Oncogene (2013) **32,** 4406–4416

www.nature.com/onc

ORIGINAL ARTICLE

A human monoclonal antibody 264RAD targeting αvβ6 integrin reduces tumour growth and metastasis, and modulates key biomarkers *in vivo*

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αvβ6 integrin expression is upregulated on a wide range of epithelial tumours, and is thought to play a role in modulating tumour growth. Here we describe a human therapeutic antibody 264RAD, which binds and inhibits αvβ6 integrin function. 264RAD cross-reacts with human, mouse and cynomolgus monkey αvβ6, and inhibits binding to all ligands including the latency-associated peptide of TGF-β. Screening across a range of integrins revealed that 264RAD also binds and inhibits the related integrin αvβ8, but not the integrins α5β1, αvβ3, αvβ5 and α4β1. *In vitro* 264RAD inhibited invasion of VB6 and Detroit 562 cells in a Matrigel invasion assay and αvβ6 mediated production of matrix metalloproteinase-9 in Calu-3 cells. It inhibited TGF-β-mediated activation of dermal skin fibroblasts by preventing local activation of TGF-β by NCI-H358 tumour cells in a tumour cell – fibroblast co-culture assay. *In vivo* 264RAD showed dose-dependent inhibition of Detroit 562 tumour growth, regressing established tumours when dosed at 20 mg/kg once weekly. The reduction in growth associated with 264RAD was related to a dose-dependent inhibition of Ki67 and phospho-ERK and a reduction of αvβ6 expression in the tumour cells, coupled to a reduction in fibronectin and alpha smooth muscle actin expression in stromal fibroblasts. 264RAD also reduced the growth and metastasis of orthotopic 4T1 tumours. At 20 mg/kg growth of both the primary tumour and the number of metastatic deposits in lung were reduced. The data support the conclusion that 264RAD is a potent inhibitor of αvβ6 integrin, with some activity against αvβ8 integrin, that reduces both tumour growth and metastasis.

Oncogene (2013) **32,** 4406–4416; doi:10.1038/onc.2012.460; published online 29 October 2012

Keywords: integrin; αvβ6; αvβ8; beta-6; beta-8; TGF-β

INTRODUCTION

The integrin αvβ6 is an epithelial restricted transmembrane cell surface receptor that has a role in promoting a number of different pathologies, including cancer and fibrosis. It is a dimeric receptor, consisting of an α and a β subunit, that binds the extracellular matrix proteins fibronectin and tenascin.[1–3] In addition, it binds the latency-associated peptide (LAP) of TGF-β1 and -β3 with high affinity, and activates TGF-β.[4] In each ligand it engages an integrin binding motif Arg – Gly – Asp (RGD).[1–3,5] In normal tissues expression is tightly regulated; however, αvβ6 is upregulated in subsets of a wide range of epithelial-derived cancers. Increased expression was originally noted in oral squamous carcinoma,[6,7] where αvβ6 was associated with tumours of greater invasive potential. These observations extend to other solid tumours, including pancreatic, colon, ovarian, breast and lung cancer.[8–12] In these diseases patients showing highest αvβ6 expression have poor prognosis.

Increased expression of αvβ6 in tumour cells is associated with activation of a number of pathways. αvβ6 engagement increases ERK activation,[13] and the secretion of proteases MMP-2 (matrix metalloproteinase-2), -9 and UPA (urokinase-type plasminogen activator).[13–17] Moreover, it drives epithelial- to -mesenchymal transition and the invasive and metastatic potential of cells.[18–21] αvβ6 expression is dynamically regulated with increased expression at the invasive edge of the tumour.[22,9] One of the key physiological functions of αvβ6 is the regulation of local TGF-β pathway activation. TGF-β forms a dimer constrained into an inactive form by the association with TGF-β LAP. αvβ6 releases this constraint, enabling TGF-β to bind TGF-β-RII on adjacent cells;[4,23] therefore, increasing αvβ6 enables tumour cells to modulate TGF-β in a paracrine manner.[24,19] TGF-β signalling in the tumour microenvironment is pleiotropic; in some tumours it acts as a tumour suppressor, while in more advanced tumours TGF-β activation promotes tumour growth and metastasis. In other disease states aberrant activation of TGF-β by αvβ6 has been associated with the development of fibrotic diseases such as kidney and liver fibrosis, and lung disease, and this fibrosis is reduced by αvβ6 inhibition.[25–30] In a pre-clinical tumour model, blocking the receptor with the human therapeutic antibody 3G9 reduces tumour growth.[31]

αvβ6 is functionally related to αvβ8 integrin, interacting with TGF-β LAP.[32,33] Less is known about αvβ8 function, particularly in tumours. Like αvβ6, αvβ8 binds TGF-β-LAP through the integrin binding motif, and activates the TGF-β-mediated signalling cascade.[32–34] αvβ8 has been implicated as a tumour suppressor in certain glioblastoma lines,[35] with downregulation increasing

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Received 17 April 2012; revised 6 August 2012; accepted 16 August 2012; published online 29 October 2012

tumour growth. It is also associated with the regulation of developing vasculature in the embryonic brain, ablation of expression resulting in embryonic lethality through vascular malformations[35] and induced ablation in the adult resulting in brain arteriovenous malformation related pathology.[36]

Here we describe a novel human therapeutic antibody 264RAD, which inhibits αvβ6 integrin with additional activity, versus αvβ8 integrin, which modulates tumour growth and metastasis.

RESULTS

264RAD binds and inhibits αvβ6 integrin

The SLAM technique in Xenomouse animals[37] was used to identify a panel of individual B-cells expressing human αvβ6 reactive antibodies from animals immunised with Chinese hamster ovary cells expressing human αvβ6. Sixteen antibodies were cloned by PCR and expressed as recombinant human immunoglobulin G1 (IgG1) antibodies. The antibodies were screened for specific binding to αvβ6, inhibition of αvβ6 mediated adhesion and selectivity over the related integrin α5β1, αvβ3 and α4β1 (Barry and Foltz, unpublished data). This identified antibody 264, which bound to purified human αvβ6 heterodimer and inhibited binding of purified αvβ6 to TGF-β LAP (data not shown), consistent with direct binding to the receptor. The CDR3 of this antibody consisted of the amino-acid sequence 'VETGRGDYHFYAMDV', which contains the common integrin binding motif RGD. Some low-affinity binding to other receptors was observed by fluorescence activated cell sorting (FACS); therefore, to reduce the potential of low-affinity interaction with other integrins, point mutations were made in the CDR3 to remove the RGD site. Mutating the glycine to alanine resulted in an antibody with the CDR3 'VETGRADYHFYAMDV', named 264RAD; it retained binding to αvβ6, but showed no low-affinity binding to other receptors by FACS. Importantly, the 264RAD mutant has similar potency compared to the parent antibody 264 (Supplementary Table 1).

To specifically assess the interaction of 264RAD with αvβ6, K562 cells were stably transfected with human αvβ6. K562 cells were selected, as they only express α5β1 integrin.[38] Immobilised 264RAD bound K562 αvβ6 cells (Figure 1a). As a positive control the β6 blocking antibody 10D5 and the β1 blocking antibody 4B4 were included. Binding was specific for αvβ6 integrin, as control un-transfected K562 cells did not bind 264RAD (Figure 1b). 264RAD also inhibits the ability of K562 αvβ6 cells to bind recombinant TGF-β LAP (Figure 1c). 264RAD did not inhibit αvβ3/β5-mediated binding to vitronectin, while L230 an αv blocking antibody, reduced adhesion (Figure 1d). 264RAD did not inhibit α5β1-mediated binding to fibronectin, while IIA1 an α5 blocking antibody, reduced adhesion (Figure 1e). 264RAD did not inhibit α4β1 binding to CS-1, while 4B4 a β1 blocking antibody reduced adhesion (Figure 1f).

To confirm selectivity for cells expressing endogenous αvβ6, binding to a panel of cell lines was determined. Cells were selected based on the differential expression of *ITGB6, ITGB8, ITGB5, ITGAV, ITGB3, ITGA5 and ITGA4* (Supplementary Table 2). Colo205, HT29, MBA-MB-231, NCI-H2122 and NCI-H358 express αvβ6, as confirmed by binding to the β6 blocking antibody 10D5 (Figure 1g). 264RAD is bound to each of these cell lines (Figure 1g). MDA-MB-231 and NCI-H1299 did not bind 10D5, suggesting lack of β6 expression. 264RAD did not bind these cell lines. As an additional control, human umbilical vein endothelial cells were included as an αvβ6 null cell type that expresses αv integrins. 264RAD did not bind human umbilical vein endothelial cells, providing further evidence of the selectivity (Figure 1g).

264RAD inhibits human, mouse and cynomolgus monkey αvβ6

αvβ6 potency was determined using cell-based assays. In a cell-based adhesion assay, 264RAD was significantly more potent than 10D5, a commercially available αvβ6 blocking antibody (Supplementary Figure 1), 264RAD bound αvβ6 on cells with an affinity of <10 pM. To compare binding of 264RAD to human, murine and cynomolgus monkey αvβ6 in the same cellular background, we stably overexpressed each respective species homologue of both αv and β6 integrin in K562 cells to generate species-specific heterodimers. K562 cells did not express human αv integrin. 264RAD bound human, murine and cynomolgus monkey αvβ6 integrin heterodimer (Figure 2a). 264RAD did not bind untransfected K562 cells (data not shown), consistent with the data presented in Figure 1. To determine whether 264RAD was able to inhibit binding of human, murine and cynomolgus monkey αvβ6 to glutathione-s-transferase-TGF-β LAP, a cell-based adhesion assay was used (Figures 2b – d). 264RAD is active against all three αvβ6 homologues.

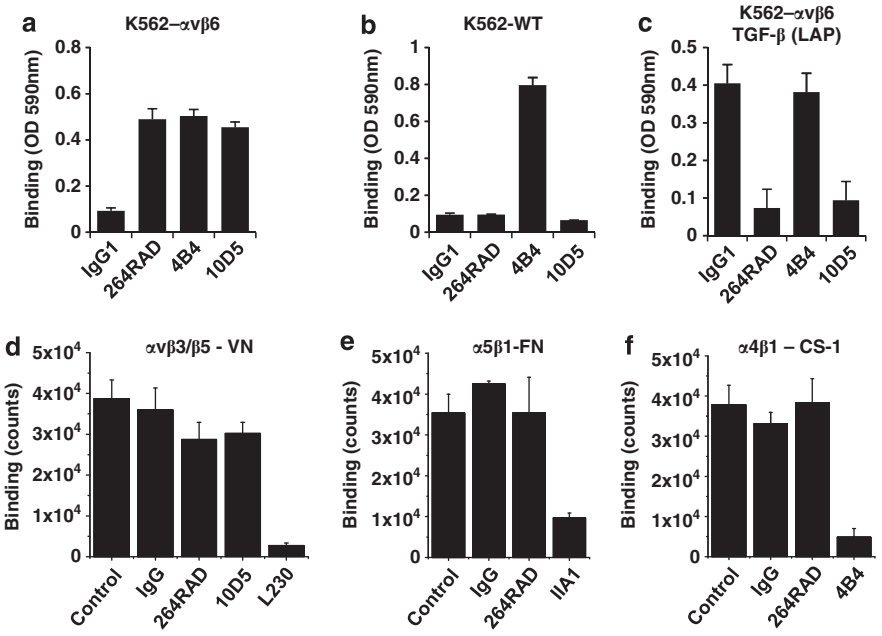
264RAD binds and inhibits αvβ8 integrin

The β6 chain of αvβ6 is related to another integrin β8 which also binds TGF-β LAP.[32] 264RAD also bound K562 cells expressing human αvβ8 by FACS (Figure 3a). αvβ6-and αvβ8-expressing K562 cell binding to 264RAD-coated plates was assessed (Figure 3b). 264RAD bound both αvβ6 and αvβ8 overexpressing cells. 264RAD also inhibited the binding of K562 cells overexpressing αvβ6 and αvβ8 to immobilised glutathione-S-transferase-TGF-β LAP (Figures 3c and d); however, 264RAD consistently did not give full inhibition of binding (Figure 3d). 264RAD binds and inhibits both αvβ6 and αvβ8 integrins.

264RAD inhibits tumour cell invasion and MMP-9 secretion *in vitro* and activation of TGF-β in a co-culture assay

Increased αvβ6 expression increases the invasive potential of tumour cells *in vitro*. At 1 μg/ml 264RAD markedly reduced the invasion of VB6 cells[16] through Matrigel (Figure 4a). 264RAD was more effective against Detroit 562 cells, giving full inhibition down to 1 μg/ml (Figure 4b). The potential for co-expression of αvβ6 and αvβ8 integrin was assessed across a number of tumour cell lines. Owing to a lack of appropriate antibodies to assess expression of β8 protein, expression was determined by reverse transcriptase–quantitative PCR (Supplementary Figure 2). Detroit cells also express *ITGB8;* therefore, it is possible that 264RAD also modulates αvβ8 function in this line. αvβ6 has previously been shown to modulate protease production.[13–17] When present at 10 μg/ml 264RAD reduced the accumulation of MMP-9 in Calu-3 conditioned media as determined by enzyme-linked immunosorbent assay (Figure 4c).

αvβ6 and αvβ8 integrins have a pivotal role in modulating activation of TGF-β.[4,32,33] 264RAD in the presence or absence of latent TGF-β reduced both basal and induced pSmad3 levels in Detroit 562 cells (Supplementary Figure 4). To assess modulation of TGF-β further a co-culture system was used. Co-culturing tumour cells with fibroblasts induces alpha smooth muscle actin (α-SMA) expression via TGFβ-mediated mechanisms.[19] Culturing NCI-H358 with fibroblasts induced activated α-SMA-positive fibroblasts (Figures 4c, i, and iii). This activation was inhibited by both a small-molecule inhibitor of Alk-5 (TGF-β RII) (Figure 4c, iv) and 264RAD (Figure 4c,ii), demonstrating effective inhibition of TGF-β activation. 264RAD inhibited activation of TGF-β mediated by NCI-H358 cells at concentrations as low as 80 ng/ml (Figure 4d). Although we have confirmed that NCI-H358 express αvβ6, αvβ8 expression is unknown. NCI-H358 had detectable *ITGB8* mRNA suggesting the potential to express β8 integrin, although lower than Detroit 562 cells (Supplementary Figure 2). Although we cannot exclude that αvβ8 makes some contribution to the activation of fibroblasts by NCI-H358 cells, downregulating β6 expression by small interfering RNA treatment of the co-culture abrogated α-SMA activation in both relatively high (Calu-3) and low NCI-H358 *ITGB8*-expressing lines (Supplementary Figure 3).



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Cell line	Capture Antibody						
	264RAD	10D5 beta-6	P5H9 beta-5	L230 alpha-v	LM609 beta-3	IIA1 alpha-5	7.2R alpha-4
Colo205	1996	1917	0	2629	28	3	0
HT-29	693	2491	5116	1305	9	0	0
MDA-MB-B231	0	0	3280	2953	5579	4431	0
MDA-MB-468	5962	4142	1415	1699	0	0	0
NCI-H1299	0	7	0	1346	73	1548	1078
NCI-H2122	2045	4553	3014	705	0	139	22
NCI-H358	741	3496	239	2367	11	8	0
HUVEC	5	24	0	267	2002	1477	0

Figure 1. 264RAD binds and inhibits αvβ6 integrin. Binding of K562 cells overexpressing αvβ6 (**a**) and K562 control cells (**b**) to wells coated with 10 μg/ml human IgG1 control, 264RAD, 4B4 and 10D5 antibodies. Cells were allowed to attach for 30 min at RT. 264RAD inhibits binding of K562 αvβ6 cells to wells coated with 0.5 μg/ml GST TGF-β LAP. K562 αvβ6 cells (**c**) adhered in the presence of 10 μg/ml of human IgG1 control antibody, 264RAD, 4B4 and 10D5 for 30 min at 37 °C. To test the selectivity over αvβ3/β5 integrins, the ability of 264RAD to block A375M adhesion to plates coated with vitronectin was tested. Cells were incubated with IgG1 isotype control, 264RAD, 10D5 (β6) and L230 (αv) at 0.5 μg/ml, and adhered at 37 °C for 40 min (**d**). To test the selectivity over α5β1 integrin, the ability of 264RAD to block K562 adhesion to plates coated with GST-FN$^{8-10}$ was tested. Cells were incubated with IgG1 isotype control, 264RAD, IIA1 (α5) at 5 μg/ml, and adhered at 37 °C for 40 min (**e**). To test the selectivity over α4β1 integrin, the ability of 264RAD to block K562 adhesion to plates coated with CS-1 was tested. Cells were incubated with IgG1 isotype control, 264RAD, 4B4 (β1) at 5 μg/ml, and adhered at 37 °C for 40 min (**f**). To assess cell binding, 264RAD and a range of integrin antibodies were immobilised on the plate (at 1 μg/ml). Cells were allowed to bind to antibodies for 30 min at RT. Green represents strong binding and red represents no binding (**g**). The data are representative of more than two similar experiments.

264RAD inhibits growth of Detroit 562 tumours

Detroit 562 tumour xenografts are dependent on αvβ6 as growth is inhibited by the β6-specific antibody 3G9.[31] First the expression of αvβ6 and αvβ8 was assessed; Detroit 562 cells expressed *ITGB6* and *ITGB8* mRNA (Supplementary Figure 2). Next, 264RAD was active *in vitro* versus Detroit 562 cells fully inhibiting adhesion to TGF-β LAP at concentrations above 5 μg/ml (Figure 5a). *In vivo* 264RAD inhibited the growth of Detroit 562 tumours. Animals bearing established tumours were dosed with 264RAD once a week at 20, 5 and 1 mg/kg (Figure 5b). At 20 mg/kg 264RAD inhibited growth of the tumours, consistently inducing a minor but clear regression of the tumours. At 5 mg/kg growth suppression was still >90%, and was marginally reduced at 1 mg/kg.

264RAD modulates key biomarkers in Detroit 562 cells

The Detroit 562 tumours were analysed with a panel of immunohistochemistry biomarkers. In control tumours, αvβ6 expression was high at the edge of the tumour nest and reduced within the core of each nest (Figure 6a). Treatment with 264RAD reduced the expression of αvβ6 integrin in a dose-dependent manner (Figures 6a and b), markedly reducing the expression at the periphery of the tumour. 264RAD gave a marked dose dependent decrease in FN in stromal cells (Figures 6a and c), and Ki67 (Figures 6a and d) and pERK (phospho-ERK) (Figures 6a ande) in tumour cells. The decrease in αvβ6 was not due to steric inhibition of the detection antibody by 264RAD, as binding of the detection antibody to NCI-H358 cells (an *ITGB6* 'high' *ITGB8* 'low' cell line) was detected in the presence of saturating amounts of 264RAD (Supplementary Figure 5). E-cadherin expression in the tumour cell compartment was also increased in those regions where αvβ6 was reduced (Supplementary Figure 6). Consistent with the change in stromal FN, 264RAD also reduced α-SMA expression in stromal cells (Figure 6f), both regulated by TGF-β.[39,40] TGF-β activation in the tumour cells was determined by assessing human *PAI-1* mRNA expression but no change was detected, nor was a change in the pSmad2/3 status detected in tumour lysates (data not shown). Vascular structures in the tumour were also assessed (Supplementary Figure 8). Microvessel density was unaffected, although there was a small decrease in

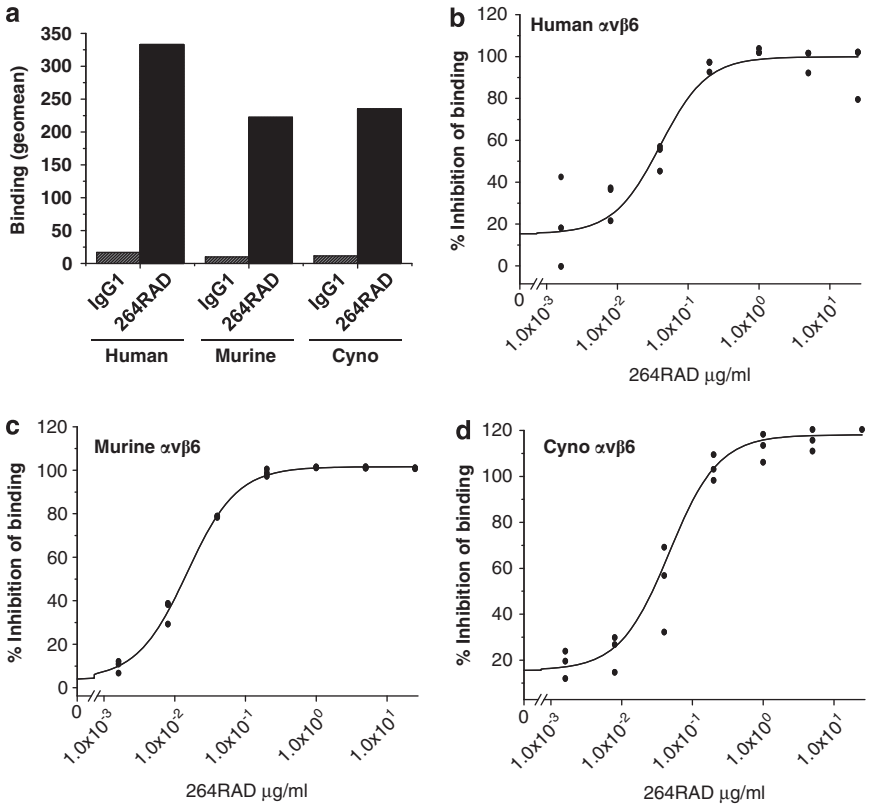


Figure 2. Cross-species binding and functional activity of 264RAD. (**a**) Binding to K562 cells overexpressing human, murine and cynomolgus monkey was assessed by FACS. 264RAD and isotype control antibody were incubated with cells at 15 μg/ml, at RT for 30 min. The geomean for each is shown. The data are representative of more than two experiments. To assess functional activity, the ability of 264RAD to inhibit cell binding to GST TGF-b LAP was assessed. K562 cells overexpressing human (**b**) murine (**c**) and cynomolgus monkey (**d**) were incubated in the presence of a range of concentrations of 264RAD, and the ability to inhibit binding to plates coated with GST TGF-b LAP at 15 μg/ml for 30 min at 37°C was studied. The percentage inhibition of binding is represented. The data are representative of three identical assays.

microvessel area (Supplementary Figure 8). At the end of the study we were unable to detect increases in caspase 3 in the 264RAD-treated tumours (Supplementary Figure 7). The changes in proliferation and pERK activation are consistent with 264RAD having an antiproliferative mode of action in this tumour model, while the changes in FN and α-SMA demonstrate additional modulation of stromal cells.

264RAD reduces growth of 4T1 murine tumour xenografts *in vivo* and reduces lung metastasis

To further assess the *in vivo* anti-tumour properties of 264RAD, the efficacy in 4T1 tumours, a murine orthotopic breast tumour model that metastases to the lung, was assessed. 4T1 expresses murine *Itgb6* and *Itgb8* (Supplementary Figure 2C). 264RAD inhibited binding of 4T1 cells to TGF-β LAP (Figure 7a). 264RAD reduced primary 4T1 tumour growth by 40 and 50% when treated twice weekly with 264RAD at 20 mg/kg (Figure 7b), and the metastatic tumour burden by >90% (Figure 7c). These data demonstrate that in addition to having potential to target the growth of primary tumours, treatment with 264RAD is associated with reduced metastasis.

DISCUSSION

264RAD is a potent inhibitor of αvβ6 that also modulates αvβ8 but does not bind or inhibit αvβ3, αvβ5, α4β1 or α5β1. The binding of 264RAD is influenced by features in the heavy chain CDR3, which consists of the amino acids 'VATGRADYHFYAMDV'. 264RAD contains a motif RADYxxY in the CDR3 (RGDYxxY in the parent antibody) that is critical for binding, as a RTD mutant was unable to bind αvβ6 (unpublished data). The motif is similar to peptides derived from the foot and mouth virus, and the integrin binding motif in TGF-β LAP that has the canonical binding sequence RGDLxxL[41,42,12,5] and that also binds αvβ8.[32–34] It is surprising that 264RAD can tolerate an alanine-to-glycine mutation retaining binding activity, as this often abrogates binding to RGD-dependent integrins. Although not tested in the current study, the tyrosine residues may also contribute to the selectivity of the antibody, holding the RAD motif in the correct conformation for binding.

The *in vitro* properties of 264RAD show the potential to reduce local cell invasion and metastasis by inhibiting protease production and cell invasion. This cell invasion is mediated by αvβ6 integrin in both lines, as invasion of VB6 is induced by overexpression of αvβ6, whereas the invasion of Detroit 562 cells was abrogated by the specific αvβ6 blocking antibody 10D5 (unpublished data). 264RAD also inhibited integrin-mediated activation of TGF-β measured in a tumour cell – fibroblast co-culture assay, reducing the induction of a tumour cell-conditioned fibroblast phenotype. This was consistent across a number of tumour cell lines tested, and was associated with modulation of Smad2/3 activation (Eberlein *et al.*, unpublished data). The role of αvβ6 as a key activator of TGF-β activation in tissues is well described.[4,23] This function can also be performed by αvβ8;[32,34] therefore, 264RAD could modify αvβ8-dependent TGF-β activation either in the tumour cells or in fibroblasts. However, in the Calu-3 (*ITGB8* 'high') and NCI-H358 (*ITGB8* 'low')

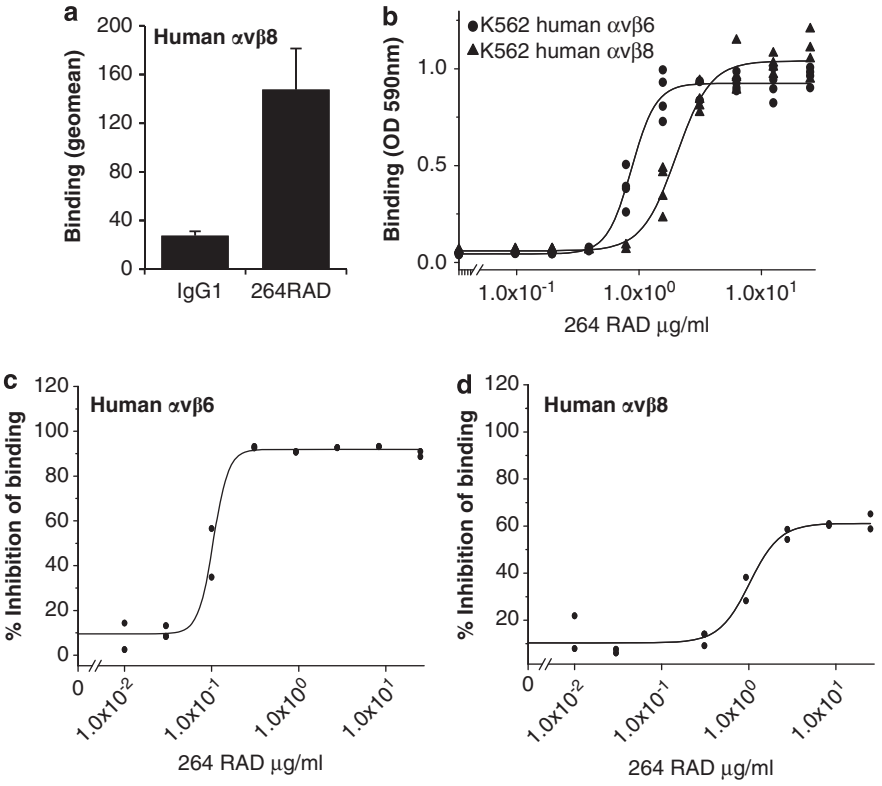


Figure 3. Inhibition of αvβ8 integrin. Binding of 264RAD to αvβ8, K562 cells overexpressing αvβ8 were incubated with 264RAD at 10 μg/ml and binding detected by FACS (**a**). To confirm binding of 264RAD to αvβ6 and αvβ8, the binding of K562 cells overexpressing each receptor to immobilised 264RAD was assessed (**b**). 264RAD was immobilised at a range of concentrations as indicated, and cells allowed to adhere for 30 min at RT. Data are representative of greater than three similar experiments. To assess functional inhibition the ability to block binding of K562 cells overexpressing αvβ6 (**c**) and αvβ8 (**d**) to plates coated with TGF-β LAP was assessed. GST TGF-β LAP was coated at 0.5 μg/ml, and cells were allowed to adhere in the presence of a range of concentrations of 264RAD for 30 min. The data are representative of three similar experiments.

cells the cross-talk was αvβ6 dependent, as small interfering RNA to β6 completely inhibited tumour cell activation of α-SMA. This does not exclude a role for β8, but does suggest that the initiating event is largely β6 dependent.

The anti-tumour effects of 264RAD were more apparent *in vivo*. In Detroit 562 we show that 264RAD influences both tumour cell proliferation and activation of stromal cells. 264RAD gave modest (30%) inhibition of Detroit 562 growth *in vitro* (data not shown); however, biomarker analysis performed on treated tumours revealed marked inhibition of pERK and Ki67 in the tumour cells. 264RAD also downregulated αvβ6 expression in the tumour cells, consistent with observations that 264RAD drives receptor internalisation *in vitro* (unpublished data). We also observed a reciprocal increase in E-cadherin expression in those regions where αvβ6 expression was reduced, consistent with the association of αvβ6 expression with epithelial–mesenchymal transition, and driving the downregulation of E-cadherin expression.[9] Although in tumours taken at the end of study no change in Smad phosphorylation was detected, modulation of both FN and α-SMA expression, both TGF-β-mediated proteins was observed,[39,40] suggesting modulation of TGF-β-dependent signalling in the stroma. From these data we conclude that 264RAD inhibits tumour cell growth by targeting both tumour proliferation signals and modulating activated stroma. Detroit 562 cells express both β6 and β8 mRNA, but we have been unable to determine whether 264RAD modulates tumour growth purely through αvβ6, and it would be informative to understand whether αvβ8 also contributes to tumour growth in this model.

αvβ6 and TGF-β regulate a number of processes involved in tumour progression and have a close functional relationship. In tumour cells αvβ6 can activate ERK,[17] increase protease expression,[14,16,17] modulate epithelial–mesenchymal transition status[9] and promote an invasive phenotype,[16,18,20] whereas in the context of the tumour microenvironment αvβ6 can induce a tumour-associated fibroblast phenotype.[19] TGF-β can suppress or promote growth, depending on cell context, drive epithelial – mesenchymal transition[43,44] and activate cancer associated-fibroblasts.[19] Given the strong disease association for αvβ6 in a number of tumour types, identifying the key features of tumours likely to be dependent on αvβ6-mediated signalling is critical to understand where agents targeting αvβ6 will give benefit in established disease. Our data suggest that αvβ6 antagonists give single-agent activity in tumours that are dependent on TGF-β. Interestingly features that confer TGF-β dependency have been suggested, such as downregulation of disabled homolog 2[43] or an association with ras or raf mutation.[44]

Increased αvβ6 expression is associated with increased invasion and metastasis, expression at the invasive edge being associated with increased protease activity. 264RAD inhibited lung metastasis *in vivo* in the metastatic 4T1 murine breast orthotopic model. Demonstrating modulation of both primary the tumours and the incidence of metastatic deposits suggest that 264RAD could retard tumour cell invasion and dissemination. To our knowledge this is the first time a therapeutic targeting αvβ6 has been shown to inhibit distant metastasis. Although 4T1 are strongly positive for αvβ6 integrin, the mRNA *Itgb8* is also expressed. Owing to a lack of suitable antibodies we were unable to confirm protein expression. Therefore it is not possible to exclude that αvβ8 forms part of this signalling complex. Interestingly, 4T1 tumour growth is also sensitive to TGF-β sequestration[45] and Alk-5 inhibition

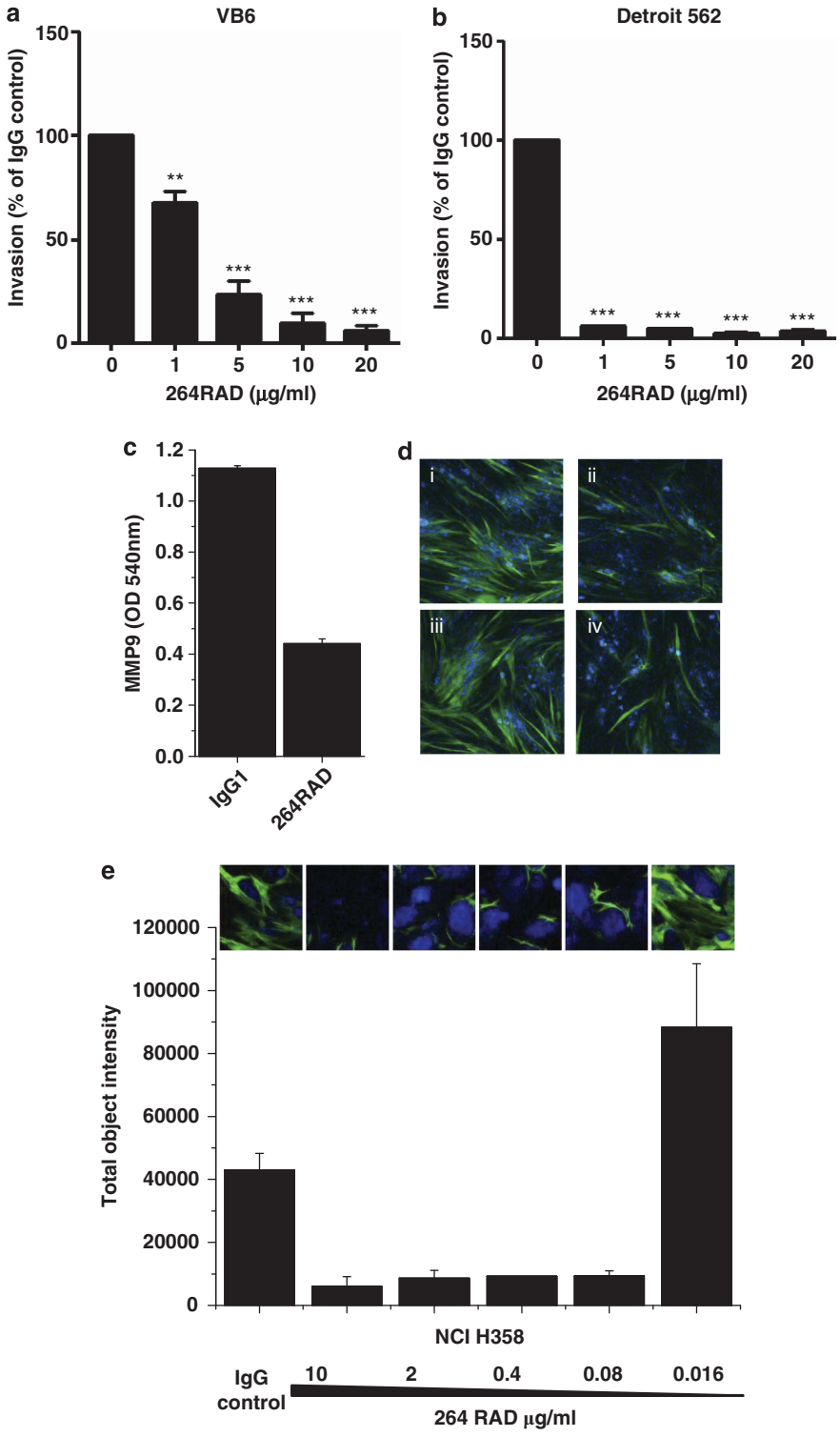


Figure 4. 264RAD inhibits invasion, protease production and activation of TGF-β. The ability of 264RAD to inhibit tumour cell invasion was tested using VB6 cells (**a**) and Detroit 562 cells (**b**). Invasion of cells through Matrigel was measured after 72 h incubation at 37 °C. Data shown are the averages of at least three separate experiments with three replicates per experiment ± s.d. Statistical differences were determined by ANOVA and Dunnett's *post-hoc* test, where $^{*}P \leqslant 0.05$, $^{**}P \leqslant 0.01$, $^{***}P \leqslant 0.001$. (**c**) To determine whether 264RAD reduces MMP-9 production, Calu-3 cells were incubated with 10 μg/ml 264RAD or IgG1 isotype control antibody for 7 days at 37 °C. (**d**) To assess inhibition of TGF-β activation NCI-H358 cells were co-cultured with fibroblast. Activation of TGF-β was monitored by staining fibroblasts for α-SMA. To demonstrate Alk-5 and αvβ6 dependency, co-cultures were treated with (i) DMSO, (ii) Alk-5 inhibitor SB-431542 (10 μM), (iii) IgG1 control (10 μg/ml) and (iv) 264RAD (10 μg/ml). α-SMA-positive fibroblasts are shown in green, and Hoescht-positive cell nuclei are shown in blue. Magnification × 50. Data are representative of three similar experiments. The potency of 264RAD was confirmed by incubating the co-culture at a range of concentrations of 264RAD as indicated. IgG1 isotype control was used at 10 μg/ml (**e**). (**e**) Quantification of the α-SMA-positive cells in the co-culture; representative images of the same treatments. This experiment is representative of three experiments.

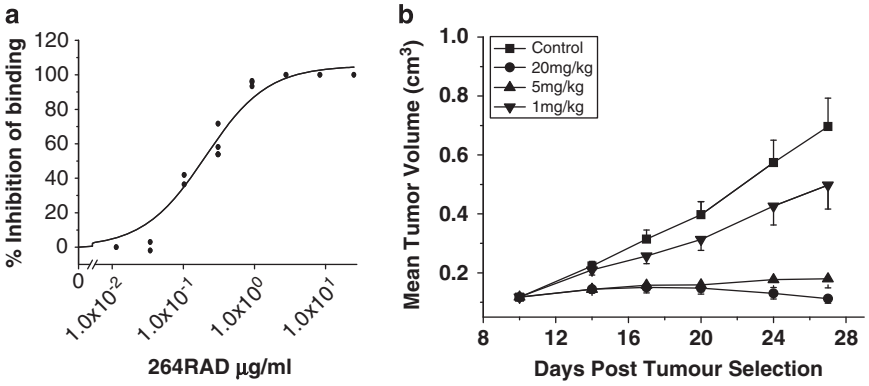


Figure 5. Inhibition of Detroit 562 tumour growth. (**a**) A cell adhesion assay was used to demonstrate functional inhibition of Detroit 562 binding to GST − TGF − LAP (coated at 0.5 μg/ml). (**b**) Established Detroit 562 tumours were treated with 264RAD at 20 mg/kg (circle), 5 mg/kg (triangle) and 1 mg/kg (inverse triangle) twice, weekly. Tumour growth relative to control is represented ± s.e.m. This is representative of three similar studies.

(unpublished data). Inhibition of TGF-β activation can additionally stimulate an immune response to the tumour; indeed sequestration of TGF-β in 4T1 tumour-bearing mice can lead to an *ex vivo* immune response to 4T1 cells;[45] this may also contribute to the anti-metastatic effect observed with 264RAD.

Given tumour models express *ITGB8,* we cannot exclude the possibility that the inhibition of αvβ8 by 264RAD contributes to anti-tumour activity, even though we have not demonstrated a role for αvβ8 *in vitro*. The role of αvβ8 in epithelial tumour cell biology is poorly understood. Increased expression of αvβ8 is reported to negatively regulate the growth of SW480 cells.[35] In U87 MG cells downregulation of β8 by microRNA-93 is associated with increased angiogenesis and tumour growth, while cells overexpressing the integrin show increased apoptosis.[46] Owing to the lack of antibodies to β8 integrin we do not know the expression of the integrin in our pre-clinical models. There is a correlation between αvβ6 expression and αvβ8 at the mRNA level in tumour array data; however, this type of disease expression analysis is complicated by the expression of αvβ8 on activated fibroblasts.[46] Interestingly, β8 expression has been associated with a metastatic breast signature, which suggests that it may make a positive contribution to epithelial tumour progression.[47,48] In some tumours αvβ6 and αvβ8 may therefore cooperate to promote tumour growth and dissemination. Further exploration of the role of αvβ8 in solid tumour biology is warranted. In the current studies we have assessed the effects of 264RAD using xenografted tumour models. Given the complexity of the biology and the limitations of xenograft tumour models it will be interesting to test 264RAD in murine transgenic or human-derived explant models that may recapitulate relevant tumour biology more faithfully.[49]

In summary, we have generated a therapeutic antibody with the unique property of targeting αvβ6 integrin with some activity versus αvβ8 integrin. This antibody has potential to continue to be developed as a therapeutic, treating tumours dependent on αvβ6 for growth and invasion.

MATERIALS AND METHODS

Immunisation and antibody generation

Antibodies to αvβ6 were generated as described in US patent Application Serial number 08/759,620 and International Patent Applications WO98/24893 and WO00/76310. Xenomouse animals were immunised with Chinese hamster ovary cells overexpressing αvβ6 integrin. Spleens were harvested from the mice showing the strongest titres and hybridomas generated by B cell fusion with myeloma P3 × 63Ag8.653 cells purchased from ATCC (cat#CRL1580) (Manassas, VA, USA). Control human IgG1 antibody was generated at MedImmune (Cambridge, UK).

Reagents

Collagen I-coated 96-well plates were obtained from BD Biosciences (Oxford UK). αvβ6 antibody (10D5) and αvβ3 antibody (LM609) were obtained from Merck and Millipore (Darmstadt, Germany); αvβ5 antibody (P5H9) and α4 antibody (7.2R) from R&D Systems (Abingdon, UK); αv antibody (L230) from Alexis (Exeter, UK); α5 antibody (IIA1) from BD Biosciences (Oxford, UK). Goat Anti-Human, Alexa Fluor 488 from Invitrogen (Paisley, UK); mouse anti α-smooth muscle actin antibody (1A4) from Sigma (Dorset, UK); and ELISA kit for MMP-9 from R&D Systems (Abingdon, UK).

Cell culture and stable cell lines

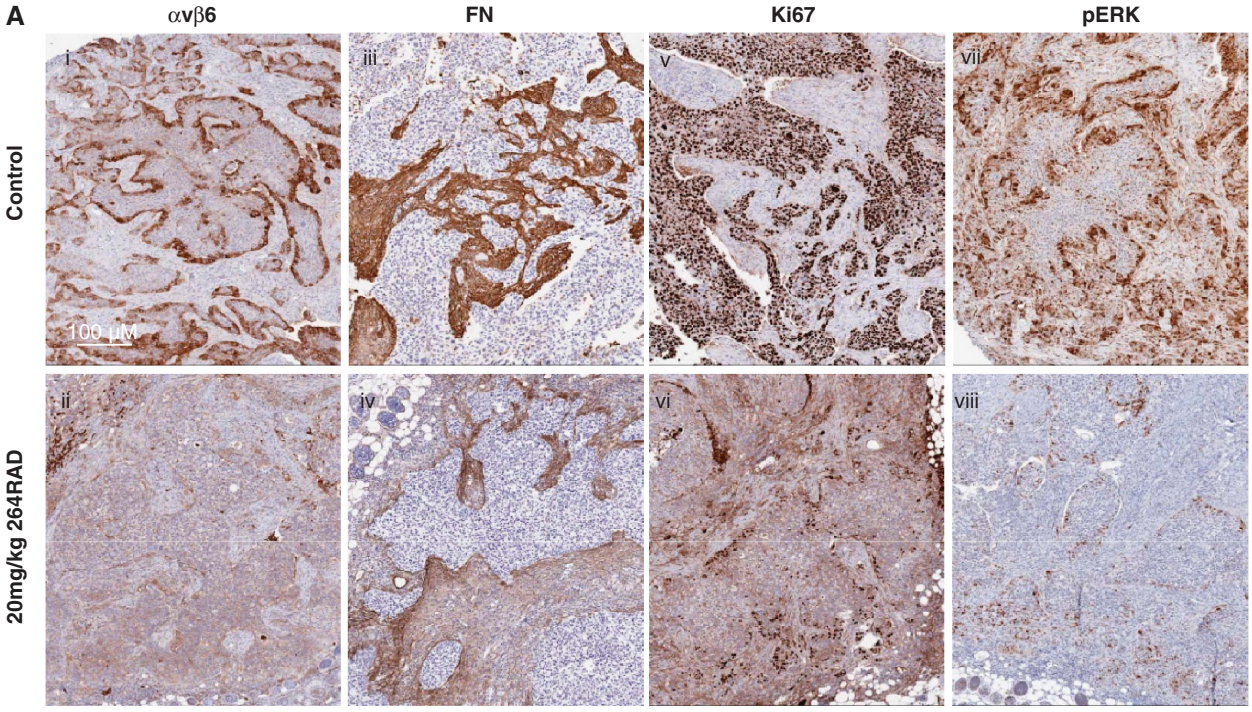
Normal human dermal fibroblasts were purchased from PromoCell, cultured in PromoCell fibroblast growth media PromoCell (Heidelberg, Germany) and maintained at 5% CO_2 at 37 °C. A549, 4T1, Detroit 562, Calu-3, MDA-MB-231, MDA-MB-468, NCI-H2122, NCI-H1299 and NCI-H358 cells were obtained from ATCC. Colo205 and HT-29 cells were obtained from the ECACC. No further authentication was performed. Human, murine and cynomolgus αv and β6 genes were cloned into pCR3.1 to generate K562 cell lines stably expressing human, murine or cynomolgus αvβ6. Human αv and β8 genes were cloned into pGenIRES constructs to create K562 cells stably expressing αvβ8. All cell lines were passaged for no longer than 3 months, cultured in RPMI media supplemented with 10% serum and 2 mM L-glutamine and maintained in 5% CO_2 at 37 °C.

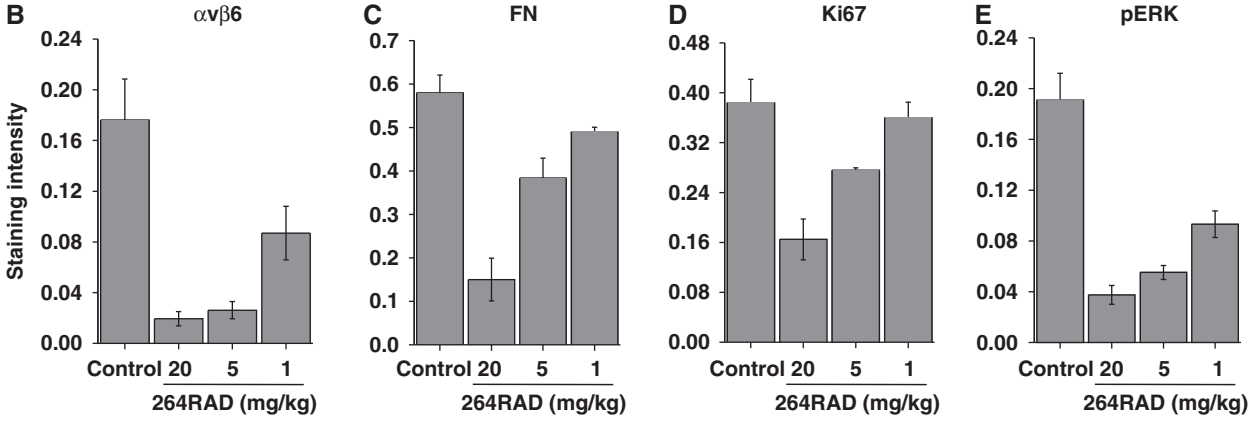
Cell adhesion assays

Detailed methods for all cell adhesion assays are shown in Supplementary Methods. Briefly, for each cell adhesion 96-well assay plates were coated with ligands at the appropriate concentration and blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). As required, cells were trypsinised to take them into suspension. All cells were then allowed to adhere to ligand-coated plates, in the presence and absence of function-blocking antibodies in Hank's balanced salt solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$ at 37 °C, 5% CO_2 for 30–60 min. K562 cells were allowed to adhere in the presence of 1 mM $MnCl_2$. Plates were then washed in PBS, fixed in ethanol and binding visualised by staining with crystal violet, solubilised in 0.1% Triton X-100 and read at optical density 570 nm.

Integrin profiling capture assays

96-well microtitre plates were coated with integrin antibody at 1 μg/ml in PBS, overnight at 4 °C. Plates were washed and blocked with PBS 3% BSA for 1 h at room temperature (RT). Cells were trypsinised and washed in Hank's balanced salt solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$ and resuspended in Hank's balanced salt solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. A total of 200 000 cells were then added per well and the plates incubated at 37 °C, 5% CO_2 for 1 h. Following incubation all





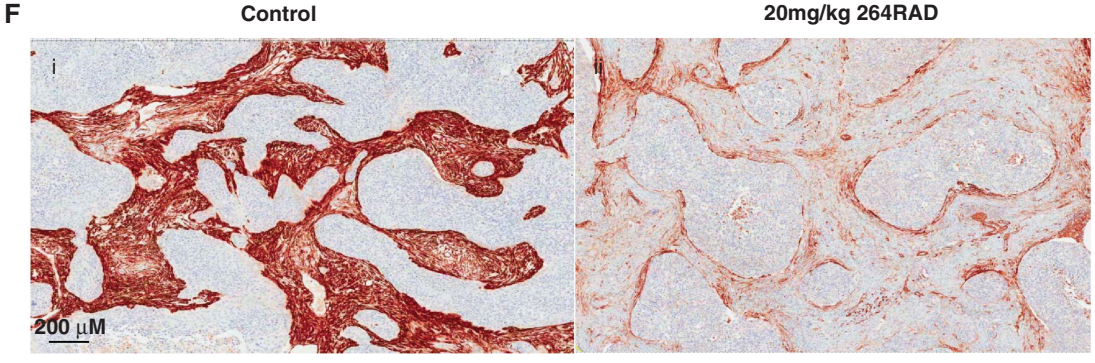


Figure 6. 264RAD induced pharmacodynamic changes in Detroit 562. To assess this mechanism, a range of histological assays were used. Representative images of αvβ6 in control (**ai**) and 20 mg/kg 264RAD (**aii**) treated tumours, FN in control (**aiii**) and 20 mg/kg 264RAD (**aiv**) treated tumours, Ki67 in control (**av**) and 20 mg/kg 264RAD (**avi**) treated tumours, and pERK in control (**avii**) and 20 mg/kg 264RAD (**aviii**) treated tumours are shown. Bar represents 20 μm. The effects on these biomarkers following treatment with 20, 5 and 1 mg/kg are shown for αvβ6 (**b**), fibronectin (**c**), Ki67 (**d**) and pERK (**e**). The mean values ± s.d. are represented. The expression of α-SMA in the stroma was assessed (**f**). Representative images of control (**i**) and 264RAD-treated tumours (**ii**) (20 mg/kg twice weekly) are shown. Bar represents 200 μm.

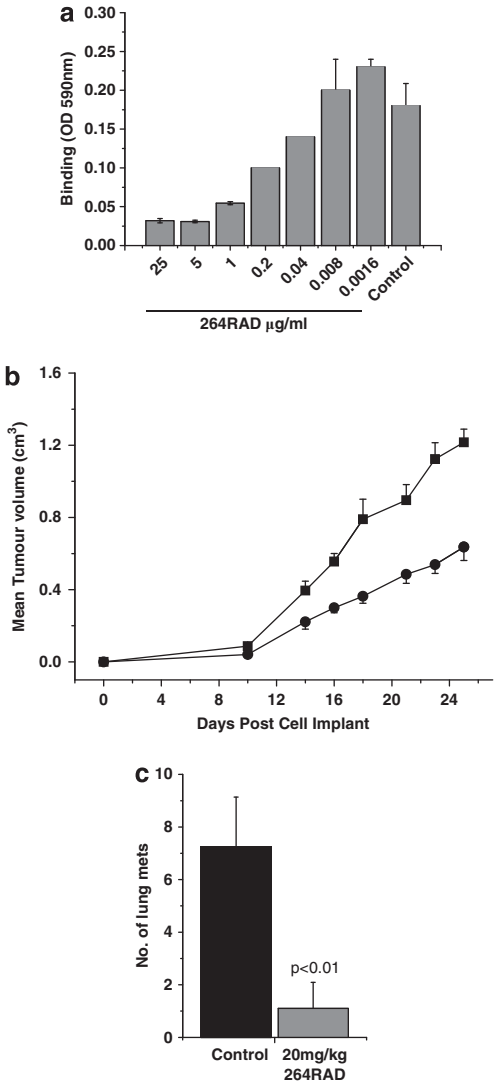


Figure 7. 264RAD inhibits growth of 4T1 tumour xenografts. (**a**) The inhibition of 4T1 binding to TGF-β LAP (coated at 0.5 μg/ml) was investigated using a cell-adhesion assay. 4T1 cells were allowed to adhere in the presence of a range of concentrations of 264RAD. Control IgG was used at 25 μg/ml. Mean binding is represented ± s.d. The data are representative of two identical experiments. (**b**) Established 4T1 tumours were treated with 264RAD at 20 mg/kg twice weekly. The mean group size is represented ± s.e.m. The data are representative of three identical experiments. (**c**) To assess the effect of 264RAD on metastasis, lungs from control and 20 mg/kg 264RAD-treated tumour-bearing animals were assessed for lung metastases. The average number of individual metastases in each group is indicated ± s.e.m.

non-adhered cells were flicked from the plates and each well was washed carefully three times with PBS. Bound cells were fixed with ethanol for 30 min RT, and the cell nuclei stained using 1 μg/ml Hoechst 33342 stain in PBS for 30 min RT. The number of nuclei in each well was counted on the Cellomics Arrayscan (Thermo Scientific, Erembodegem, Belgium).

FACS-based binding assays

The relative binding of 264RAD to human αvβ6 and αvβ8 and to murine and cynomolgus αvβ6 was measured using FACS. Cells were harvested and resuspended at 2×10^6 cells/ml in ice-cold PBS/1% BSA containing 2 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Two hundred microltres of cell suspension was incubated with dilutions of either 264RAD or IgG1 isotype control in FACS tubes BD Biosciences, for 1 h on ice. The cells were washed with 4 ml of PBS/1% BSA, and incubated with 200 μl of PBS/1% BSA containing 10 μg/ml of goat anti-human, Alexa 488 antibody at 4 °C for 30 min and washed as above. Cells were finally resuspended in 500 μl of PBS and binding of 264RAD measured using a FACS Calibur BD Biosciences. Histograms of fluorescence intensity (X-axis) against the number of fluorescent cells (Y axis) were plotted.

Transwell-Matrigel 2D invasion assay

A volume of 70 μl Matrigel BD Biosciences, diluted 2:1 in serum-free media (α-MEM) was added to the polycarbonate filter Transwell (6.5 mm diameter, 8 μm; Corning BV, Amsterdam, Netherlands) for 1 h at 37 °C. A single-cell suspension of cells (VB6 or Detroit 562; 5×10^4) in α-MEM (200 μl) were added to the Matrigel and 0.5 ml fetal calf serum containing α-MEM placed in the lower chamber. The Transwell was incubated at 37 °C. After 72 h, cells that had invaded to the bottom were collected as follows. The 0.5 ml of media in the lower chamber was collected and placed in 9 ml of filtered Isoton (BD Biosciences). Trypsin-EDTA (0.5 ml) was added to the lower chamber and incubated at 37 °C (45 min), the insert agitated to release cells attached to its under-surface and then removed to the same Isoton suspension. The total 10-ml cell suspension was counted on a Casy 1 cell counter (Sharfe System GmbH, Reutlingen, Germany). Results were expressed relative to the invasion of control treatments. In some experiments αvβ6-blocking 264RAD or control antibody (human IgG1) was added (10 μg/ml) to cells for 30 min at 4 °C prior to cell plating onto Matrigel.

MMP-9 protease expression assay

Calu-3 cells were seeded on collagen-I-coated 96-well tissue culture plates at 7500 cells per well in RPMI supplemented with 10% fetal calf serum. Cells were incubated in the presence of 264RAD or control human IgG1 for 7 days at 37 °C. Condition media was then harvested and analysed for the presence of MMP-9 by ELISA.

Normal human dermal fibroblasts/tumour cell co-culture assays

Normal human dermal fibroblasts were seeded on collagen I-coated 96-well plates at 2000 cells per well in PromoCell fibroblast growth media. Following 3 days incubation at 37 °C, 5% CO_2, the media was aspirated from the wells and tumour cells seeded onto the normal human dermal fibroblasts monolayer at 1000 cells per well in 100 μl of RPMI, 10% fetal calf serum, 2 mM L-glutamine per well. The following day the antibody was added in RPMI, 2% fetal calf serum, 2 mM L-glutamine. Fresh media and antibody were added after 4 days incubation at 37 °C, 5% CO_2. After a total of 8 days cells were fixed with 4% formaldehyde solution in PBS containing 0.1% Triton X-100 for 30 min at RT and then washed with PBS and blocked for 1 h at 37 °C with PBS/1% BSA. The cells were incubated with α-SMA antibody diluted 1 in 2000 in PBS/0.05% Tween 20/1%BSA for 1 h at 37 °C. Cells were then washed and incubated with PBS/1%BSA containing 4 μg/ml anti-human Alexa 488 antibody and 1 μg/ml Hoechst 33342 Stain for 30 min at 37 °C. The plates were washed twice with PBS and imaged on the Cellomics Arrayscan.

Anti-tumour activity studies

The detailed method is shown in Supplementary Methods. All mice were maintained in accordance with specific institutional guidelines. Detroit 562 human tumour xenografts were established in female athymic mice by subcutaneous implantation into the hind flank. Mice were randomised into groups of 15 when tumours reached a volume of $\sim 0.1\ cm^3$. 4T1 syngeneic tumours were established in Balb-C mice by orthotopic implantation into the mammary fat pad. Groups of 12–14 were dosed 24 h post cell implantation. 264RAD or vehicle was administered intraperitoneally as indicated for each experiment. Tumour volumes were assessed twice weekly. Statistical significance was evaluated using a one-tailed two-sample *t*-test. Tumours and lungs (4T1 studies only) were collected into buffered formalin on the day of study termination and processed to paraffin block for histological evaluation.

Immunohistochemistry PD analysis

Detailed methods for each analysis are provided in Supplementary Methods. Briefly, immunohistochemistry analysis was performed on

formalin-fixed, paraffin-embedded tissue sections at RT unless otherwise stated. Four micrometre sections were used and subjected to different antigen retrieval methods as appropriate for each antibody. Antibodies used were mouse anti-integrin beta6 (442.5C4) (Calbiochem, Nottingham, UK), rabbit anti-human fibronectin A0245 (Dako, Ely, UK), rabbit anti-human pERK (Cell Signalling Technology, Danvers, MA, USA), mouse anti-Ki-67 MIB-1 M7240 Dako and mouse anti-human α-SMA (α-SMA; Sigma; A2547).

Metastatic tumour analysis

To assess the number of lung metastases in the 4T1 study, following formalin fixation each lobe was sectioned, five serial sections per lobe, 200 μm apart. The sections were then stained with hematoxylin and eosin stain and the number of metastases counted using light microscopy.

ABBREVIATIONS

α-SMA, alpha smooth muscle actin; GST, glutathione-s-transferase; MVD, microvessel density; MVA, microvessel area; TGF-β LAP, transforming growth factor β latency-associated peptide.

CONFLICT OF INTEREST

CE, JK, KM, VNJ, NRS, HMW, DCB, VB, SJR CR STB are current employees of AstraZeneca. AA, JSK, JR and INF are current or former employees of Amgen/Abgenix. The remaining authors declare no conflict of interest.

ACKNOWLEDGEMENTS

Adrian Churchman was funded by a MRC Pilot Award.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)