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Acetazolamide serves as selective delivery vehicle for dipeptide-linked drugs to renal cell carcinoma

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Abstract

In most cases, cytotoxic drugs do not preferentially accumulate at the tumor site, causing unwanted toxicities and preventing dose escalation to therapeutically active regimens. Here, we show that acetazolamide derivatives, which bind to carbonic anhydrase IX (CAIX) on the surface of kidney cancer cells, selectively deliver payloads at the site of disease, sparing normal organs. Biodistribution studies, performed in tumor-bearing mice with acetazolamide derivatives bearing a technetium-99m chelator complex or a red fluorophore as payload, revealed a preferential tumor accumulation of the compound at doses up to 560 nmol/Kg. The percentage of injected dose per gram in the tumor was dose-dependent and revealed optimal tumor:organ ratios at 140 nmol/Kg, with a tumor:blood ratio of 80:1 at 6 h. Acetazolamide, coupled to potent cytotoxic drugs via a dipeptide linker, exhibited a potent antitumor activity in nude mice bearing SKRC-52 renal cell carcinomas, while drug derivatives devoid of the acetazolamide moiety did not exhibit any detectable anticancer activity at the same doses. The observation of tumor regression with a noninternalizing ligand and with different cytotoxic moieties (MMAE and PNU-159682) indicates a general mechanism of action, based on the selective accumulation of the product on tumor cells, followed by the extracellular proteolytic release of the cytotoxic payload at the neoplastic site and the subsequent drug internalization into tumor cells. Acetazolamide-based drug conjugates may represent a promising class of targeted agents for the treatment of metastatic kidney cancer, as the majority of human clear cell renal cell carcinomas are strongly positive for CAIX.

Keywords

Carbonic Anhydrase IX; Tumor Targeting; Small Molecule Drug Conjugates; Renal Cell Carcinoma; Therapy Studies

Introduction

The majority of cytotoxic agents used for the pharmacotherapy of cancer do not preferentially accumulate at the tumor site, leading to potential toxicities and to suboptimal therapeutic efficacy [1–4]. In tumor-bearing mice, unfavorable tumor:organ ratios have been

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reported for many drugs, including doxorubicin [5], paclitaxel [6], cisplatin [7], cyclophosphamide [8], sunitinib [9], and different fluorinated pyrimidines [10], to name just a few. For example, the relative uptake of doxorubicin in the tumor (expressed as percentage injected dose per gram, or %ID/g) was found to be only 5-10% of the corresponding values in lung, heart or liver, in the time period 0.5 – 8 hours [5]. Importantly, similar trends are emerging from the positron emission tomography study of cancer patients, who had received radiolabeled preparations of cytotoxic drugs [3, 4].

The coupling of potent cytotoxic payloads to antibodies or small organic molecules, acting as selective pharmacodelivery vehicles, has been proposed as a general strategy to improve the therapeutic index of anticancer drugs. Indeed, certain antibody-drug conjugates (ADCs) [11–14] and small molecule-drug conjugates (SMDCs) [2] have exhibited promising activity in preclinical models of cancer. Recently, two ADC products (Adcetris™ and Kadcyla™) have gained marketing authorization for oncological applications.

Some ADCs cured tumors in mice at doses of 0.5 – 3 mg/Kg, while the same products could be administered at 60-fold greater dose with acceptable safety profiles [15]. Unfortunately, such a high therapeutic index is rarely observed in the clinic, suggesting that the tumor targeting properties of the antibody, the drug release process and/or the intrinsic sensitivity of tumor cells may be dramatically different in preclinical models and in cancer patients. Nuclear medicine studies with radiolabeled antibody preparations have previously shown that lower tumor:organ ratios are often observed in humans compared to tumor-bearing mice, possibly reflecting differences in antigen abundance and vascular permeability [16, 17]. SMDCs may represent an attractive alternative to ADC products, as smaller pharmaceuticals extravasate more rapidly and diffuse more homogeneously within the neoplastic mass [2, 18–22]. Unlike antibodies (which can easily be raised against the majority of target proteins of interest), it is not always easy to generate high-affinity small molecule ligands to tumor-associated antigens. However, excellent tumor-targeting results have been reported for folate analogues targeting folate-receptor positive tumors [23, 24], substituted urea derivatives targeting prostate-specific membrane antigen [25], somatostatin antagonists targeting the somatostatin receptor [26] and for carbonic anhydrase IX (CAIX) ligands [16–18, 27].

It has generally been assumed that ADC and SMDC products would crucially rely on the use of ligands, capable of selective internalization of the conjugate into the tumor cells, followed by an intracellular liberation of the cytotoxic payload [11–14]. This concept, however, has recently been challenged, as potent and selective anticancer activity has been observed with ADCs and SMDCs specific to antigens, which do not internalize [19, 20, 28–31].

CAIX is a membrane protein, expressed in the majority of clear cell renal cell carcinomas [32–34]. CAIX is virtually undetectable in most normal adult tissues, except for certain structures in the gastrointestinal tract (stomach, duodenum, gallbladder), which are strongly positive for the antigen. Anti-CAIX monoclonal antibodies efficiently localize to metastatic renal cell carcinoma lesions in patients but, surprisingly, not to CAIX-positive stomach, duodenum and gallbladder [35], suggesting that differences in vascular permeability may account for the process.

To the best of our knowledge, CAIX is a tumor-associated target which does not internalize [19], even though at least one anti-CAIX ADC product has been studied in the clinic in patients with cancer [36]. CAIX-positive tumors can be efficiently targeted using certain small aromatic sulfonamides [18–20, 33, 37], including acetazolamide.

We have previously reported that acetazolamide can be used to deliver disulfide-linked derivatives of DM1 (a potent cytotoxic maytansinoid) to CAIX-positive tumors, leading to a potent antitumor effect [19, 20]. We hypothesized that the extracellular drug release process at the tumor site would be facilitated by the death of tumor cells and the subsequent release of glutathione into the tumor extracellular space, which can work as a reducing agent on disulfide bonds, favoring an amplified cascade of drug release and tumor cell death. Since disulfide bonds can be unstable *in vivo* and since most anticancer drugs (unlike DM1) do not contain thiol groups suitable for coupling purposes, it would be interesting to learn whether potent non-internalizing SMDCs can be generated with stable peptidic linkers and with other classes of drugs. In this article, we describe that potent anticancer SMDCs can be generated by coupling acetazolamide to highly cytotoxic drugs (e.g., MMAE and PNU-159682), using a linker containing a valine-citrulline moiety and a self-immolative spacer. These findings reinforce the concept that potent antitumoral activity can be generated by the efficient release of toxic payloads in the tumor extracellular space, without ligand internalization into the target cells of interest.

Materials and Methods

Detailed synthetic procedures and characterization of the presented compounds (i.e., HPLC purity of final products, MS and NMR data, SPR assays) are described in the Supplementary Information [Supplementary Figures S1–11], together with additional biological data (i.e., stability of **4a** in the presence of Cathepsin B and CAIX, biodistribution in CAIX-negative lesions) and protocols (i.e., expression and purification of recombinant human CAIX) [Supplementary Figures S12–15].

Peptide Synthesis

Peptidic precursors of compounds **1–5** [Figure 1] were synthesized by solid phase peptide synthesis (SPPS), using Fmoc-protected amino acids. Chlorotrityl resin (500 mg), pre-loaded with Fmoc-Cys(Trt)-OH, was swollen with DMF (10 ml) for 15 min inside a syringe equipped with a filter pad. Fmoc deprotection was achieved by shaking the resin with 20% v/v piperidine in DMF (5 ml) for 10 min for three times. After deprotection, the resin was washed with DMF (4 × 10 ml). Fmoc-protected amino acids (3 eq) were activated with HATU (3 eq) and DIPEA (6 eq) in DMF (5 ml) for 15 min in ice bath. After this time the solution was allowed to react with the syringe for 1 h, discarded and the resin washed with DMF (4 × 10 ml). Coupling and deprotection steps were alternated, in order to obtain the desired peptide sequence.

Acetazolamide or free amide moieties were then introduced by performing a CuAAC “click” reaction [38] with the corresponding peptide derivatives (carrying an azide moiety) on solid phase, followed by side chain deprotection, cleavage from the resin and RP-HPLC purification.

Radiolabelling

Radiolabeling procedures with technetium-99m were performed essentially as described [18]. Briefly, compound **1** (60 nmol) in TBS pH 7.4 (50 µl) was mixed with SnCl₂ (Sigma Aldrich, 200 µg) and sodium glucoheptonate (TCI, 20 mg) in H₂O (150 µl). Tris-buffered saline at pH 7.4 (600 µl) was added and the resulting solution degassed for 5 min by bubbling with nitrogen gas. The eluate from a ^{99m}Tc-generator (200 µl, ca. 200 MBq, Mallinckrodt) was added and the reaction mixture heated to 90 °C for 20 min. After cooling to room temperature, an aliquot was analyzed by RP-HPLC (XTerra C18, 5% MeCN in 0.1% aq. TFA to 80% over 20 min on a Merck-Hitachi D-7000 HPLC system equipped with a Raytest Gabi Star radiodetector). Technetium-99m incorporations >95% were routinely achieved. The radioactive solution was then diluted to the desired concentrations with a molar excess of compound **1**, dissolved in TBS, pH 7.4.

Conjugates Preparation

Peptide precursors of compounds **2-5** were dissolved in phosphate buffered saline (PBS; 50 mM phosphate, 100 mM NaCl, pH 7.4) and a solution of maleimidocaproyl-linker-*p*-aminobenzylalcohol-Drug in DMF was added at suitable molar ratios. The mixtures were stirred at room temperature until completion and the solvents were removed under vacuum. The conjugates were purified from the crude material via RP-HPLC and lyophilized to obtain the solid products. Purity was checked by UPLC on a Waters Acquity UPLC H-Class System using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm column. Purities higher than 95% were routinely achieved. Details related to specific conjugates are reported in the Supplementary Information.

Cell Cultures

The human renal cell carcinoma cell line SKRC-52 was kindly provided by Professor E. Oosterwijk in 2008 (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands) and subsequently stored in liquid nitrogen. Upon thawing, cells were kept in culture for no longer than 14 passages, tested for CAIX expression by immunofluorescence and not further authenticated. SKRC-52 and HEK293 (ATCC, CRL-1573) were maintained in RPMI medium (Invitrogen) supplemented with fetal calf serum (10%, FCS, Invitrogen) and Antibiotic-Antimycotic (1%, AA, Invitrogen) at 37 °C and 5% CO₂. For passaging, cells were detached using Trypsin-EDTA 0.05% (Invitrogen) when reaching 90% confluence and re-seeded at a dilution of 1:6.

Ligand Internalization Analysis by Confocal Microscopy

SKRC52 or HEK 293 cells were seeded into 4-well cover slip chamber plates (Sarstedt) at a density of 10⁴ cells per well in RPMI medium (1 mL, Invitrogen) supplemented with 10% FCS, AA and HEPES (10 mM) and allowed to grow for 24 h under standard culture conditions. The medium was replaced with medium containing **2a** or **2b** (120 nM), after 30 min or 1 h Hoechst 33342 nuclear dye (Invitrogen) was added and randomly selected colonies imaged on a SP8 confocal microscope equipped with an AOBs device (Leica Microsystems).

Linker Stability Assays

Compounds **4a** and **5a** were dissolved in PBS (100 µg/ml) and incubated at 37 °C in a shaking incubator. Aliquots (150 µl) were taken at different time points (0, 15', 1h, 6h, 24h, 48h) and frozen at -20°C. Standard solutions of the conjugates were prepared in PBS at different concentration (25, 50, 100, 150 µg/ml) and stored at -20 °C. The standards and the samples were analyzed in triplicate by Liquid Chromatography - Mass Spectrometry (LC-MS) on a Waters Xevo G2-XS QTOF coupled to a Waters Acquity UPLC H-Class System using a ACQUITY UPLC BEH C18 Column, 130 Å, 1.7 µm, 2.1 mm × 50 mm column. Peaks relative to the intact SMDCs were integrated and area values from the standards were used to obtain calibration curves. Concentration of intact test compounds in the samples was determined at the different time points to obtain a stability profile of the conjugates.

To measure mouse serum stabilities of the conjugates, the compounds were dissolved in freshly thawed mouse serum (Invitrogen) (100 g/ml) and incubated at 37 °C in a shaking incubator. Aliquots of 150 µl were taken at different time points (0, 20', 1h, 3h, 6h, 24h) and frozen at -20°C. Standard solutions were prepared in mouse serum (Invitrogen) at different concentration (25, 50, 100, 150 µg/ml) and stored at -20°C. All the samples and the standards were thawed and diluted with four volumes of MeOH. After vigorous vortex agitation for 1 min, the protein precipitate was spun down and 400 µl of the supernatant was lyophilized overnight. The resulting solid material was re-dissolved in 100 µl of Millipore water and analyzed as described above.

In Vitro Cytotoxicity Assay

SKRC-52 cells were seeded in 96-well plates in RPMI added with 10% FCS (100 µl) at a density of 5000 cells per well and allowed to grow for 24 h. The medium was replaced with medium containing different concentrations of test substance (1:3 dilution steps) and plates were incubated under standard culture conditions. After 72 h the medium was removed, MTS cell viability dye (20 µl, Promega) in 150 µl of the medium was added, the plates were incubated for 2 h under culture conditions and the absorbance at 490 nm measured on a Spectra Max Paradigm multimode plate reader (Molecular Devices). Experiments were performed in triplicate and average cell viability calculated as measured background corrected absorbance divided by the absorbance of untreated control wells. IC50 values were determined by fitting data to the four-parameter logistic equation, using a Prism 6 software (GraphPad Software) for data analysis.

Animal Studies

All animal experiments were conducted in accordance with Swiss animal welfare laws and regulations under the license number 27/2015 granted by the Veterinäramt des Kantons Zürich.

Implantation of Subcutaneous SKRC-52 Tumors

SKRC-52 cells were grown to 80% confluence and detached with Trypsin-EDTA 0.05% (Life Technologies). Cells were washed with Hank's Balanced Salt Solution (HBSS, pH 7.4) once, counted and re-suspended in HBSS to a final concentration of 3.4×10^7 cells ml⁻¹.

Aliquots of 5×10^6 cells (150 μ l of a suspension) were injected subcutaneously in the right flank of female athymic Balb/c nu/nu mice (8–10 weeks of age, Janvier).

Quantitative Biodistribution Studies

SKRC-52 xenografted tumors were implanted into female Balb/c nu/nu mice (Janvier) as described above, and allowed to grow for three weeks to an average volume of 0.25 ml. Mice were randomized (n=5 per group) and injected intravenously with different doses of radiolabelled preparations (15–25 MBq, 70–560 nmol/Kg). Mice were sacrificed 6h after the injection by CO₂ asphyxiation and organs extracted, weighted and radioactivity measured with a Packard Cobra γ -counter. Values are expressed as %ID/g $\pm$ SD.

IVIS Imaging

Female Balb/c nu/nu mice bearing subcutaneous SKRC-52 tumors were injected intravenously with Compound **3a**, containing the near infrared dye moiety IRDye680RD (LI-COR Biosciences) (250 nmol/Kg), dissolved in sterile PBS (100 μ l). Mice were anesthetized with isoflurane and fluorescence images acquired on an IVIS Spectrum imaging system (Xenogen, exposure 1s, binning factor 8, excitation at 675 nm, emission filter at 720 nm, f number 2, field of view 13.1). Images were taken before the injection and after 10 min, 1 h, 3 h and 6 h. Food and water was given ad libitum during that period.

Dose Escalation Study

Recommended dose of compound **4a** suitable for therapy experiments was determined by dose escalation in wild type female athymic Balb/c nu/nu mice (8–10 weeks of age, Janvier). A schedule of five injections on five consecutive days was used to compare increasing doses (250 nmol/Kg or 500 nmol/Kg) of the targeted derivative **4a** with untargeted compound **4b** [Supplementary Figure S13]. Three mice were used for each group. Tolerated dose was defined when animals did not loose more than 5% of their initial body weight over the duration of the experiment after the initial injection.

Therapy Experiments

SKRC-52 xenografted tumors were implanted into female Balb/c nu/nu mice (Janvier) as described above, and allowed to grow for two weeks to an average volume of 0.1 ml. Mice were randomly assigned into therapy groups of 4 or 5 animals and treatment started by injecting a solution of the targeted drugs, untargeted drugs or vehicle (PBS only or PBS containing 1% of DMSO) intravenously (tail vein) at the doses and with the schedules indicated in the text. Compounds **4a,b** were injected as solutions in sterile PBS. Compounds **5a,b** were injected as solutions in sterile PBS containing 1% DMSO. Animals were weighed and tumor sizes measured daily with an electronic caliper. The tumor volume was calculated according to the formula (long side) $\times$ (short side) $\times$ (short side) $\times$ 0.5. Animals were sacrificed when the termination criteria were reached. Prism 6 software (GraphPad Software) was used for data analysis (regular two-way ANOVA with the Bonferroni test).

Immunofluorescence Studies

SKRC-52 tumors were excised from the animals treated with conjugate **5a** or alternatively with vehicle during the therapy experiment, embedded in OCT medium (Thermo Scientific), and cryostat sections (10 μ m) were cut. Slides were stained using the following antibodies: mouse antihuman CAIX GT12 (Thermo Scientific), to detect the antigen, and rat anti-mouse CD31 (BD Biosciences) to detect endothelial cells. Nuclear staining was performed with DAPI. Anti-mouse IgG-AlexaFluor488 (Molecular Probes by Life Technologies) and anti-rat IgG-AlexaFluor594 (Molecular Probes by Life Technologies) were then used as secondary antibodies for microscopic detection.

Results

Synthesis and tumor-targeting properties of acetazolamide derivatives

Acetazolamide was coupled to a Lys-Asp-Cys-based ^{99m}Tc -chelating moiety, which had previously been used for the imaging of cancer patients with folate derivatives [39], using a “click” chemistry reaction [Figure 1]. The resulting conjugate **1** could be labeled with ^{99m}Tc (radiolabel incorporation >95% routinely achieved; 160-210 MBq/mL) for quantitative biodistribution analysis in tumor-bearing mice. Click chemistry was also used to install the acetazolamide moiety onto Asp-Arg-Asp-Cys peptidic derivatives, which were subsequently coupled to a red Alexa fluorophore (Compound **2a**), a near-infrared dye (Compound **3a**) or cytotoxic drugs, based on the monomethyl auristatin E (MMAE; Compound **4a**) or PNU-159682 (Compound **5a**) active moieties [Figure 1]. As negative controls devoid of CAIX binding, derivatives containing an amide function instead of the acetazolamide moiety were used [Compounds **2b**, **4b** and **5b**].

Confocal microscopy experiments were performed to investigate the internalization of our acetazolamide derivatives upon ligand binding to CAIX. SKRC-52 cancer cells (expressing high levels of CAIX) were incubated with compound **2a** and the fluorescence derived from Alexa594 labeled acetazolamide was imaged after incubation for 30 and 60 min [Figure 2]. Compound **2a** showed selective accumulation on the membrane SKRC-52 cells, as compared to CAIX-negative HEK 293 cells. No intracellular fluorescence was observed throughout the experiment, indicating that the AAZ binding to CAIX was not followed by a significant receptor-mediated endocytosis. In a control experiment, compound **2b**, devoid of the targeting moiety, showed no interaction with CAIX-positive SKRC-52 cells [Figure 2].

The tumor-targeting properties of acetazolamide derivatives were characterized using both radioactive and near-infrared fluorescence methods in nude mice bearing subcutaneously-grafted SKRC-52 tumors [40]. Compound **1**, labeled with ^{99m}Tc , was injected intravenously at doses ranging between 70 nmol/Kg and 560 nmol/Kg. A preferential tumor uptake was observed 6 hours after intravenous administration at all doses [Figure 3A]. However, a progressive decrease in percent injected dose per gram (%ID/g) of tumor was detected, reflecting target saturation at the highest dose. Interestingly, compound uptake in normal organs (e.g., liver, lung, kidney, intestine, stomach) also decreased at higher doses. These biodistribution results suggested that doses around 200-300 nmol/Kg may represent a good compromise between high tumor uptake and low accumulation in normal tissues. The near-

infrared fluorescent derivative **3a**, administered at 250 nmol/Kg, was shown to preferentially accumulate in the tumor at various time points, between 5 min. and 6 hours [Figure 3B]. These results are in keeping with the ones previously reported by our group with similar fluorescently-labeled derivatives [17, 18, 30], indicating that the acetazolamide moiety can deliver different types of payloads to SKRC-52-positive tumors.

Prior to therapy studies, the stability of acetazolamide-drug conjugates **4a** and **5a** was investigated *in vitro* at 37 °C, both in phosphate-buffered saline solution (PBS) and in mouse serum [Figure 4]. For both compounds, a half-life in PBS > 48 hours was observed. A lower stability was seen in mouse serum, but >70% of both conjugates were intact after 6 hours (i.e., a sufficiently long time, compared to the circulatory half-life of acetazolamide derivatives in blood, which is typically shorter than 15 min) [16]. Mass spectrometric analysis of compound **4a** revealed free MMAE as the main release product, while in the degradation profile of compound **5a** in mouse serum free PNU-159682 was not identified, probably due to low sensitivity of the mass spectrometer to a such hydrophobic moiety.

An *in vitro* toxicity study, performed with SKRC-52 cells incubated with the conjugates **4a,b-5a,b** or with the corresponding unmodified drugs, confirmed that the acetazolamide coupling had led to the formation of prodrugs. Indeed, all the conjugates resulted to be less toxic compared to the parental cytotoxic moieties [Figure 5].

Therapy experiments

Compounds **4a** and **5a** were used in therapy experiments with nude mice, bearing subcutaneous SKRC-52 tumors. The maximum tolerated doses of the two prodrugs were determined by dose escalation experiments and found to be 500 nmol/Kg for compound **4a** [Supplementary Figure S13] and 50 nmol/Kg for compound **5a**. The MMAE-conjugate **4a**, used at a dose of 250 nmol/Kg with ten daily administrations, exhibited a potent antitumor activity effect ($p < 0.0001$ at day 26, compared to the control groups), while compound **4b** (devoid of the acetazolamide moiety and serving as negative control) did not retard tumor growth [Figure 6A]. In these experimental conditions, no acute toxicity and no significant weight loss was observed for the group of mice treated with the acetazolamide-MMAE conjugate **4a** [Figure 6B]. One out of five mice enjoyed a durable complete response (with no tumor regression for the next four months), while lesions started to regrow in the remaining four mice one week after the last injection.

The antitumor efficacy of the PNU-159682 conjugate **5a** was investigated at a dose of 25 nmol/Kg [Figure 6C]. Also in this case, the conjugate **5a** exhibited a potent antitumor effect, while the negative control compound **5b** did not inhibit tumor growth, confirming the crucial role of the acetazolamide moiety for the potentiation of drug activity. An additional group of animals was injected with equimolar doses of compound **5a** and the acetazolamide derivative **3a**, leading to a slight decrease in therapeutic activity and tolerability [Figure 6C]. The treatment of mice with PNU-159682 derivatives was associated with higher toxicity compared to the results obtained with MMAE conjugates, but the **5a** treatment group completely recovered from a reversible 10% body weight loss [Figure 6D]. At day 40, a second therapeutic cycle of four injections was performed, which did not lead to the

disappearance of neoplastic lesions. In order to investigate the reasons for the absence of therapeutic activity in the second treatment cycle, mice were injected with the fluorescently-labeled acetazolamide derivative **3a** (250 nmol/Kg) which exhibited only a faint tumor accumulation [Figure 7A], in stark contrast to pre-dosing results [Figure 3B]. An immunofluorescence analysis of resected tumors confirmed that cancer cells remained strongly positive for CAIX [Figure 7B], suggesting that a reduction in vascular permeability after therapy may have impaired drug accumulation in the neoplastic lesions.

Discussion

Acetazolamide derivatives selectively localize to renal cell carcinoma xenografts in mice, which express CAIX as membrane protein. The interaction of these compounds with CAIX-expressing cells has been investigated at the cellular level, by means of fluorescence microscopy experiments. The latter showed that acetazolamide-bearing conjugates bind to CAIX on the cell membrane without triggering a receptor-mediated endocytosis, in agreement with previous findings [19, 20].

A pool of AAZ-based small molecule-drug conjugates has been prepared by our group in recent years: different types of cytotoxic drugs (DM1, MMAE or PNU-159682) were attached to acetazolamide via cleavable linkers (i.e., disulfide bond or valine-citrulline peptide). These SMDCs were found to have similar serum half-lives, which were sufficiently high to exhibit therapeutic benefits *in vivo*. In particular, the new valine-citrulline-bearing compounds **4a** and **5a** exhibited a half-life of ~ 7 hours in serum, which is substantially longer than the circulatory half-life of the radiolabeled analogue **1**.

While drugs commonly used for the treatment of metastatic kidney cancer (e.g., sorafenib, sunitinib) did not display any detectable activity against SKRC-52 tumors [20], acetazolamide derivatives of MMAE (a tubulin inhibitor) and of PNU-159682 (a nemorubicin metabolite) could suppress tumor growth, sometimes mediating a complete tumor eradication. The removal of the acetazolamide moiety from the linker-payload combinations abrogated therapeutic activity in all experimental systems, confirming that the ligand-based delivery of cytotoxic drugs to the extracellular tumor environment represents a strict requirement for anticancer efficacy. Both compounds **4a** and **5a** described in this article feature a linker containing a valine-citrulline moiety, which was previously believed to be particularly suited for the intracellular release of drug payloads, due to the action of lysosomal cathepsin B [41]. This protein is a primarily intracellular protease that can be secreted extracellularly by dying cells, but also by living tumor cells to initiate extracellular proteolytic cascades and to enable tumor cell proliferation [42]. Our therapy data suggest that the presence of cathepsin B in the extracellular tumor environment is sufficient for the efficient liberation of cytotoxic drug moieties, which can then diffuse into surrounding tumor cells.

Nude mice do not represent a perfect model for the assessment of anticancer agents for a number of reasons [43], including their lack of T cells. It has recently been shown that certain cytotoxic agents can mediate an immunogenic tumor cell death [44, 45]. In addition,

certain immunostimulatory agents (e.g., antibody-cytokine fusion proteins, immunological check-point inhibitors) may potentially synergize with some cytotoxic agents [46–48].

In spite of the fact that a strong suppression of tumor growth was observed both for compounds **4a** and **5a**, cancer cures were rare. Interestingly, treatment of mice with a second cycle of **5a** therapy did not cause a second tumor regression [Figure 6]. Macroscopic and microscopic imaging data suggest that the tumor uptake of acetazolamide derivatives is reduced after therapy, probably as a result of changes in vascular permeability at the neoplastic site, while cancer cells remain CAIX-positive. It will be interesting to investigate whether similar findings can be observed with other classes of targeted cytotoxics (e.g., with ADC products).

The potent therapeutic activity observed with compounds **4a** and **5a** suggests that these products may provide a benefit to patients with kidney cancer, as the majority of clear cell renal cell carcinomas are strongly positive for this target [32–34]. The biodistribution profiles of acetazolamide derivatives compares favourably with the ones previously reported for other ligands [23–26]. In particular, we were pleased to see that %ID/g values in the tumor were higher than the corresponding values in the kidney (the main organ for drug clearance), even as early as one hour after intravenous administration of the product [18]. A nuclear medicine trial in patients with metastatic renal cell carcinoma is currently planned for ^{99m}Tc-labeled acetazolamide **1**. That study will provide essential information, regarding the tumor:organ ratios that can be expected in cancer patients and in mouse models of the disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Chemical structures of CAIX-targeting acetazolamide derivatives. Compound **1** features a ^{99m}Tc-chelating moiety, while compounds **2a** and **3a** contain respectively an Alexa594 or a IRdye680RD fluorophore moiety. Cytotoxic drug derivatives featured an acetazolamide moiety (**a** series) or an amide (**b** series, serving as negative control). Products containing the MMAE (compounds **4a** and **4b**) or PNU-159682 payload (compounds **5a** and **5b**) featured a Asp-Arg-Asp-Cys spacer, a valine-citrulline dipeptide cleavable linker, as well as a self immolative linker.

Figure 2.

Confocal microscopy image of CAIX-expressing SKRC52 cells after exposure to targeted dye conjugate **2a** (120 nM) at different incubation times. The conjugate is mainly bound to the cell surface. Alternatively SKRC52 cells were exposed to untargeted dye conjugate **2b** (120 nM) for 30 min. CAIX-negative HEK 293 cells were also exposed to targeted dye conjugate **2a** (120 nM) for 30 min. No cell surface binding can be detected for both the negative controls. Red = Alexa594 derivatives staining; Blue = Hoechst 33342 staining.

Figure 3.

(A) Organ distribution of ^{99m}Tc radiolabeled compound **1** injected at different doses in Balb/c nu/nu mice bearing SKRC-52 xenografts ($n = 5$ per group). The data, expressed as mean % Injected Dose/gram of tissue $\pm$ SD, correspond to the 6 hours time point after the intravenous administration of the radiolabeled compound; (B) Near-infrared fluorescence imaging evaluation of the targeting performance of the IRDye680RD conjugate **3a** in Balb/c nu/nu mice bearing SKRC-52 xenografts.

Figure 4. Stability of prodrugs **4a** and **5a** in PBS, pH 7.4 (A) and in mouse serum (B) at 37°C as determined by LC-MS. Dipeptide derivatives of both types of payload were found to be highly stable in saline solution ($t_{1/2} > 48$ h). A lower stability was observed in mouse serum, but at least 70% of the compounds were still intact after 6 hours of incubation.

Figure 5.

Toxicity of (A) MMAE derivatives **3a** and **3b**, (B) PNU-159682 derivatives **4a** and **4b** and the corresponding unmodified payloads towards CAIX-expressing SKRC-52 cells. Cells were incubated for 72 h in the presence of various concentrations of the test compound at 37°C. Data points are averages of three experiments. Error bars indicate standard deviations. Cytotoxicity values for targeted and non-targeted conjugates are comparable in these *in vitro* experiments (IC₅₀ values: 485 nM for **4a**, 204 nM for **4b**, 25 nM for **5a**, 26 nM for **5b**), confirming the absence of efficient internalization. In all cases, prodrugs were found to be

less potent *in vitro* than the parental free cytotoxic compound (IC₅₀ values: 1.5 nM for **MMAE**, 0.16 nM for **PNU-159682**).

Figure 6.

Therapeutic activity of compound **4a** (A; 250 nmol/Kg) and **5a** (C; 25 nmol/Kg) in Balb/c nu/nu mice bearing SKRC-52 xenografts. In the experiments, drug derivatives devoid of the acetazolamide moiety were used as negative controls (**4b** and **5b**). The graphs in (B) and (D) show the changes in body weight for the treated animals. The statistical analysis of the therapy results, comparing the therapeutic outcome with acetazolamide derivatives and the data obtained with **4b** and **5b** compounds, indicate superior efficacy of the acetazolamide-based conjugates. **** indicates $p < 0.0001$; *** indicates $p < 0.001$; ** indicates $p < 0.01$; * indicates $p < 0.05$.

Figure 7.

Analysis of tumor targeting after therapy experiments. (A) Imaging of a mouse, carrying a subcutaneously-grafted SKRC-52 tumor, with 250 nmol/Kg of the fluorescent acetazolamide derivative **3a**, performed 34 days after the beginning of therapy with compound **5a**. A striking difference in tumor uptake compared to the data of Figure 2B can be observed. (B) Microscopic analysis of representative sections of SKRC-52 tumors, before and after therapy with the acetazolamide derivative **5a**. Green = CAIX staining; Red = CD31 staining; Yellow = overlap of the two colors; Blue = DAPI staining.