Quantity and accessibility for specific targeting of receptors in tumours

Sajid Hussain1,2, Maria Rodriguez-Fernandez3, Gary B. Braun1,2, Francis J. Doyle, III3 & Erkki Ruoslahti1,2

1Cancer Research Center, Sanford-Burnham Medical Research Institute, La Jolla, California 92037, USA, 2Center for Nanomedicine, and Department of Cell, Molecular and Developmental Biology, University of California Santa Barbara, Santa Barbara, CA 93106-9610, USA, 3Department of Chemical Engineering, University of California Santa Barbara, Santa Barbara, CA 93106-5080, US.

Synaphic (ligand-directed) targeting of drugs is an important potential new approach to drug delivery, particularly in oncology. Considerable success with this approach has been achieved in the treatment of blood-borne cancers, but the advances with solid tumours have been modest. Here, we have studied the number and availability for ligand binding of the receptors for two targeting ligands. The results show that both paucity of total receptors and their poor availability are major bottlenecks in drug targeting. A tumour-penetrating peptide greatly increases the availability of receptors by promoting transport of the drug to the extravascular tumour tissue, but the number of available receptors still remains low, severely limiting the utility of the approach. Our results emphasize the importance of using drugs with high specific activity to avoid exceeding receptor capacity because any excess drug conjugate would lose the targeting advantage. The mathematical models we describe make it possible to focus on those aspects of the targeting mechanism that are most likely to have a substantial effect on the overall efficacy of the targeting.

Results

Biotinylation of accessible proteins in vivo. To assess the fraction of tumor proteins accessible to a blood-borne probe, we first determined the total amount of the two receptors we had chosen for this study: the first was the αv integrins, which are highly expressed in tumor vasculature, tumor cells and tumor fibroblasts4 and much used in synaphic drug targeting1,5. The second receptor was the HER2 tyrosine kinase receptor, the gene which is frequently amplified in breast cancer and drives tumor development and progression6,7. HER2 is also commonly used as a target for drug delivery. ELISA quantification provided a baseline value on the amount of αv integrins and HER2 in tumors (Tables 1 and 2).

To determine the fraction of αv integrins accessible to a blood-borne probe, we perfused tumor-bearing mice with the amine reactive ester derivative of biotin (sulfo-NHS-LC-biotin)8. We obtained high degree of labeling of tumor vessels in 4T1 and M21 tumors (Figures 1 and S1), higher than what was seen in Rybak et al. (2005). A possible explanation is that we used orthotopic tumors, which are thought to have a better perfused vasculature than the subcutaneous xenograft tumors used by Rybak et al.9. In addition, we examined tumor periphery, which is relatively well vascularized.
there are 109 cells in a gram of tumor\textsuperscript{10,11} this translates to an access-
of iRGD into mice bearing 4T1 tumors (\textit{7.5 tumor the fraction of biotin-accessible}

6

accessible quantity of 6.3
13 of a total 1850
biotinylation compound was found to be approximately 10-fold
tributed by the non-tumor cells in the tumors (Table S4). In BT474, a
as an underestimate since it does not include the mouse
(\textit{Table 1}), 31
3 being labeled in M21
(a
v integrin antibodies) or

Tissue biotin distribution was visualized in tumor sections using
Tissue vascular permeability, P, and the diffusion coefficient of
through the non-vascular tumor tissue (Figure 3). We used our

The accessible and total receptors were measured by ELISA as described under “Materials and Methods”. Data are presented as the mean ± SD (n = 3).
\textsuperscript{h} denotes human component; \textsuperscript{m} denotes mouse component.
* Assuming 10\textsuperscript{6} cells per gram of tumor tissue\textsuperscript{10}.

Table 1 | Total tumor cell integrin αvβ3 and those accessible to in vivo biotinylation in 4T1 and M21 tumors, with (+) and without (−) iRGD pre-injection

| Tumor  | Integrin αvβ3 pmol/g tumor\textsuperscript{a} | # receptors/cell \(\times10^6\) | % accessible receptors |
|--------|-------------------------------------------|----------------|-----------------------|
| 4T1    | Total                                     | 170 ± 15      | 102 ± 8                | -                      |
|        | Biotin-labeled (−iRGD)                     | 7.5 ± 0.7     | 4.4 ± 0.4              | 4.3                   |
|        | Biotin-labeled (+iRGD)                     | 33 ± 1.6      | 20 ± 1                | 20                    |
| M21    | Total                                     | 340 ± 20 (h + m) | 205 ± 3            | -                      |
|        | Biotin-labeled (−iRGD)                     | 31 ± 3.6 (h) + 4.7 (m) | 18.9 ± 2.1 (h) + 2.8 (m) | 9                   |
|        | Biotin-labeled (+iRGD)                     | 130 ± 5 (h)  | 78 ± 3                | 38                    |

Effect of iRGD on receptor biotinylation. The tumor-penetrating
peptide, iRGD (sequence: CRGDK/RGPDG), is capable of enhancing
the delivery of co-administered drugs to tumors\textsuperscript{5}. We next tested
whether iRGD would increase accessibility of αvβ3 and HER2 to the
circulating biotinylation probe. The iRGD effect is transient; it peaks
at 30 min and is essentially over at 1 h, presumably because the
half-life of the peptide in the circulation is short, about 10 min. We
limited our analysis to one administration of the peptide to emphasize
that we are measuring accessibility and that it can be modulated.
Repeated administration of iRGD has been used previously and does enhance long-term antibody accumulation in
tumors\textsuperscript{5}. iRGD was intravenously injected into mice bearing 4T1 or
BT474 tumors, followed 30 min later by perfusion with the biotiny-
lation reagent and comparison to control-injected tumor mice. Tissue
biotin distribution was visualized in tumor sections using streptavidin-Alexa Fluor 488 (Figure S1a). The results revealed 4 to
6-fold increased biotinylation of tumor αvβ3 and HER2 when iRGD
was pre-injected (Tables 1 and 2).

Effect of iRGD on antibody accumulation in tumors. Pre-injection
of iRGD into mice bearing 4T1 tumors (αv integrin antibodies) or
BT474 tumors (anti-HER2 trastuzumab) 30 min prior to the antibody
greatly increased antibody accumulation in the tumors as detected by
fluorescent microscopy (Figure 2a). Quantification of the antibodies
in tumor homogenates by ELISA showed that iRGD increased the
accumulation of both antibodies by 4 to 5-fold (from 16.6 to 77.4 pmol/g in 4T1 and from 27.1 to 128 pmol/g in BT474)
(\textit{Table 3}). Converted to percentage of accessible receptors, it increased from 10% to 43% in the 4T1 model and from 1.5% to
6.8% in the BT474 model.

Effect of iRGD on antibody accumulation in tumors. Pre-injection
of iRGD into mice bearing 4T1 tumors (αv integrin antibodies) or
BT474 tumors (anti-HER2 trastuzumab) 30 min prior to the antibody
greatly increased antibody accumulation in the tumors as detected by
fluorescent microscopy (Figure 2a). Quantification of the antibodies
in tumor homogenates by ELISA showed that iRGD increased the
accumulation of both antibodies by 4 to 5-fold (from 16.6 to 77.4 pmol/g in 4T1 and from 27.1 to 128 pmol/g in BT474)
(\textit{Table 3}). Converted to percentage of accessible receptors, it increased from 10% to 43% in the 4T1 model and from 1.5% to
6.8% in the BT474 model.

Effect of iRGD on antibody accumulation in tumors. Pre-injection
of iRGD into mice bearing 4T1 tumors (αv integrin antibodies) or
BT474 tumors (anti-HER2 trastuzumab) 30 min prior to the antibody
greatly increased antibody accumulation in the tumors as detected by
fluorescent microscopy (Figure 2a). Quantification of the antibodies
in tumor homogenates by ELISA showed that iRGD increased the
accumulation of both antibodies by 4 to 5-fold (from 16.6 to 77.4 pmol/g in 4T1 and from 27.1 to 128 pmol/g in BT474)
(\textit{Table 3}). Converted to percentage of accessible receptors, it increased from 10% to 43% in the 4T1 model and from 1.5% to
6.8% in the BT474 model.

Effect of iRGD on antibody accumulation in tumors. Pre-injection
of iRGD into mice bearing 4T1 tumors (αv integrin antibodies) or
BT474 tumors (anti-HER2 trastuzumab) 30 min prior to the antibody
greatly increased antibody accumulation in the tumors as detected by
fluorescent microscopy (Figure 2a). Quantification of the antibodies
in tumor homogenates by ELISA showed that iRGD increased the
accumulation of both antibodies by 4 to 5-fold (from 16.6 to 77.4 pmol/g in 4T1 and from 27.1 to 128 pmol/g in BT474)
(\textit{Table 3}). Converted to percentage of accessible receptors, it increased from 10% to 43% in the 4T1 model and from 1.5% to
6.8% in the BT474 model.

Table 2 | Total and in vivo biotin accessible HER2 receptor in BT474 tumors, with (+) and without (−) iRGD pre-injection

| HER2  | pmol/g tumor | # receptors/cell \(\times10^6\) | % accessible receptors |
|-------|-------------|----------------|-----------------------|
| Total | 1850 ± 150  | 111.4 ± 9.1    | -                      |
| Biotin-labeled (−iRGD) | 105 ± 13   | 6.3 ± 0.8     | 5.7                   |
| Biotin-labeled (+iRGD) | 613 ± 85   | 36.8 ± 4.8    | 33.1                  |

The accessible and total receptors were measured by ELISA as described under “Materials and Methods”. Data are presented as the mean ± SD (n = 3).
permeability, and estimated the change in P for the iRGD experiments. We also estimated the radius of the Krogh cylinder (R_{Krogh}), the amount of tissue supplied by a capillary, since the degree of vascularization can vary between tumor types (Figure 3). In the fitting of P and R_{Krogh} we included data corresponding to the %ID of antibody per gram tumor from both conditions with iRGD and without iRGD and assumed that the geometry, tissue density, and total blood volume remain constant for each tumor type and condition (Tables S1, S2). The optimized model (Supporting Information, Table S2) shows good agreement with the complete set of experimental data (Table 4), with iRGD causing a 4.5-fold increase in P (Table S3).

To validate this model we used an independent set of data, i.e., the availability of receptors to the biotinylation probe. The sulfo-NHS-LC-biotin used in this study is considered to be membrane impermeable, passing through pores in or between cells to reach tissue targets beyond the endothelial layer. This reagent is frequently used for the identification and quantification of proteins on cell membranes, although proteins annotated to subcellular localizations may also be labeled. In our study, the data from M21 tumors show that 14% of the accessible receptors without using iRGD belong to host mouse endothelial cells while the rest are from tumor cells (Table S4). This indicates that the biotinylation reagent is indeed able to cross the endothelial layer. The large excess of biotinylation compound (~2 mM) in the perfusion solution over vascular receptors promotes the diffusion and reaction in the perivascular tissue, and mimics certain aspects of a high concentration of targeting probe binding to a receptor. Using the rest of the parameters from the antibody simulations we estimated P for the sulfo-NHS-LC-biotin from the biotinylation experimental data. The percentage of labeled antigen predicted by the fitted model is in good agreement with the experimental data for the three tumor types (Table 4). The fitted P and R_{Krogh} values are summarized in Table S3. P for biotinylation (P = 7.9e-9 m/s) is much greater than for the antibody transport, indicating faster movement of lower molecular weight biotin. However, it is slower than reported for other small molecules, a deviation attributed to the negative charge and limited membrane permeability characteristics of sulfo-NHS-LC-biotin, and consumption of the probe by amine-richly present in the interstitial space.

**Simulation results.** Using the model we plotted the antibody accumulation profile in a tissue cross-section (Figure 4), illustrating how the antibody penetrates deeper into the tumor when iRGD is present. In Figure 4B,C, we compare the effect of the dose of antibody on the

---

**Figure 1 | Schematic representation of the analysis of accessible receptors in tumor bearing mice by using the in vivo biotinylation method.**

**Figure 2 | Effect of iRGD on antibody accumulation in tumor tissue.** (A) Fluorescence microscopy images of biotin-labeled rat anti-mouse zv with and without prior injection of the tumor penetrating peptide, iRGD. Images representative of at least five sections from each tumor (n = 3 mice per group) are shown. Scale bars = 100 μm. (B) Percentage of injected dose of the antibody per gram tumor (%ID/g) measured by sandwich ELISA. The inset shows the ELISA standard curve.
%ID/g, plotting simulation results for 4T1 and BT474 tumors. In the case of 4T1 tumors, the %ID/g decreases slightly for doses above 100 μg when iRGD is present, meaning that saturation of the target is occurring (Ab > Ag, Figure 4B). When iRGD is not included, the initial stage of saturation occurs well above 1000 μg indicative of limited accessibility. For BT474 tumors the saturation with iRGD administration occurs at higher dosage than for 4T1 since BT474 has a greater amount of receptors per tumor volume (Figure 4C). In this tumor model, iRGD increases the %ID/g by 4 to 5 fold across the entire therapeutically relevant dosage window.

### Discussion

Information on the quantity and accessibility of targeting receptors in intact tissues is an increasingly important factor to consider in developing targeted therapies. Here we develop methods for both quantifying accessible receptor in tissues and for computational modeling of the kinetics of drug permeation. The αv integrin and HER2 amount in tumors was as expected; the 40,000 receptors per cell number we measured for αv integrins is typical of cell surface proteins, including integrins15,16, and the amplification of the HER2 gene in the BT474 cells agreed with the 10 fold higher expression of this protein17. Nonetheless, the amount of total receptor available for tumor targeting of drugs is quite limited, only 200–2,000 picomoles per gram of tumor.

An even more sobering number is the relatively small fraction of the receptors that our results show to be available for blood-borne probes, around 5%. Taken together with the total number of receptors in a tumor, it means that only a few picomoles of a drug conjugate can be specifically targeted to a tumor using tumor-specific targeting probes. This figure is likely modified over time by factors such as diffusion of the drug conjugate into the tumor, receptor turnover, modification of entry into cells and other factors, some of which have been modeled in our simulation studies. These factors are likely to have a greater effect when the probe has a long half-life in circulation, such as an antibody.

We found greater availability for the αvβ3 integrin than HER2, despite the 10-fold higher expression of HER2. The likely reason is that the integrin is also expressed in tumor vessels, where all cell surface receptor should be available, whereas HER2 binding requires access to tumor cells. This comparison emphasizes the utility of vascular tumor markers in drug targeting. The differences in the accessibility of the same receptor in different tumors, which we also observed, is likely to be caused by differences in the density of the tumors, which is known to affect drug penetration18,19. In addition, the amount of intracellular receptor, which constitutes a substantial fraction of the total receptor20,21, may vary from tumor to tumor.

Higher receptor saturation densities than ours have been observed, particularly with antibodies22,23. However, these authors used the test antibodies in large excess relative to the receptor, which is not advisable in synaptic targeting because exceeding the capacity of the receptors will defeat the purpose of the targeting. We also used the biotinylation probe in excess to detect available receptor. Thus, it should be noted that the biotinylation analysis probably overestimates the number of receptors readily available for synaptic targeting, particularly in the relatively short time frame we used.

A recently identified tumor-penetrating peptide, iRGD, promotes the entry of coupled and co-injected compounds into tumor tissue through a transport pathway specifically activated in tumors24–26. Our modeling results identify vascular permeability as the modeling parameter most relevant to iRGD-enhanced treatment. Permeability is used here as an operational term that describes the transport across the endothelial layer separating the blood volume from the tissue. It does not include details involving the biological mode of the transport, e.g. endocytosis, transcytosis, or convection flow.

One finding of this study is that the increase in tumor accumulation for the targeted drugs by iRGD was effective for both biotin and antibody penetration. Earlier studies have shown that tumor accumulation of compounds ranging from simple drugs to nanoparticles is enhanced by iRGD co-administration2. Thus, the iRGD effect is general, and minimally dependent on the size and hydrophilicity of the drug. Here, we examined the effects of a single injection of iRGD in the one-hour window that has been observed for iRGD2. The pharmacokinetic model we have developed can be used to model drug transport, and this model suggests that iRGD enhances the effective vascular permeability, which increases receptor accessibility and drug accumulation in the tumor. This effect is likely to be particularly pronounced for compounds with a short half-life in the circulation because they have a limited time window to penetrate into extravascular tumor tissue.

Even with the aid of approaches such as using iRGD, these numbers still mean that only picomoles of a drug can be specifically delivered per gram of tumor using targeting probes. Few drugs have specific activities compatible with these numbers, which emphasizes

| Tumor   | Antibody Dose (μg) | Predicted (%ID/g) | Experiment (%ID/g) | Predicted (pmol/g) | Experimental (pmol/g) | Experimental (% receptors) |
|---------|--------------------|-------------------|--------------------|-------------------|----------------------|---------------------------|
| 4T1     | 100 (−iRGD)       | 2.64              | 2.50               | 17.6              | 16.6                 | 10.3                      |
|         | 100 (+iRGD)       | 11.2              | 11.6               | 74.4              | 77.4                 | 43.3                      |
|         | 60 (−iRGD)        | 2.64              | 3.07               | 10.6              | 12.3                 | 6.22                      |
|         | 60 (+iRGD)        | 11.2              | 10.4               | 44.8              | 41.8                 | 26.4                      |
| BT474   | 100 (−iRGD)       | 4.15              | 3.94               | 28.6              | 27.1                 | 1.53                      |
|         | 100 (+iRGD)       | 18.4              | 18.4               | 126               | 128                  | 6.75                      |

The amount of antibody in the tumor tissue after an injection of anti-mouse αv (4T1 tumor mice) or Trastuzumab (BT474 tumor mice) at different concentrations was determined experimentally after 1 h circulation as described in the “Materials & Methods” section. The effect of iRGD on antibody uptake was evaluated. Data represent the mean %ID/g tissue (n = 3–5 mice per group).

**Figure 3** | The geometry of the vascular-tissue model, the Krogh cylinder. A hollow inner cylinder with radius R_cap represents a capillary that carries the blood plasma and probe molecules while a semi-permeable endothelial barrier restricts passage between the plasma and the surrounding tissue. Each section of capillary vessel is responsible for supplying blood and the small molecule biotinylation probe, or antibody, to the surrounding cylindrical section of tissue with radius R_Krogh.
the importance of selecting drugs that have the highest possible specific activities for synaptic targeting. These quantitative aspects of targeting have generally been underappreciated, and exceeding the receptor capacity is likely to be the most common reason for failures in attempt to deliver drugs using the targeting approach. Using multiple receptors at the same time would be one solution, but it would not change the fact that most of the available receptors will not be available for the drug conjugate to bind to. Our experimental data provide a quantitative basis for assessing the magnitude of the problem and the mathematical model we have developed allows the assessment of the parameters that are most likely to help deal with it.

**Methods**

**Antibodies and reagents.** The anti-integrin αvβ3 monoclonal antibody LM609 was purchased from Millipore (Hayward, CA). The goat anti-human integrin αv polyclonal antibody was obtained from R&D systems (Minneapolis, MN), and the biotin-labeled, rat anti-mouse integrin αv monoclonal antibody was from eBioscience (San Diego, CA). The anti-biotin BN34 mouse monoclonal antibody was from Sigma Life Sciences (St. Louis, MO), and trastuzumab (Herceptin) was kindly provided by Dr. Daniel Greenwald of the Cancer Center of Santa Barbara. Horseradish peroxidase (HRP) conjugated goat anti-rat and donkey anti-goat secondary antibodies were from Millipore and R&D systems, respectively. Recombinant human integrin αvβ3 and HER2-Fc chimeras were purchased from R&D systems. Sulfo-NHS-LC-biotin and biotin quantitation kit were from Pierce (Rockford, IL). The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and all other reagents of analytical and molecular biology grade were obtained from Sigma-Aldrich. iRGD peptide with sequence Ac-{CRGDKGPDC}-NH2 was synthesized in house as described2.

**In vivo tumor models.** The M21 human melanoma cancer cell line was cultured in Dulbecco’s Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FCS) and penicillin/streptomycin. The BT474 human breast cancer cell line was cultured in DMEM/F12 (1:1) medium with 10% FCS and penicillin/streptomycin. The mouse 4T1 breast cancer cells were cultured in Iscove’s Modified Dulbecco Medium (IMDM; Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. The mouse 4T1 breast cancer cells were cultured in Iscove’s Modified Dulbecco Medium (IMDM; Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. The mouse 4T1 breast cancer cells were cultured in Iscove’s Modified Dulbecco Medium (IMDM; Invitrogen) supplemented with 10% FCS and penicillin/streptomycin. For the 4T1 xenografts, 1 × 10⁶ cells were injected orthotopically into BALB/c mice (Charles River, Wilmington, MA). The M21 model was generated by injecting 1 × 10⁶ cells subcutaneously (s.c.) into the flanks of female nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN). For the BT474 xenografts, 17β-estradiol pellets (Innovative Research of America, Sarasota, FL) were implanted s.c. into the back of the nude mice one day prior to the orthotopic inoculation of 5 × 10⁶ cells in matrigel (BD Biosciences, San Jose, CA). We avoided using tumours that had grown big enough to have necrosis. All animal experimentation was performed according to procedures approved by the Animal Research Committee at the University of California, Santa Barbara.

**In vivo biotinylation.** To specifically quantify accessible αvβ3 integrin and HER2 receptors we labeled proteins accessible from the blood circulation with perfusion of amine-reactive biotin8,24 and quantified labeled target and total target protein by ELISA. Anesthetized tumor mice were terminally perfused, first with a solution of 10% dextran-40 (w/v) in PBS at 37°C to remove blood and then with 1 mg/mL sulfo-NHS-LC-biotin in PBS containing 10% Dextran-40 (w/v), followed by neutralization of the unreacted biotinylation reagent with 50 mM Tris(hydroxymethyl)aminomethane hydrochloride in PBS (Tris, pH 7.4). Tissues and tumors were excised, and either snap-frozen to prepare homogenates or embedded in Tissue-Tek O.C.T (Fisher Scientific, USA) for cryopreservation and sectioning.

| Tumor | iRGD | Predicted % labeled Ag | Experimental % labeled Ag |
|-------|------|------------------------|--------------------------|
| 4T1   | -    | 4.2                    | 4.3                      |
|       | +    | 18.7                   | 20                       |
| M21   | -    | 9.0                    | 9                        |
|       | +    | 39.8                   | 38                       |
| BT474 | -    | 7.7                    | 5.7                      |
|       | +    | 33.8                   | 33.1                     |

**Figure 4 |** (A) Tumor total antibody concentration profile after 1 h circulation for BT474 illustrating how the antibody penetrates deeper into the tumor when iRGD is present. (B) Plot of simulation %ID/g vs Antibody (Ab) dose after 1 h circulation with and without iRGD for the 4T1 tumor model and (C) for BT474 tumor model.
Preparation of protein extracts and receptor quantification. Tumours from in vivo biotinylated mice were homogenized in lysis buffer (2% NP-40, 50 mM Tris, 10 mM EDTA, protease inhibitor cocktail) and lysates were examined and photographed under a Fluoview 500 confocal laser-scanning microscope (Olympus).

Biotinylation of recombinant αvβ3 proteins. For quantifying accessible receptors for antibody injection experiments, recombinant αvβ3 monoblonal antibody LM609 and rat anti-mouse αv polyclonal antibodies, goat anti-integrin αvβ3, were used. The biotinylation of HER2 receptor in the BT474 model was similarly quantified by direct sandwich ELISA using anti-human HER2 (Herceptin) as the capture antibody. Total number of αvβ3 or HER2 receptors in tumour extracts was quantified by competition ELISA (refer to Figure S1C). ELISA plates were coated with the respective capture antibodies, goat anti-integrin αvβ3 polyclonal antibody (4T1), anti-human integrin αvβ3 monoclonal antibody LM609 (M21), or Herceptin (BT474). Serially diluted standards (αvβ3 or HER2), or tumour extracts, were pre-mixed with biotinylated recombinant αvβ3 or HER2 in 1% BSA/PBST, and added to their respective wells. After incubation for 1 h at 37 °C, wells were washed and streptavidin–HRP was added. Following incubation and washing, the plates were developed with 2.2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and absorbance measured at 405 nm. To further quantify human and mouse αvβ3 receptors, ELISA plates were coated with 50 μg of goat anti-human αv polyclonal antibody at a concentration of 5 μg/mL for 18 h at 4 °C. The wells were washed three times with PBS/0.05% Tween-20, and blocked with 1% biotin-free BSA. Diluted tumor lysates or biotinylated standards of αvβ3 and HER2 (described in the following section) were added to the wells and incubated for 2 h at room temperature with shaking. After washing, 100 μL of streptavidin–HRP (diluted 1 : 1000) in 1% BSA/PBST (PBS with 0.05% Tween 20) was added to the wells and incubated with shaking for 30 min at RT. The plates were then developed with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and absorbance measured at 405 nm. To separately quantify human and mouse αvβ3 integrins in 2M1 xenograft tumor lysates, sandwich ELISA was performed with species-specific anti-human αvβ3 or HER2 (described in the following section) and anti-human αvβ3 (Herceptin) as capture antibody. To determine the number of biotinylated αvβ3 receptors, ELISA plates were coated with 50 μg of goat anti-human αv polyclonal antibody at a concentration of 5 μg/mL for 18 h at 4 °C. The wells were washed three times with PBS/0.05% Tween-20, and blocked with 1% biotin-free BSA. Diluted tumor lysates or biotinylated standards of αvβ3 and HER2 (described in the following section) were added to the wells and incubated for 2 h at room temperature with shaking. After washing, 100 μL of streptavidin–HRP (diluted 1 : 1000) in 1% BSA/PBST (PBS with 0.05% Tween 20) was added to the wells and incubated with shaking for 30 min at RT. The plates were then developed with 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and absorbance measured at 405 nm.

Injection of biotinylated antibodies. To study the effect of the tumor-penetrating peptide iRGD on receptor availability towards antibodies, 100 μL of 200 μM iRGD or PBS were intravenously injected into mice bearing 4T1 or BT474 tumors. Thirty minutes later, biotinylated rat anti-mouse αv (for 4T1 tumor mice) or trastuzumab (for BT474 tumor mice) was injected at different concentrations. After 1 h incubation, the mice were perfused with PBS, tumors excised and either snap-frozen or embedded in O.C.T for cryopreservation and sectioning. For quantifying accessible receptors for antibody injection experiments, recombinant αvβ3 integrin and HER2-Fc chimeric domain (aD) were biotinylated using the micro-sulf-NHS-biotinylat ion kit (Pierce) according to the manufacturer’s protocol, and the biotinylated protein was purified with a desalting column. The degree of biotinylation was determined using the 2-(4-Hydroxyphenylazo)benzoic acid (HABA) assay (Pierce). The proteins biotinylated by this procedure carried on average 2.4 ± 3.1 biotin molecules per molecule of protein.

In silico modeling. A computational model based on partial differential equations (PDEs) was developed to describe the diffusion of probes through the blood vessels and the non-vascular tissue tumor. The model builds on the work of Thurber et al., which is relevant for antibody uptake and retention in vascularized tumors. The foundation of the model includes the major processes affecting the time course of antibody concentration delivered to a tumor (see Supporting Information). The complete set of processes includes local and systemic clearance, extravasation, diffusion into tumor cells, binding, and degradation and accumulation in the tumor cell. The model was validated by comparing the simulation results with the experimental data. The model was used to predict the optimal therapeutic strategy for the treatment of tumors.
(including patents) in the same. E.R. is also a consultant/advisory board member, and major shareholder of EnduRx Pharmaceuticals Inc., and has ownership interest (including patents) in the same. No potential conflicts of interest were disclosed by the other authors.

How to cite this article: Hussain, S., Rodriguez-Fernandez, M., Braun, G.B., Doyle, F.J. & Ruoslahti, E. Quantity and accessibility for specific targeting of receptors in tumours. Sci. Rep. 4, 5232; DOI:10.1038/srep05232 (2014).