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In vivo targeting of tumor-associated carbonic anhydrases using acetazolamide derivatives

Julia K. J. Ahlskog [a,†], Christoph E. Dumelin [b,†], Sabrina Trüssel [a], Jessica Mårlind [a], Dario Neri [a,*]

[a] Department of Chemistry and Applied Biosciences, Institute of Pharmaceutical Sciences, ETH Zurich, Wolfgang-Pauli-Str.10, CH-8093 Zurich, Switzerland
[b] Philochem AG, c/o ETH Zurich, Wolfgang-Pauli-Str.10, CH-8093 Zurich, Switzerland

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ABSTRACT

We describe the synthesis and characterization of two acetazolamide derivatives containing either a charged fluorophore or an albumin-binding moiety, which restrict binding to carbonic anhydrase IX and XII present on tumor cells. In vivo studies showed the preferentially targeting of tumor cells by the fluorescent acetazolamide derivative and the ability of the albumin-binding acetazolamide derivative to cause tumor retardation in a SK-RC-52 xenograft model of cancer.

Virtually all tumors require new blood vessels, which provide nutrients and oxygen to the cancer cells, thus sustaining growth and offering an avenue for metastatic spread.[1] However, not all cells within the tumor mass receive a sufficient oxygen supply and cancer cells at a distance of 100–200 μm from the nearest blood vessel become hypoxic.[2] This pattern is particularly pronounced not only in experimental rodent models of cancer, but also in aggressive human solid tumors such as glioblastoma multiforme, where hypoxic cells form a 'palisade', delineating a border between cancer cells in proliferation around pseudoglomerular blood vessels and necrotic areas.[3]

Carbonic anhydrase IX (CA IX), a membrane-anchored enzyme, is one of the most prominent proteins, which become over-expressed in hypoxic conditions.[4,5] In addition oxygen availability also regulates the catalytical activity of CA IX to reversible converse CO_2 to carbonic acid, thereby leading to an acidification of the extracellular tumor milieu under hypoxic conditions.[6,7] Additionally, it has been proposed that transport of bicarbonate into the cytoplasm of tumor cells through a Cl^-/HCO_3^- exchanger leads to the increase of the intracellular pH of cancer cells,[6] resulting in the generation of alternatively spliced components of the extracellular matrix,[8,9] which are now frequently used as targets for antibody-mediated pharmacodelivery strategies.[10] Furthermore, by regulating the intracellular pH, CA IX and CA XII confer a growth and survival advantage to tumor cells exposed to the hypoxic and acidic microenvironment.[11]

The overexpression of CA IX in breast cancer has been found to be associated with reduced survival.[12] Furthermore, renal cell carcinoma (RCC) frequently carry a loss-of-function mutation in the *VHL* gene, the product of which is responsible for targeting the oxygen sensor HIF-1α for proteasomal degradation, thus leading to increased CA IX levels on all tumor cells, due to HIF-1α stabilization.[13] Indeed, CA IX has been used in the past as antigen for the generation of tumor targeting monoclonal antibodies[14–18] and the chimeric antibody cG250 is currently being studied by Wilex in Phase III clinical trials for the therapy of RCC.

Cells may express a number of different carbonic anhydrases. Carbonic anhydrase I, II, III, VA, VB, VII and XIII are expressed intracellularly, while carbonic anhydrase IV, IX, XII, XIV and XV are membrane-bound.[19] In tumors, not only CA IX but also CA XII is strongly over-expressed. Indeed, recent RNAi experiments indicate that only a simultaneous blockade of CA IX and CA XII leads to tumor growth retardation, as a result of impaired acidification of the tumor environment.[11] Furthermore expression as well as activation of CA IX is required for the successful inhibition of the catalytic activity which only occurs under hypoxic conditions.[20]

Based on these considerations, it is not surprising that the development of carbonic anhydrase inhibitors has been a matter of intense medicinal chemical research, in an attempt to design selective inhibitors for the different CA isoforms.[19] As CA IX and CAXII are the most relevant carbonic anhydrases for the development of anti-cancer strategies, the use of bioreductive prodrugs[21]

* Corresponding author. Tel.: +41 44 6337401; fax: +41 44 6331358.
E-mail address: neri@pharma.ethz.ch (D. Neri).
† These authors contributed equally to the article.

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or of membrane-impermeable glycoconjugates[22] and CA inhibitors[23] has been proposed. Checchi et al. demonstrated in vitro that the conjugation of sulfonamides to charged moieties displayed a decreased membrane permeability and selective inhibition of CA IX, thereby leading to a reversion of the acidification under hypoxic conditions.[23] To our knowledge, none of these therapeutic approaches has been tested in vivo so far.

Dennis et al. have recently described a general strategy for the reduction of blood clearance of therapeutic proteins (such as tumor-specific antibody fragments) by means of a C-terminal albumin-binding peptide.[24] Interestingly, the longer circulatory half-life of anti-HER2-neu antibodies in stable non-covalent association with mouse serum albumin (MSA) led to a homogenous decoration of tumor cells in vivo, which could not be obtained with the same antibody (Trastuzumab) in Fab or IgG format, that were confined to perivascular tumor cells.[25] In principle, the same strategy could be used for the modification of small organic enzyme inhibitors, with the additional advantage of preventing internalization, thus restricting their scope to membrane-bound and secreted enzyme. Recently, our lab has described derivatives of 2-amino-6-(4-(4-iodophenyl)butanamido)hexanoic acid as a general class of stable and portable albumin-binding moieties, which can be conjugated to a variety of different drugs and which prolong serum half-lives in vivo.[26]

In this Letter, we describe the synthesis and characterization (in vitro and in vivo) of a novel bispecific acetazolamide derivative, capable of simultaneous binding to carbonic anhydrases and serum albumins ('Albu-acetazolamide'). We used acetazolamide as a building block, since this compound is able to inhibit a broad spectrum of carbonic anhydrases in the low nanomolar range, including CA IX and CA XII.[19] Furthermore, we investigated the conjugation of acetazolamide to a charged fluorophore ('FAM-acetazolamide') as an alternative avenue to prevent internalization. FAM-acetazolamide binding to tumor cells was confirmed by fluorescence-activated cell sorting, while preferential in vivo tumor targeting was assessed by fluorescence microscopy analysis of tissue sections, 1 h and 2 h after intravenous (i.v.) administration. The use of acetazolamide as targeting moiety for pharmacodelivery applications is attractive since most tumors express CA IX and CA XII either at sites of hypoxia or on pVHL-defective tumor cells. Small organic ligands may be advantageous for tumor targeting applications, compared to monoclonal antibodies, in view of their rapid extravasation and homogenous tissue distribution properties.[27]

A charged fluorophore moiety or the stable non-covalent binding to serum albumin prevents the internalization of the derivatives, thus restricting binding of the acetazolamide moiety to the membrane-bound carbonic anhydrase isoforms CA IX and CA XII. Figure 1A presents two-color fluorescence microscopy images of tumor sections, showing CA IX staining in green and blood vessels in red. Two extreme situations are presented. On one hand, virtually all cancer cells express CA IX in SK-RC-52 renal tumor cells due to a defective pVHL tumor suppressor.[28] By contrast, CA IX is only detectable at sites of hypoxia (i.e., at >100 μm from tumor blood vessels) in the LS174T human colorectal adenocarcinoma xenograft model.

The structures of the two acetazolamide derivatives investigated in this study are depicted in Figure 1B and C. FAM-acetazolamide [2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)-5-(5-sulfamoyl-1,3,4-thiadiazol-2-ylcarbamoyl)benzoic acid] is an amide derivative of the green fluorophore 5-carboxyfluorescein (FAM), and is negatively charged at neutral pH. The carboxylic acid of 5-carboxyfluorescein (266 μmol) was activated with *N*-hydroxysuccinimide (NHS, 292 μmol) and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl, 292 μmol) in 3 ml of dry *N*,*N*-dimethylformamide (DMF) and stirred for 4 h at 25 °C. The reaction mixture was then added to 5-amino-1,3,4-thiadiazole-2-sulfonamide (Ramidus AB, 319 μmol) and triethylamine (3.2 mmol) dissolved in 3 ml of dry DMF and stirred protected from light at 25 °C for 16 h followed by addition of Tris/Cl, pH 8 (1.05 mmol). After stirring for 30 min at 25 °C protected from light, HPLC purification was performed running a linear gradient from 40–60% acetonitrile. The eluting FAM-acetazolamide was detected at $\lambda = 470$ nm, the desired fractions were collected, dried under vacuum and the concentration was determined by UV/VIS spectrome-

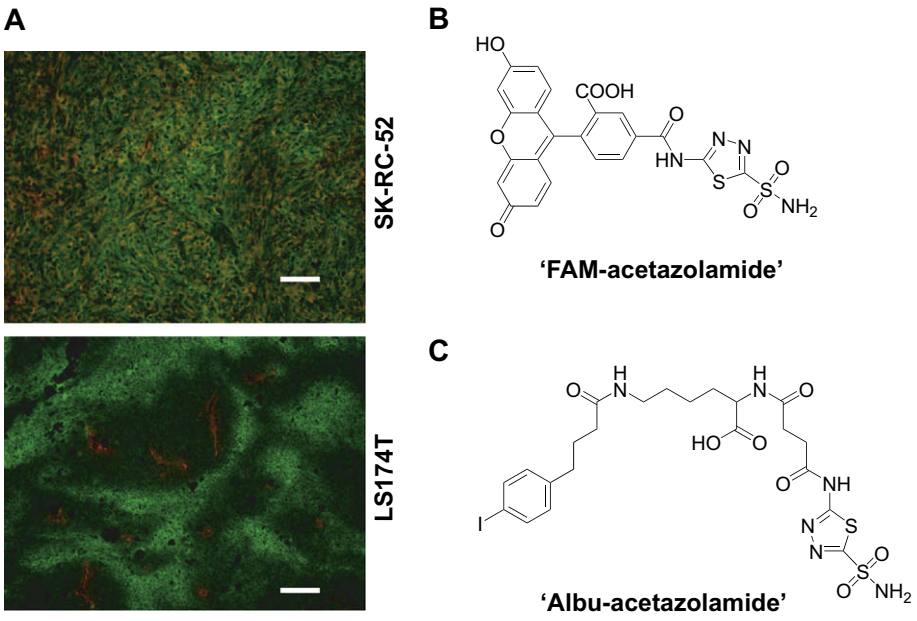

Figure 1. (A) Immunofluorescence analysis performed on human RCC SK-RC-52 (upper panel) and human colorectal adenocarcinoma LS174T (lower panel) xenografted tumor tissue sections. Overlay of red (endothelial cells, i.e., anti-CD31 staining) and green (anti-CA IX staining) fluorescence. In the pVHL-defective SK-RC-52 model CA IX is expressed constitutively, whereas in the LS174 T model the expression occurs in hypoxic regions in distance to blood vessels. Scale bar = 100 μm. (B) Structure of FAM-acetazolamide, used in this study for biodistribution analysis. (C) Structure of Albu-acetazolamide. This bispecific acetazolamide derivative is able to simultaneously bind to carbonic anhydrases and serum albumin, thereby preventing its internalization and restricting it to the extracellular space. In this study, Albu-acetazolamide was used for therapy experiments in tumor-bearing mice.

try at 495 nm (ε = 72,000 M^{-1} cm^{-1}). ESI-MS *m/z* 538.71 ([M+H$^+$], 100%), calcd: 538.03 Da. Albu-acetazolamide [6-(4-(4-iodophenyl)butanamido)-2-(4-oxo-4-(5-sulfamoyl-1,3,4-thiadiazol-2-ylamino)butanamido)hexanoic acid] is a bispecific compound containing the acetazolamide moiety linked to the 2-amino-6-(4-(4-iodophenyl)butanamido)hexanoic acid moiety, which confers stable albumin binding and a slow blood clearance profile to the molecule.[26] 5-amino-1,3,4-thiadiazole-2-sulfonamide (Ramidus AB, 611 μmol) was stirred with succinic anhydride (611 μmol) in 1 ml of dry *N,N*-dimethylformamide (DMF) for 24 h at 50 °C followed by HPLC purification running a linear gradient from 0% to 100% acetonitrile. After collection of the desired fractions, solvents and buffer were removed under vacuum. The obtained intermediate 4-oxo-4-(5-sulfamoyl-1,3,4-thiadiazol-2-ylamino)butanoic acid (366 μmol), *N*-ethyldiisopropylamine (DIPEA, 366 μmol) and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU, Novabiochem, 366 μmol) were stirred for 10 min at 25 °C in 3 ml of dry DMF followed by addition of 4-(-*p*-iodophenyl)butyl lysine (366 μmol) and triethylamine (732 μmol) in dry DMF. The reaction was stirred for 24 h at 30 °C and purified by HPLC running a linear gradient from 40% to 60% acetonitrile. The eluting product Albu-acetazolamide was collected and solvents and buffer were removed under vacuum. ESI-MS *m/z* 680.86 ([M+H$^+$], 100%), calcd: 680.06 Da. ^{1}H NMR (DMSO-d_6): 7.61 (d, 8.3 Hz, 2H), 7.00 (d, 8.3 Hz, 2H), 4.14 (m, 1H (αH)), 3.01 (m, 2H) 2.74 (t, 6.6 Hz, 2H), 2.52 (m, 4H), 2.05 (m, 2H), 1.81–1.50 (m, 4H), 1.43–1.21 (m, 4H) (Bruker 400 avance instrument).

The ability of FAM-acetazolamide to bind to tumor cells was assessed by fluorescence-activated cell sorting, revealing a >10-fold average fluorescence increase upon incubation of LS174T cells with 3 μM FAM-acetazolamide solution (Fig. 2). In order to evaluate whether this compound was also able to reach tumor cells in vivo, 680 μg (1.26 μmol) FAM-acetazolamide were injected in the tail vein of Balb/c nu/nu mice bearing LS174T tumors of a size of ~500 mg grafted subcutaneously. Figure 3 depicts fluorescence microscopy images of 10 μm-thick tissue sections, obtained from mice sacrificed 1 h and 2 h after i.v. injection. A strong and heterogeneous tumor uptake was observed at both time points, in agreement with the immunohistochemical staining patterns presented in Figure 1A. At 1 h negligible background fluorescence was detected for heart, lung, spleen and muscle, while organs involved in the clearance of the compound (kidney, liver and intestine) were homogenously bright. As expected, fluorescence signals in all organs but tumors substantially decreased at 2 h.

The ability of Albu-acetazolamide to display high-affinity binding to both CA IX and human serum albumin (HSA) was first confirmed by isothermal titration calorimetry (Supplementary Fig. 1). Albu-acetazolamide exhibited dissociation constants K_d = 3.2 nM towards the recombinant catalytic domain of human CA IX and K_d = 820 nM towards HSA. These values are comparable to the K_d constants of the individual binding moieties,[19,26] thus confirming that the construction of the bispecific molecule did not adversely affect binding.

To show that Albu-acetazolamide was able to inhibit the enzymatic activity of CA IX alone and in the presence of serum albumin, we used a colorimetric assay based on the conversion of 4-nitrophenylacetate.[29] The albumin-binding moiety displays comparable affinities towards both human and murine serum albumin[26] and we used MSA in this assay, in view of the subsequent in vivo cancer therapy studies in tumor-bearing mice. As expected, Albu-acetazolamide displayed a comparable inhibitory activity when used alone or in the presence of 10 μM MSA with K_i values of 18 nM and 8.8 nM, respectively. The K_i values of FAM-acetazolamide and acetazolamide were determined to be 220 and 230 nM, respectively (Supplementary Fig. 2).

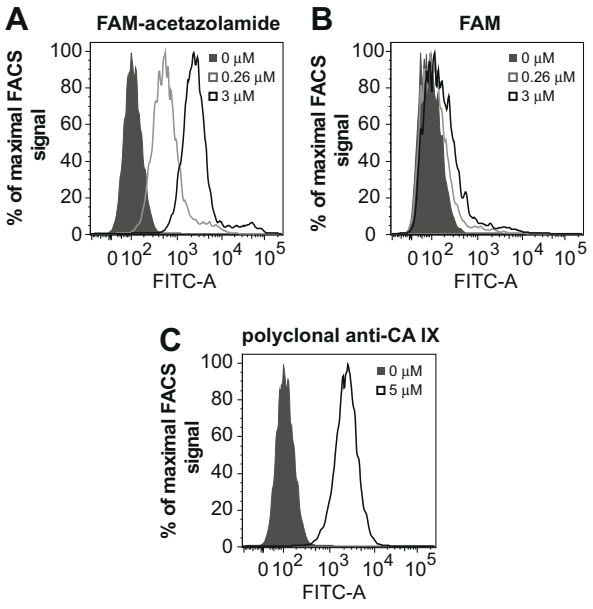

Figure 2. FACS histogram plots of the LS174T cell line. (A) FACS histogram plots of cells stained with different concentrations of FAM-acetazolamide (open gray and black curves) in comparison to non-stained cells (solid curve). (B) FACS histogram plots of cells stained with different concentrations of FAM (open gray and black curves) in comparison to non-stained cells (solid curve). (C) FACS histogram plot of cells stained with a polyclonal anti-CA IX (open curve). The solid curve represents the plot where the antiserum was omitted.

In order to demonstrate that the stable binding of Albu-acetazolamide to serum albumin prevents internalization of the drug into tumor cells, we used mass-spectrometric methodologies to compare drug uptake in non-transfected HEK EBNA 293, full-length CA IX (aa 1-459)-transfected HEK EBNA 293 and in LS174T tumor cells, in the absence or presence of 900 μM HSA. Upon the incubation of 5×10^6 cells with 500 μM of Albu-acetazolamide, followed by tandem mass-spectrometric quantitation of the internalized fraction, a dramatic reduction of drug uptake in the presence of albumin was observed for all three cell lines (Fig. 4).

We assessed the therapeutic activity of Albu-acetazolamide in mice bearing subcutaneously grafted SK-RC-52 or LS174T human tumors. While SK-RC-52 is a human RCC cell line, which displays a homogenous overexpression of CA IX due to the loss of pVHL, LS174T is a human colorectal cancer in which CA IX expression is confined to the membrane of hypoxic cells at a given distance to tumor blood vessels (Fig. 1A). In view of the over-expression of surface-associated CA, both colorectal cancer and renal cell carcinoma appear to be promising indications for the study of CA inhibitors. Furthermore, the LS174T tumor model has recently been used to assess the anti-cancer activity of RNAi-based invalidation of CA IX and CA XII.[11]

Figure 5 shows the results of therapy experiments performed in the two tumor models, using Albu-acetazolamide alone or in combination with other drugs. We used 5-fluorouracil (5-FU) for the therapy of LS174T tumors, since this drug is the mainstay of colorectal cancer in most clinical protocols,[30] while we used sunitinib (*N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-1,2-dihydro-2-oxo-3*H*-indol-3-ylidine)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide) for the therapy of SK-RC-52, since this compound is used as first-line therapy in kidney cancer.[31] For both therapy experiments, a dose of 340 μg (17 mg/kg or 0.5 μmol) of Albu-acetazolamide was used for each injection, which was found to be well tolerated in preliminary mouse treatment studies (data not shown) and which corresponds to a 250 μM concentration in blood immediately at the end of the bolus i.v. injection (Supplementary Fig. 3).

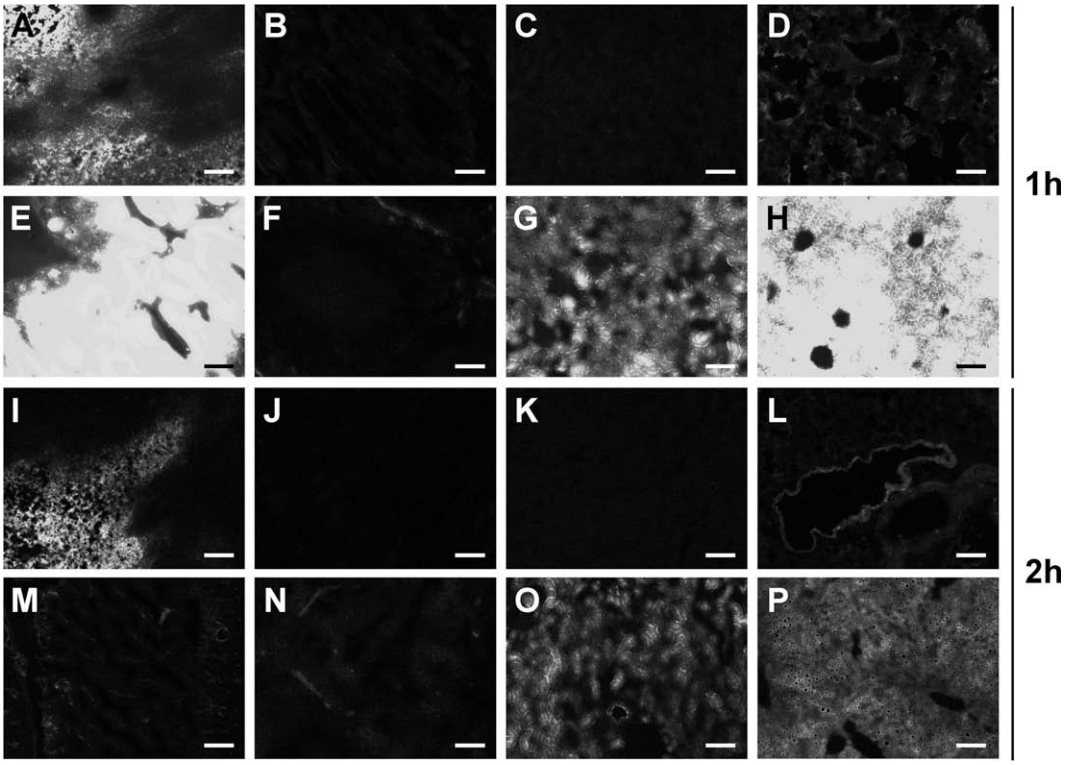

Figure 3. Biodistribution analysis with FAM-acetazolamide. LS174T xenograft-bearing nude mice were injected i.v. with FAM-acetazolamide (**A**–**H**) 1 h or (**I**–**P**) 2 h prior to sacrifice. The accumulation of the fluorescent acetazolamide derivative in (**A**, **I**) tumor, (**B**, **J**) muscle, (**C**, **K**) heart, (**D**, **L**) lung, (**E**, **M**) intestine, (**F**, **N**) spleen, (**G**, **O**) kidney and (**H**, **P**) liver is shown in gray scale. Exposure times were 900 ms for (**A**–**H**) and 1100 ms for (**I**–**P**). Scale bar = 100 μm.

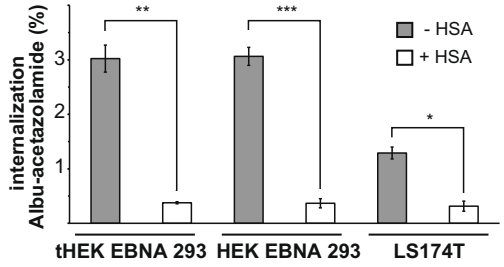

Figure 4. Cell internalization assay with tandem mass-spectrometric quantitation of Albu-acetazolamide. Three different cell lines [full-length CA IX-transfected HEK EBNA 293 (tHEK EBNA 293), HEK EBNA 293 and LS174T] were incubated with a 500 μM solution of Albu-acetazolamide, both in the absence and presence of 900 μM HSA. The assay was performed in triplicate and internalization is expressed as % of the initial amount of compound subjected to the cells. Significant differences are based on Student's *t* test (two-tailed, two-typed), with $^{*}P < 0.0005$, $^{**}P < 0.0001$ and $^{***}P < 0.00005$.

In the SK-RC-52 therapy experiment, using a 5-weekly injection schedule for three consecutive weeks, we observed only a partial inhibition of tumor growth with Albu-acetazolamide (Fig. 5A). Sunitinib treatment showed a stronger inhibition of tumor growth. Tumor stabilizations for at least 20 days could be achieved with a combination of the two compounds. Both sunitinib and Albu-acetazolamide, alone and in combination, were well tolerated as reflected by the mouse weights, without evidence of cumulative toxicity. Only mice in the control treatment group lost weight, when the tumor burden became greater than 500 mg (Fig. 5B).

The therapeutic results in the LS174T model were less favorable. This tumor model grows much more rapidly, thus forcing a treatment regimen of only two weeks (Fig. 5C). In this model, Albu-acetazolamide used as single agent led to a slight increase of tumor growth, which however was not statistically significant, compared to the control group of mice treated with vehicle alone. 5-FU, which was administered at the 25 mg/kg/day dose commonly used in rodent therapy studies[32,33] yielded only a modest tumor growth retardation, yet at the expense of a substantial toxicity (Fig. 5D). The combination of Albu-acetazolamide with 5-FU showed no evidence of cumulative toxicity, but unfortunately did not offer a therapeutic synergy.

In summary, we have described the synthesis and properties of two novel acetazolamide derivatives, which preferentially target membrane-associated carbonic anhydrases on the surface of tumor cells.

For pharmacodelivery applications, small organic molecules are increasingly being considered as suitable delivery vehicles, in parallel to the more extensively characterized antibody-based approaches.[34,35] However, while human monoclonal antibodies can be raised against virtually any antigen of choice,[36] there are only few small organic molecules which can be used as portable tumor-targeting moieties for pharmacodelivery applications. In our study, the combined use of Albu-acetazolamide with standard chemotherapeutic agents led to substantial tumor growth retardation, yet without a substantial improvement compared to chemotherapy alone. It is not clear at present whether this situation may reflect a lack of functional activity of carbonic anhydrases for the tumors which we have investigated or rather an inability for the albumin-bound acetazolamide derivative to homogenously distribute within the tumor mass. RNAi-based therapy studies in stably-transfected LS174T tumors have shown that the simultaneous invalidation of both CA IX and XII is needed in order to achieve an 85% tumor growth retardation.[11] However, in that experimental setting, all tumor cells were genetically equivalent and pharmacokinetic considerations did not apply. Albumin-binding antibody fragments have been shown to homogenously reach tumor cells,

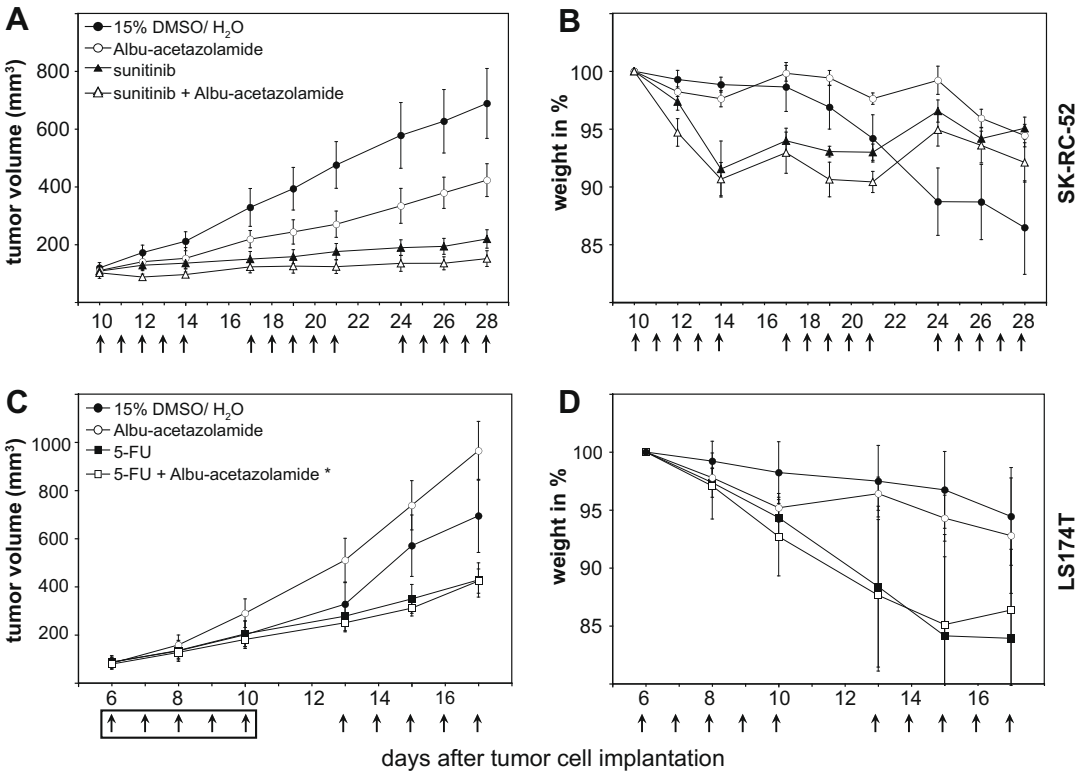

Figure 5. Therapy experiments with Albu-acetazolamide alone or in combination with standard of care chemotherapeutics. (A) Tumor growth curve expressed as mean tumor volume ± SE of SK-RC-52 in Balb/c nude mice after treatment with saline containing 15% (v/v) DMSO (i.v.), 340 μg Albu-acetazolamide (i.v.), 20 mg/kg sunitinib (i.p.) or 340 μg Albu-acetazolamide (i.v.) + 20 mg/kg sunitinib (i.p.) ($n = 5$). (B) Monitoring of the weight loss during therapy for the SK-RC-52 model expressed as% of body weight on day 1 of treatment. (C) Tumor growth curve expressed as mean tumor volume ± SE of LS174T in Balb/c nude mice after treatment with saline containing 15% (v/v) DMSO (i.v.), 340 μg Albu-acetazolamide (i.v.), 25 mg/kg 5-FU (i.p.) or 340 μg Albu-acetazolamide (i.v.) + 25 mg/kg 5-FU (i.p.) ($n = 5$). The combination subgroup comprised only $n = 4$ for the LS174T study, due to the unexpected death of one mouse ( ). (D) Monitoring of the weight loss during therapy for the LS174T model expressed as % of body weight on day 1 of treatment. Arrows indicate days of treatment. Boxed arrows indicate 5-FU treatment, which was only administered in the first of the two consecutive weeks, due to its negative effect on the body weight.

while the parental IgG molecule was trapped on perivascular tumor cells.[25] Similar localization studies are more difficult to perform with Albu-acetazolamide and will require either microautoradiographic investigations with radiolabeled analogues or the use of trifunctional acetazolamide derivatives, capable of albumin binding and carrying, in addition, a suitable detection agent (e.g., a fluorophore, biotin). In spite of these considerations, it was reassuring to observe that Albu-acetazolamide treatment was well tolerated at doses up to 17 mg/kg/d and led to stabilizations of tumors, which are difficult to cure. This warrants future preclinical therapy studies, using different doses and schedules.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.06.022.

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