdominal cavity wall in proximity of spleen. This lesion was completely eradicated by viral treatment. *C* and *D,* tumor lesion at the left kidney area (left kidney had been removed previously). Tumor size before treatment was 36 mm x 30 mm and after treatment 24 mm x 5 mm. Location of the tumors are indicated by arrowheads in each image.

Figure 2.

Patient survival after ICOVIR-7 treatment. Median survival in this study was 92 days and longest follow-up 371 days (patient N90).

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Table 1.

Sex, no. of patients (tot. N=21)	
Male	10
Female	11
Age (years)	
Median	56
Range	9-68
WHO performance status (0-5), no. of patients	
0	1
1	12
2	5
3	3
Tumor type, no. of patients	
Bladder cancer	1
Breast cancer	3
Cholangio carcinoma	1
Colon cancer	2
Gastric cancer	1
Head and neck cancer	4
Pancreatic cancer	3
Prostate cancer	1
Ovarian cancer	3
Leiomysarcoma	1
Wilms tumor	1
Previous treatments, no. of patients	
Surgery	17
Chemotherapy (mean: 5 regiments, range 2-13)	21
Radiotherapy	14
Autologous Stem Cell Transplantation	1

Table 2.

Hematological:	Grade 1 (no. of patients)	Grade 2 (no. of patients)	Grade 3 (no. of patients)	Grade 4 (no. of patients)
- anemia	6	6	1	
- elevated liver transaminases	9	4		
- hypokalemia	7			
- hyponatremia	12			
Pain:				
- injection site		1		
- chest	2			
- abdominal	5	6		
- muscular/extremity		5		
- back	1	1		
- head	1	1		
Gastrointestinal system:				
- constipation		4		
- diarrhoea	1			
- heartburn	1			
- loss of appetite	3			
- nausea, vomiting	6	2		
- abdominal swelling	3	1		
Respiratory system:				
- dyspnea	3	1		
- cough	2	1		
- sore throat	2			
- speech difficulties	1			
Immunological system:				
- fever	11	7		
- chills	7	3		
Others:				
- fatigue	3	13		
- sweating	2			
- edema (lower extremity)		1		
- itching	2			
- thirstiness		1		

Table 3.

Type of cancer, patient ID code	**Treatment dose x 10^{11} (VP)**				
		Baseline pre-treatm.	1st week	2nd week	3-4 weeks
Breast, R39	0.2	1		16384	16384
Pancreatic, H41	0.7	1			
Wilms tumor, U157	1				
Bladder, V45	2	0	1024		4096
Prostate, P44	2	1	16384		
Breast, R55	3	0		256	
Breast, R85	3	4096	16384		16384
Head & Neck, N56	3	1			1024
Ovarian, 048	3	1024			16384
Colon, C93	4	0	16		
Colon, C104	4	16	64		
Gastric, G59	4	64			
Ovarian, O53	4	4		16384	
Ovarian, O92	4	64	64		
Head & Neck, N90	5	0	1		64
Head & Neck, N106	6	4	16384		
Pancreatic, H107	6	4	4096		
Leiomyosarcoma, S102	6	16			16384
Cholangio, Y120	7	16	64		16384
Pancreatic, H111	7	1	4096		
Head & Neck, N127	10	256			

Table 4.

Type of cancer, patient ID code	Treatment dose x 10^{11} (VP)	Virus in blood (VP/ml)						Tumor markers	RECIST	Tumor density (HU)	Survival (days)
		day 0 pre-treatment	day 1	days 2-7	days 8-14	days 15-21	days 21-28				
Breast, R39	0.2	neg.	neg.	63424	< 500		< 500	**MR: CEA↓** PD: CA15-3 ↑			58
Pancreatic, H41	0.7	neg.	neg.	6791	< 500		< 500	PD: CA19-9 ↑ CEA ↑	PD		92
Wilms tumor, U157 [§]	1	neg.	neg.	2450			neg.		**PR (- 37 %)**		192
Bladder, V45[‡]	2	neg.	< 500	814	< 500		neg.		PD (+29%)	73 → 60 (-18%)	320
Prostate, P44	2	neg.	1144	4038049	2580			**SD: PSA↓**			51
Breast, R55 [‡]	3	neg.	neg.	17465	neg.		neg.	PD: CA15-3 ↑ CEA ↑	PD (24%)	79 → 57 (-28%)	142
Breast, R85 [‡]	3	neg.	neg.	neg.			neg.	PD: CA15-3 ↑			79
Head & Neck, N56	3	neg.			804	< 500		PD: CEA ↑	PD		113
Ovarian, 048 [‡]	3	neg.				neg.		**MR: CA12-5 ↓**	**MR (- 17 %)**		268[*]
Colon, C93	4	neg.		68831					PD		62
Colon, C104	4	neg.	< 500	96689							34
Gastric, G59	4[†]	neg.	< 500	neg.		neg.		PD: CA12-5 ↑			109
Ovarian, O53 [‡]	4	neg.	< 500		neg.	neg.		PD: CA12-5 ↑			79
Ovarian, O92	4	neg.	neg.	neg.				PD: CA12-5 ↑	PD		73
Head & Neck, N90 [‡]	5	neg.	< 500	< 500			< 500				371[*]
Head & Neck, N106 [‡]	6[†]	neg.	< 500	< 500							49
Pancreatic, H107	6	neg.	neg.	< 500			< 500		**SD (+ 9 %)**		93
Leiomyosarcoma, S102 [‡]	6	neg.	< 500				neg.		PD (+ 30%)		56
Cholangio, Y120	7	neg.	neg.	1413			neg.		**SD (+ 13 %)**	93 → 78 (-16%)	316[*]
Pancreatic, H111 [‡]	7	neg.	< 500	2528							60
Head & Neck, N127	10[†]	neg.	< 500						**MR (- 10 %)**		167

Supplementary Table 1.

	Pre-treat	Post-treatment (days)				
	0 (N = 21)	1 (N = 17)	2-7 (N = 16)	8-14 (N = 7)	15-21 (N = 4)	>22 (N = 16)
IL-6 (min-max)	115 (1-653)	89 (6-472)	59 (0-388)	80 (7-345)	15 (6-22)	62 (0-294)
IL-8 (min-max)	126 (5-626)	129 (12-689)	184 (8-1159)	217 (38-898)	48 (11-72)	182 (10-1001)
IL-10 (min-max)	38 (0-116)	30 (2-75)	43 (0-109)	34 (6-78)	13 (9-18)	29 (0-83)
TNF-α (min-max)	41 (0-97)	35 (0-91)	40 (0-88)	47 (20-85)	23 (15-32)	36 (0-91)

mean (range: min-max), unit: pg/ml

Figure 1.

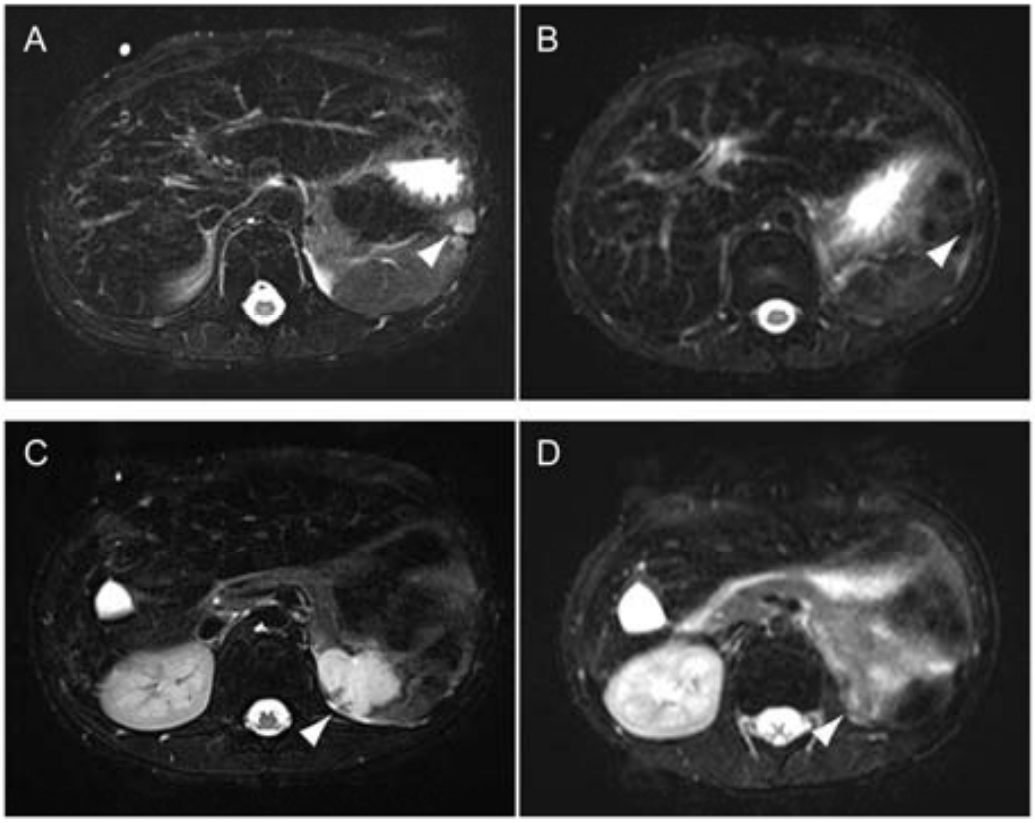
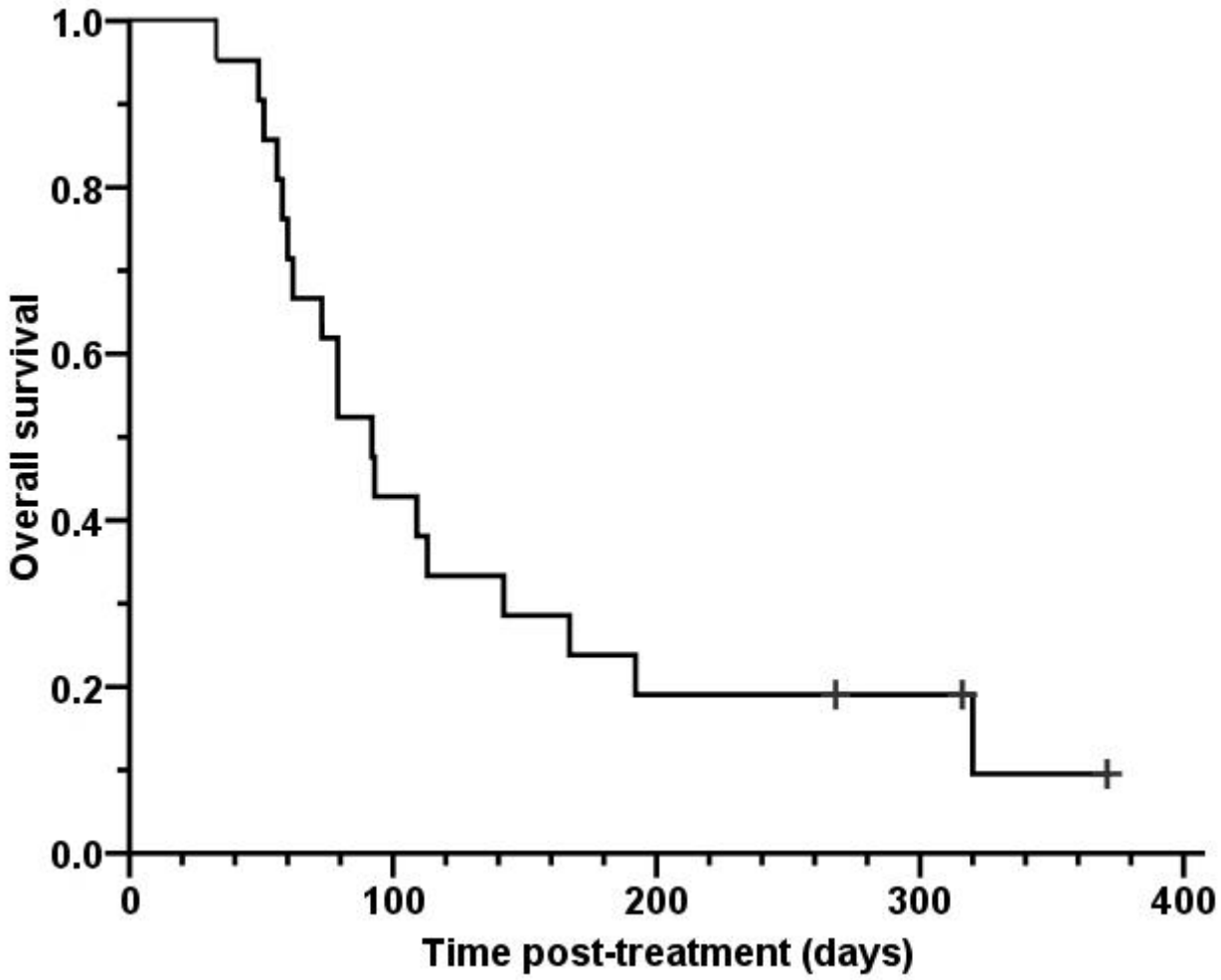


Figure 2.


1.0
0.8
0.6
0.4
0.2
0.0
Overall survival
0
100
200
300
400
Time post-treatment (days)

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INSTANCIA DE SOLICITUD

NUMERO DE SOLICITUD

P200600216

FECHA Y HORA DE PRESENTACIÓN EN O.E.P.M.

FECHA Y HORA PRESENTACIÓN EN LUGAR DISTINTO O.E.P.M.

(1) MODALIDAD

[X] PATENTE DE INVENCIÓN [ ] MODELO DE UTILIDAD

(2) TIPO DE SOLICITUD

[ ] ADICIÓN A LA PATENTE
[ ] SOLICITUD DIVISIONAL
[ ] CAMBIO DE MODALIDAD
[ ] TRANSFORMACIÓN SOLICITUD PATENTE EUROPEA
[ ] PCT: ENTRADA FASE NACIONAL

(3) EXPED. PRINCIPAL O DE ORIGEN
MODALIDAD
NUMERO SOLICITUD
FECHA SOLICITUD

(4) LUGAR DE PRESENTACIÓN: MADRID — CÓDIGO: 28

(5) SOLICITANTE(S). APELLIDOS O DENOMINACIÓN SOCIAL	NOMBRE	NACIONALIDAD	CÓDIGO PAIS	DNI/CIF	CNAE	PYME
INSTITUT CATALA D'ONCOLOGIA		ESPAÑOLA	ES	Q5856383D		

(6) DATOS DEL PRIMER SOLICITANTE

DOMICILIO: AV. GRAN VIA, S/N, KM. 2,7
LOCALIDAD: L'HOSPITALET DE LLOBREGAT
PROVINCIA: BARCELONA
PAIS RESIDENCIA: ESPAÑA
NACIONALIDAD: ESPAÑOLA

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CORREO ELECTRONICO
CÓDIGO POSTAL: 08907
CÓDIGO PAIS: ES
CÓDIGO NACION: ES

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ROJAS EXPOSITO	JUAN JOSE	ESPAÑOLA	ES

(8)
[ ] EL SOLICITANTE ES EL INVENTOR
[X] EL SOLICITANTE NO ES EL INVENTOR O ÚNICO INVENTOR

(9) MODO DE OBTENCIÓN DEL DERECHO
[X] INVENC. LABORAL [ ] CONTRATO [ ] SUCESIÓN

(10) TÍTULO DE LA INVENCIÓN
"ADENOVIRUS ONCOLÍTICOS PARA EL TRATAMIENTO DEL CANCER"

(11) EFECTUADO DEPÓSITO DE MATERIA BIOLÓGICA: [ ] SI [ ] NO

(12) EXPOSICIONES OFICIALES: LUGAR ____ FECHA ____

(13) DECLARACIONES DE PRIORIDAD PAIS DE ORIGEN	CÓDIGO PAÍS	NÚMERO	FECHA

(14) EL SOLICITANTE SE ACOGE AL APLAZAMIENTO DE PAGO DE TASAS PREVISTO EN EL ART. 162. LEY 11/86 DE PATENTES [ ]

(15) AGENTE/REPRESENTANTE. NOMBRE Y DIRECCIÓN POSTAL COMPLETA (SI AGENTE P.I., NOMBRE Y CÓDIGO) (RELLÉNESE ÚNICAMENTE POR PROFESIONALES):
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(16) RELACIÓN DE DOCUMENTOS QUE SE ACOMPAÑAN.

[X] DESCRIPCION. Nº DE PÁGINAS: 35
[X] Nº DE REIVINDICACIONES. 12
[X] DIBUJOS Nº DE PÁGINAS: 10
[X] LISTA DE SECUENCIAS Nº DE PÁGINAS. 4
[X] RESUMEN
[ ] DOCUMENTO DE PRIORIDAD
[ ] TRADUCCION DEL DOCUMENTO DE PRIORIDAD
[X] DOCUMENTO DE REPRESENTACION
[X] JUSTIFICANTE DEL PAGO DE TASAS DE SOLICITUD
[ ] HOJA DE INFORMACIÓN COMPLEMENTARIA
[ ] PRUEBAS DE LOS DIBUJOS
[ ] CUESTIONARIO DE PROSPECCIÓN
[ ] OTROS

FIRMA DEL SOLICITANTE O REPRESENTANTE

JORGE ISERN JARA
Colegiado Nº 515

(VER COMUNICACIÓN)

FIRMA DEL FUNCIONARIO

NOTIFICACIÓN DE PAGO DE LA TASA DE CONCESIÓN:

Se le notifica que esta solicitud se considerará retirada si no procede al pago de la tasa de concesión; para el pago de esta tasa dispone de tres meses a contar desde la publicación del anuncio de la concesión en el BOPI, más los diez días que establece el art. 81 del R.D. 2245/1986

ILMO. SRA. DIRECTORA DE LA OFICINA ESPAÑOLA DE PATENTES Y MARCAS

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