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Research paper

New approach of delivering cytotoxic drugs towards CAIX expressing cells: A concept of dual-target drugs

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ABSTRACT

Carbonic anhydrase IX (CAIX) is a hypoxia-regulated and tumor-specific protein that maintains the pH balance of cells. Targeting CAIX might be a valuable approach for specific delivery of cytotoxic drugs, thereby reducing normal tissue side-effects. A series of dual-target compounds were designed and synthesized incorporating a sulfonamide, sulfamide, or sulfamate moiety combined with several different anti-cancer drugs, including the chemotherapeutic agents chlorambucil, tirapazamine, and temozolomide, two Ataxia Telangiectasia and Rad3-related protein inhibitors (ATRi), and the anti-diabetic biguanide agent phenformin. An ATRi derivative (**12**) was the only compound to show a preferred efficacy in CAIX overexpressing cells versus cells without CAIX expression when combined with radiation. Its efficacy might however not solely depend on binding to CAIX, since all described compounds generally display low activity as carbonic anhydrase inhibitors. The hypothesis that dual-target compounds specifically target CAIX expressing tumor cells was therefore not confirmed. Even though dual-target compounds remain an interesting approach, alternative options should also be investigated as novel treatment strategies.

1. Introduction

Solid tumors are characterized by a hypoxic microenvironment caused by their immature and inadequate vascular supply of oxygen and nutrients. These hostile hypoxic conditions result in a phenotype that is associated with a worse prognosis [1] and resistance to standard treatment options such as radiotherapy, chemotherapy, and surgery [2] [3], and [4]. Several different approaches are currently being investigated to target these hypoxic areas to make tumors more sensitive to standard treatment modalities [5] [6], and [7].

Carbonic anhydrase IX (CAIX) can be a valuable therapeutic target since it plays an important role in maintaining the intracellular pH homeostasis [8] and [9]. Furthermore its expression is

Abbreviations: ATRi, Ataxia Telangiectasia and Rad3 related inhibitor; CAIX, Carbonic anhydrase IX; CAIXi, Carbonic anhydrase IX inhibitor; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, Dimethylsulfoxide; FDA, Food and Drug Administration; HIF, Hypoxia-inducible factor; MDCK, Madin-Darby Canine Kidney; MTIC, 3-methyl-(triazen-1-yl)imidazole-4-carboxamide; OCR, Oxygen Consumption Rate.

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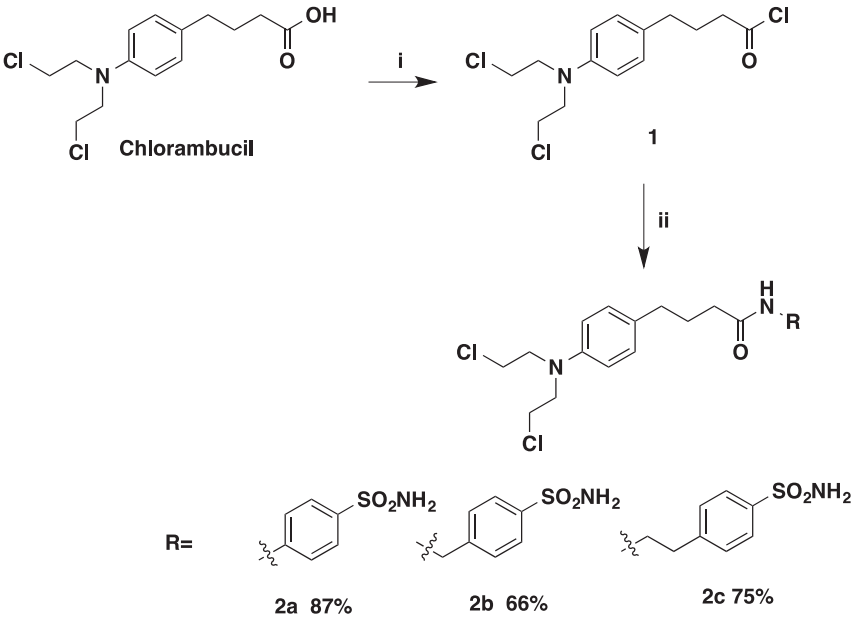
predominantly tumor specific [5] [8], and [10] and directly regulated via the hypoxia-inducible factor (HIF) pathway [11]. Even though alternative pathways are also able to modulate CAIX expression [12] [13], and [14], its significant prognostic value in many different tumor types [15] has promoted investigations in its use as an imaging agent for diagnostic and prognostic purposes [5] [16], [17] [18], and [19]. Together these characteristics of CAIX support investigations into the therapeutic targeting of CAIX to improve efficacy of standard treatments. The function of CAIX is evolutionary conserved and catalyzes the hydration of carbon dioxide to bicarbonate at the cell membrane. The bicarbonate is transported back intracellular from the extracellular space, whereas the free proton is extruded in the extracellular space. CAIX thereby maintains the balance between an acidic extracellular and alkaline intracellular pH of tumor cells, the latter of which would otherwise acidify due to the increased acid production resulting from their glycolytic metabolism [8] and [9]. Many different inhibitors are currently being developed to specifically target the tumor-associated CAIX isoform and have shown promise in reducing tumor cell survival, migration, invasion, and reduce tumor xenograft growth and metastases formation [20] [21], [22], and [23]. Furthermore, the combination therapy of CAIX inhibitors (CAIXi) with standard treatment options was previously found to increase the efficacy of radiotherapy [24] and of weakly basic chemotherapeutic agents such as doxorubicin [25].

The predominant expression of CAIX on hypoxic tumor cells can also be exploited to direct cytotoxic agents specifically to those CAIX expressing cancer cells thereby possibly minimizing normal tissue toxicity. This can be achieved by conjugating anti-cancer drugs with CAIX inhibiting molecules that bind to the Zn^{2+} active site of CAIX and hence inhibit its enzymatic function [8] [26], and [27], *i.e.* a so-called dual-targeting approach. Our group showed previously that such a dual-target approach with a sulfamide CAIXi moiety coupled to the radiosensitizing compound nitroimidazole to be a more effective radiosensitizer than an indanesulfonamide CAIXi [28]. Alternative novel dual-target compounds have been developed to investigate this strategy of dual-targeting further in the context of anti-cancer agents to target CAIX. Here we have designed five different classes of dual-target compounds conjugated to CAIXi (sulfonamide, sulfamide, or sulfamate), which included the chemotherapeutic anti-cancer agents chlorambucil, tirapazamine, and temozolomide, two ataxia telangiectasia and Rad3-related protein inhibitors (ATRi), and the biguanide agent phenformin, previously used in diabetes treatment. We hypothesize that these new dual-target compounds will have the ability to specifically target CAIX expressing cells and modulate their efficacy in a CAIX-dependent manner.

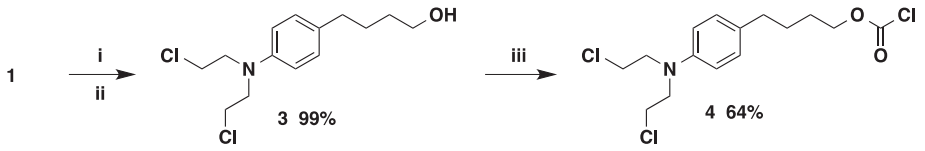
2. Results and discussion

2.1. Chemistry

Chlorambucil was converted to its acid chloride [29] **1** by using oxalyl chloride. This chlorambucil acid chloride reacted with different benzene sulfonamides under basic condition to obtain good yields of chlorambucil derivatives **2a**, **2b** and **2c**. Chlorambucil carbamate derivatives were obtained by converting compound **1** into a methyl ester [30] using methanol. This ester was reduced to alcohol [31], *i.e.* compound **3**, after treating with lithium aluminium hydride. Compound **3** was treated with triphosgene to obtain its respective chloroformate [32], *i.e.* compound **4** (Scheme 2). The reaction of chlorambucil chloroformate (**4)** with different benzene



Scheme 1. Reagents and conditions: (i) $(COCl)_2$, DMF, DCM, 0 °C–rt; (ii) DIPEA, THF, 0 °C–rt.



Scheme 2. Reagents and conditions: (i) MeOH, DCM; (ii) LAH, THF; (iii) Triphosgene, Na_2CO_3, Toluene, DMF.

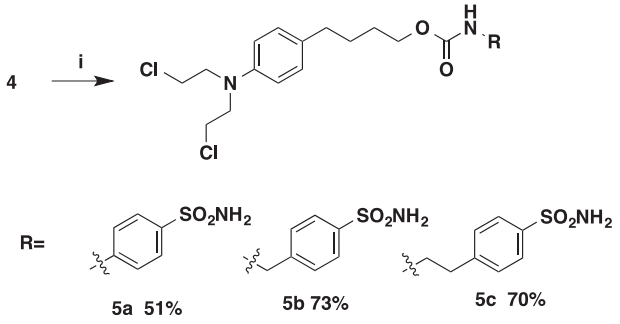
sulfonamides resulted in compounds **5a**, **5b** and **5c** (Scheme 3).

Tirapazamine derivatives **8** and **11** were synthesized from **6** and **9** with the previously described procedure [33]. In short, **6** and **9** reacted with 4-(2-aminoethyl) benzene sulfonamide under reflux conditions and was followed by oxidation of the mono-oxides (Scheme 4).

The ATRi derivatives **12** and **13** were synthesized from commercially available VE-821 and VE-822 (MedChemTronica) using a classical synthetic strategy described previously [25] (Scheme 5).

Commercially purchased temozolomide (SelleckChem) was converted into its respective acid by treating with concentrated sulfuric acid and sodium nitrate at 0 °C–15 °C (Scheme 6) [34]. Reacting the temozolomic acid with different benzenesulfonamides, aminoxysulfonamide [35] and 5-amino-1, 3,4-thiadiazole-2-sulfonamide hydrochloride under known amide bond formation conditions [34] resulted in compounds **15a, 15b, 15c** and **15d** (Scheme 7).

The compound **18** was obtained by a slight modification based on the method reported by Kelarev et al. [36]. The commercially available compounds 4-(2-aminoethyl) benzenesulfonamide **16** and cyanoguanidine **17** were coupled in *n*-butanol using a stoichiometric amount of hydrochloric acid (Scheme 8).



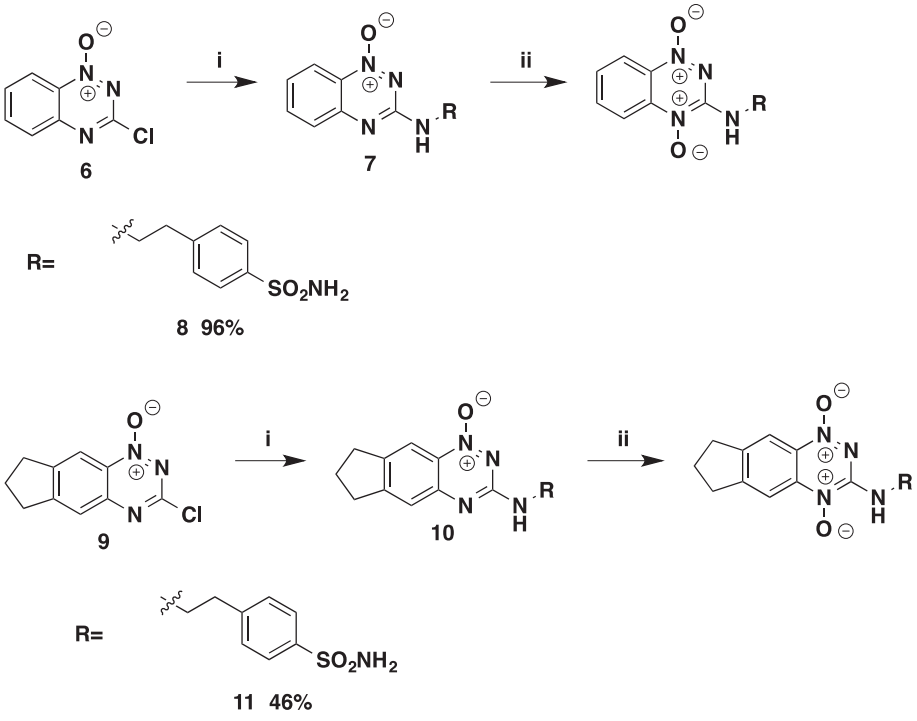
Scheme 3. Reagents and conditions: (i) DIPEA, THF, 0 °C–rt.

2.2. Binding affinity human CAs

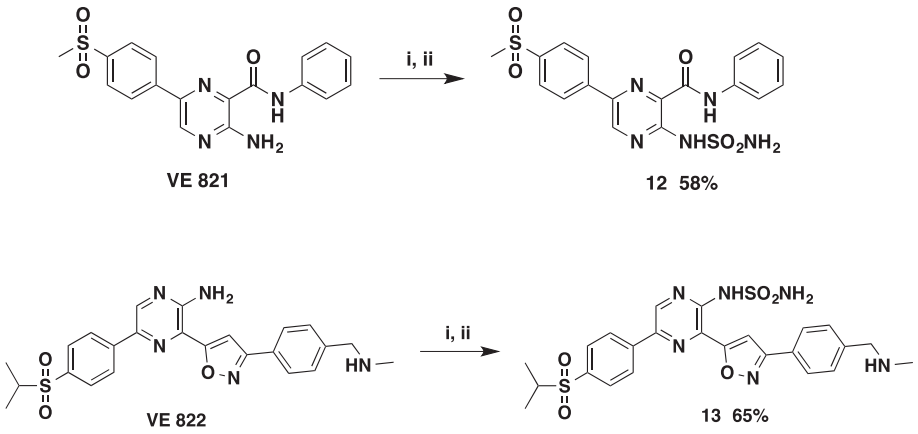
Increased binding affinity to human carbonic anhydrases (CAs) as compared with their respective parental compound are observed (Table 1) for most of the compounds, except for the CAIXi conjugated ATRi (**12** and **13**), which do not bind to any of the four tested human CA isoforms included (K_i > 50000 nM). The K_i values of the other dual-targeting compounds are higher than of the previously reported CAIXi [20] with **15a** showing relatively good K_i for the CAII and CAIX isoforms, but not for CAXII. Only the phenformin derivative **18** was found to be selective for the transmembrane CAIX and CAXII isoforms. To investigate whether the biological efficacy of the functionalized compounds is dependent on CAIX expression, canine kidney epithelial (MDCK) cells without CAIX (CAIX$^-$), *i.e.* both human and canine [37], or MDCK cells transfected with human CAIX [37], i.e. overexpressing CAIX (CAIX$^+$), were used. Western blotting confirmed differential expression of CAIX in these cells both under normoxic and hypoxic conditions (Supplementary Fig. S1).

2.3. Chlorambucil derivatives

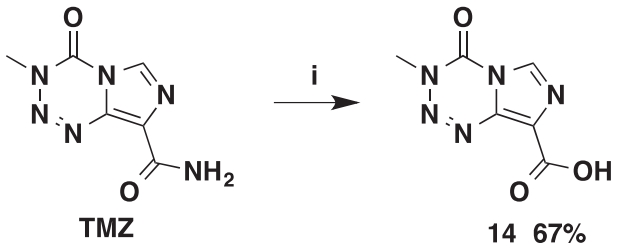
Chlorambucil (4-[p-[bis(2-chloroethyl)amino]phenyl]butyric acid) is a nitrogen mustard that acts as a bifunctional alkylating agent used for decades to treat cancers originating in the blood and lymphatic system, *e.g.* chronic lymphocytic leukemia and lymphomas [38]. Even though reported data suggest chlorambucil efficacy to increase in an acidic microenvironment [39], the CAIXi moiety (benzenesulfonamides) with different linkers (*i.e.* amide, carbamate) might allow for specific targeting of the compounds to these areas in the tumor. The six CAIXi conjugated chlorambucil derivatives (**2a**, **2b**, **2c**, **5a**, **5b**, and **5c**, Schemes 1 and 2) lead to reduced cell viability as compared to the parental compound,



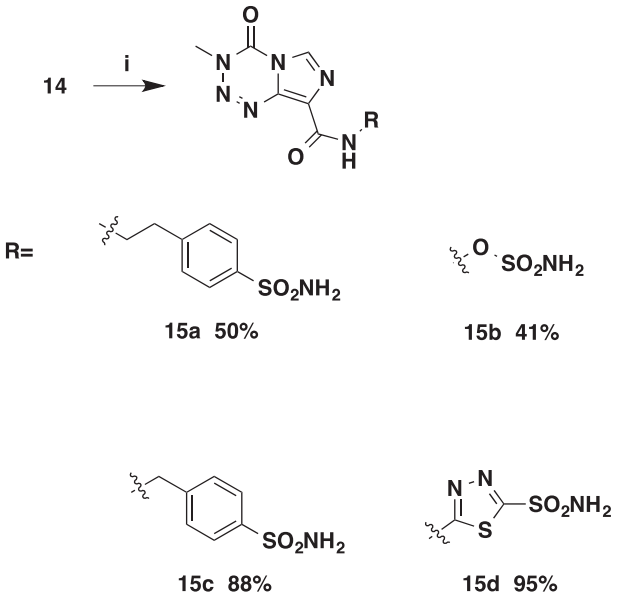
Scheme 4. Reagents and conditions: (i) RNH_2 (3 equiv.), DME, reflux; (ii) TFAA, H_2O_2, DCM, rt.



Scheme 5. Reagents and conditions: (i) $ClSO_2NCO$, tBuOH, NEt_3, DCM, 0 °C to rt; (ii) 20% TFA-DCM.



Scheme 6. Reagents and conditions: (i) Con. H_2SO_4, $NaNO_2$, 0 °C–15 °C.

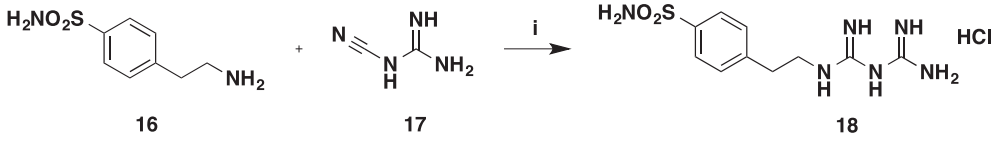


Scheme 7. Reagents and conditions: (i) BOP, NEt_3, DCM, rt.

which was only marginally effective (Table 2, Supplementary Fig. S1). The therapeutic efficacy of these compounds however was not increased in the $CAIX^+$ MDCK cells as compared with the $CAIX^-$ MDCK cells. Furthermore, none of the six compounds showed an increased efficacy upon hypoxia exposure (0.2% O_2). In contrast, some of the chlorambucil dual-target derivatives were less cytotoxic (*i.e.* higher IC_{50}, Table 2) in CAIX expressing cells independent of oxygen levels, which contradicts the studies demonstrating an increased efficacy of chlorambucil in an acidic micromilieu [39]. All together from these results it can be concluded that the CAIXi conjugated chlorambucil derivatives do not show an increased efficacy in a CAIX or hypoxia dependent manner.

2.4. Tirapazamine derivatives

The hypoxia-activated prodrug tirapazamine (3-amino-1,2,4-benzotriazine-1,4-dioxide) has been tested in several clinical trials in combination with chemo- and/or radiotherapy [40]. The cytotoxicity of tirapazamine results from activation by reductive enzymes that add an electron to the parent drug to produce a radical species that causes DNA damage. Nevertheless, no definitive conclusions regarding its clinical efficacy can be drawn since addition of tirapazamine to standard treatment (*i.e.* radiochemotherapy) did not result in an increased benefit in phase III clinical trials. In addition, tirapazamine treatment was often characterized by toxic side-effects, such as nausea, vomiting, and diarrhea, which limited its therapeutic gain [40]. Targeting tirapazamine towards the CAIX expressing (hypoxic) areas in tumors by conjugating tirapazamine with the benzenesulfonamide CAIXi might thereby prove a valuable approach to reduce normal tissue toxicity and increase the efficacy of the compounds (**8** and **11**) in CAIX expressing (hypoxic) cells. Cell viability assays (Table 2, Supplementary Fig. S2) confirmed that the parental compound was specifically effective in hypoxic cells, and more effective in $CAIX^-$



Scheme 8. Reagents and conditions: (i) 6.0 M HCl aq, nBuOH, reflux.

Table 1
Binding affinity (K_i) to human CAI, CAII, CAIX, and CAXII of the parental compounds (bold) and their CAIXi conjugated derivatives.

Compound	K_i (nM)[a]				Selectivity Ratios[b]	
	hCA I	hCA II	hCA IX	hCA XII	K_i hCA II/K_i hCA IX	K_i hCA II/K_i hCA XII
Chlorambucil	>50000	>50000	>50000	>50000		
2a	73.0	9.0	172	689	0.05	0.01
2b	5950	747	8970	7340	0.08	0.10
2c	8400	450	4610	10160	0.10	0.04
5a	5580	553	2740	9380	0.20	0.06
5c	6140	265	4130	9570	0.06	0.03
5c	5670	504	3850	13600	0.13	0.04
Tirapazamine	>50000	>50000	>50000	>50000		
8	567	7.1	383	14600	0.02	<0.01
11	428	8.1	307	624	0.03	0.01
Temozolomide	>50000	>50000	>50000	>50000		
15a	91.3	9.2	37.1	9300	0.25	<0.01
15b	>50000	>50000	>50000	>50000		
15c	539	90.5	271	12400	0.33	0.01
15d	743	15.7	176	92.7	0.09	0.17
ATRi VE-821	>50000	>50000	>50000	>50000		
12	>50000	>50000	>50000	>50000		
ATRi VE-822	>50000	>50000	>50000	>50000		
13	>50000	>50000	>50000	>50000		
Phenformin	>50000	>50000	>50000	>50000		
18	4435	501	20.2	1.7	24.8	295
Acetazolamide[c]	250	12.1	25.3	5.7	0.48	2.12

[a] Values reported (in nM) are the average of three different estimations with errors between 5 and 10% of the reported values. Reported values > 50000 indicates no binding of the compound towards the CA isoforms.
[b] Selectivity ratios of the cytosolic hCAII over the tumor-associated hCAIX and hCAXII isoforms.
[c] Non-specific CAi acetazolamide is included as a reference.

Table 2
Estimated IC_{50} of the cytotoxic parental compounds (bold) and their CAIXi conjugated derivatives obtained with cell viability assays for MDCK $CAIX^-$ and MDCK $CAIX^+$ cells exposed to normoxic and hypoxic conditions.

Compound	Normoxia (μM)[a]		Hypoxia (μM)[a]	
	$CAIX^-$	$CAIX^+$	$CAIX^-$	$CAIX^+$
Chlorambucil[b]	93	>100	~100	>100
2a	~100	87	~100	95
2b	18	98	14	92
2c	18	~100	18	~100
5a	8	56	8	62
5b	86	98	52	100
5c	89	99	81	~100
Tirapazamine[c]	>300	>300	<50	95
8	>300	>300	~300	>300
11	>300	>300	>300	>300
Temozolomide[d]	775	>1000	~1000	>1000
15a	>1000	>1000	>1000	>1000
15b	719	>1000	~1000	>1000
15c	>1000	>1000	>1000	>1000
15d	>1000	>1000	>1000	>1000

[a] No IC_{50} reached is indicated with >. Estimated IC_{50} value higher than the maximum concentration included is indicated with ~.
[b] Included concentrations for chlorambucil were 1, 10, and 100 μM.
[c] Included concentrations for tirapazamine were 50, 100, 200, and 300 μM.
[d] Included concentrations for temozolomide were 100, 400, 700, and 1000 μM.

cells (IC_{50} < 50 versus 95 μM, p = 0.037). The CAIXi conjugated tirapazamine derivatives however abrogated the effect observed for the parental compound, both during hypoxia and normoxia, which was independent of CAIX levels (Table 2).

2.5. Temozolomide derivatives

The current treatment of glioblastoma is based on radiotherapy combined with temozolomide, which has been shown to increase survival in phase III clinical trials [41]. Temozolomide is a methylating agent that spontaneously hydrolyzes to its active metabolite 3-methyl-(triazen-1-yl)imidazole-4-carboxamide (MTIC) at physiological pH [42]. The acidic extracellular pH in tumors might therefore reduce spontaneous temozolomide conversion and thereby decrease its efficacy. Inhibiting CAIX function is known to decrease extracellular acidification *in vitro* [24] [25], [28], and [43] and we hypothesized that conjugating temozolomide with a sulfonamide or sulfamate moiety (**15a**, **15b**, **15c**, and **15d**) will specifically target hypoxic tumors and increase temozolomide conversion and thereby its efficacy. Nevertheless, while temozolomide resulted in lower cell viability in $CAIX^-$ cells, consistent with the pH-dependent mechanism of activation, the CAIXi conjugated temozolomide derivatives **15a**, **15c**, and **15d** were ineffective in reducing cell viability in both MDCK cell lines during normoxic and hypoxic conditions within the concentration range tested in the present study (Table 2, Supplementary Fig. S3). In contrast, **15b** was similarly effective as the parental temozolomide compound (Table 2).

This dual-target compound was therefore investigated further in clonogenic survival assays in which the medium of the cells was acidified because of CAIX function during hypoxic conditions (Supplementary Fig. S1) [44]. Temozolomide was again more effective in reducing clonogenic cell survival in the $CAIX^-$ MDCK cells as compared with the $CAIX^+$ MDCK cells (Fig. 1), similarly to its efficacy on cell viability. During hypoxia however temozolomide caused no difference in clonogenic survival as compared to normoxia, even though hypoxia is required to activate CAIX and cause extracellular acidification [43] and [45] and is therefore hypothesized to reduce temozolomide conversion and efficacy. In contrast, the CAIXi conjugated derivative **15b** significantly reduced clonogenic cell survival in hypoxic versus normoxic conditions in the $CAIX^+$ cells (surviving fraction is 46.1 ± 3.1 versus 26.1% ±7.9 during normoxia and hypoxia respectively, p < 0.05). Nevertheless, the effect of **15b** on survival was not significantly different from the parental temozolomide compound. In addition, the low binding affinity of the compound (Table 1) combined with its relatively low efficacy in the $CAIX^+$ as compared to the $CAIX^-$ cells minimizes its

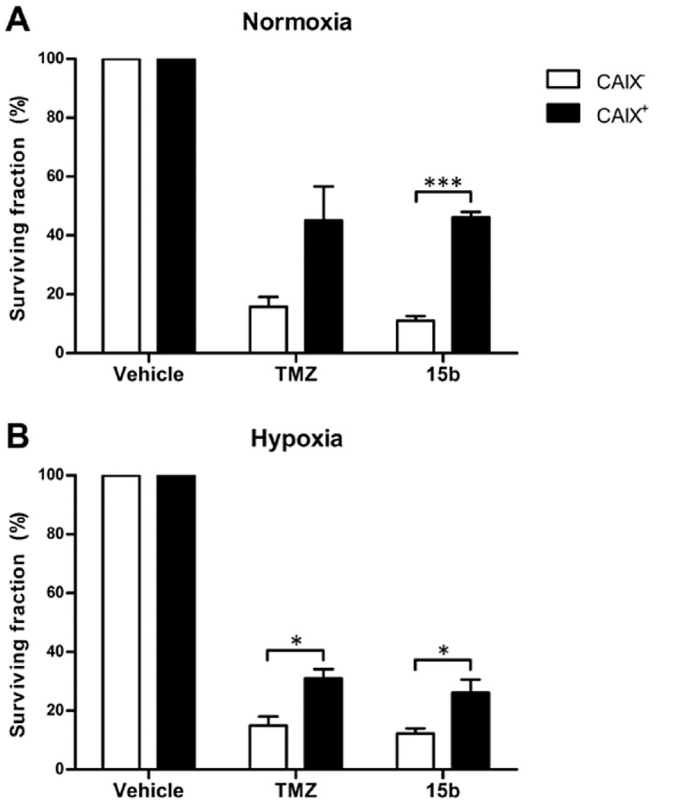


Fig. 1. Clonogenic cell survival of confluent MDCK CAIX$^-$ and CAIX$^+$ cells during normoxia (21% O_2) and hypoxia (0.2% O_2) when exposed to temozolomide (TMZ) and the CAIXi conjugated derivative **15b**. Surviving fraction (%) was normalized to vehicle control. Average ± SEM of three independent biological repeats is shown. Asterisks indicate statistical significance (*p < 0.05; ***p < 0.001).

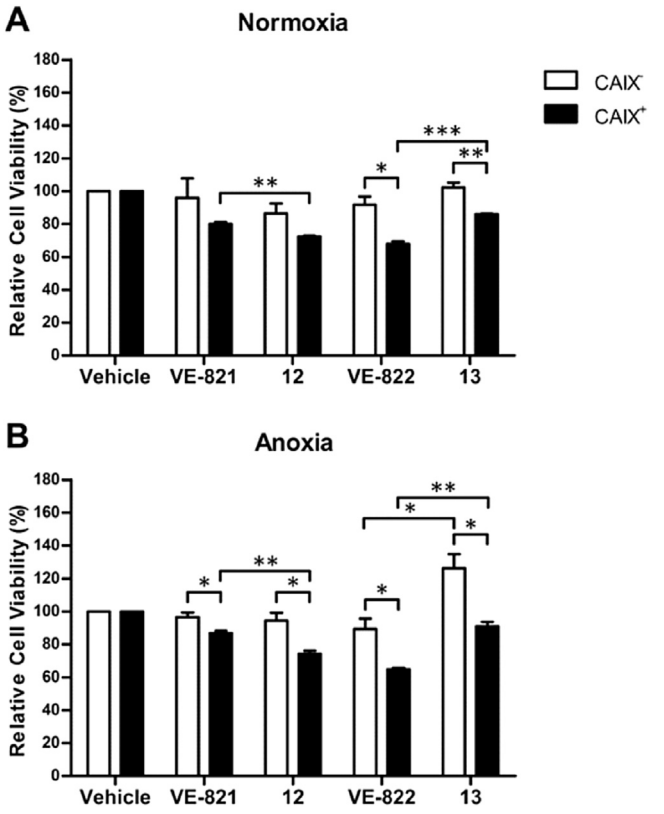


Fig. 2. Relative cell viability (%) in MDCK CAIX$^-$ and CAIX$^+$ cells exposed to ATR inhibitors (VE-821 and VE-822) or the CAIXi conjugated derivatives (**12** and **13**) in combination with radiation during normoxia (21% O_2) and anoxia (≤0.02% O_2). Normoxic cells were irradiated with 2 Gy and anoxic cells with 4 Gy to induce similar effects on cell viability. Cells were exposed to 500 nM VE-821 and **12**, and to 50 nM VE-822 and **13**. Average ± SEM of three independent biological repeats is shown. Asterisks indicate statistical significance (*p < 0.05; **p < 0.01; ***p < 0.001).

potential for further development. These results furthermore suggest that temozolomide efficacy is not affected by CAIX dependent changes in extracellular pH during hypoxia. A reduction of temozolomide conversion and efficacy might require lower pH levels, *i.e.* pH < 6.6, which may have not been achieved in the present experiments [8] [9], and [39].

2.6. ATR inhibitor derivatives

Preclinical experiments have shown that Ataxia Telangiectasia and Rad3-related protein inhibitors (ATRi) reduce the DNA repair capacity resulting in enhanced cell death and decreased tumor growth when combined with either chemo- or radiotherapy [46] [47], and [48]. However ATRi are not highly tumor specific, thus targeting these compounds towards the CAIX expressing areas of a tumor might increase their therapeutic benefit. The effect on cell viability of the parental ATRi (VE-821 and VE-822) and their CAIXi conjugated derivatives (**12** and **13**) was tested in combination with radiotherapy to induce DNA damage where a higher radiation dose was applied to anoxic cells, since those are more radioresistant [49] and [50]. The parental ATRi and the CAIXi conjugated derivatives in combination with radiation decreased cell viability as compared to radiation only in the CAIX$^+$ cells ($p < 0.05$) under both normoxic and anoxic conditions, but not in the CAIX$^-$ cells (Fig. 2). The only exception is the derivative **13**, which had no significant effect on cell viability during anoxic conditions in both cell lines as compared to radiation alone ($p = 0.09$ and $p = 0.08$ for CAIX$^-$ and CAIX$^+$ cells, respectively). More importantly, the CAIXi conjugated derivative **12** was more effective than its respective parental ATRi (VE-821) in the CAIX$^+$ ($p < 0.01$ during normoxia and anoxia), but not the CAIX$^-$ cells ($p = 0.52$ and $p = 0.72$ for normoxia and anoxia, respectively), suggesting a CAIX specific effect. In contrast, the CAIXi conjugated derivative **13** in combination with radiation was less effective in reducing cell viability than the parental compound VE-822 in CAIX$^+$ cells ($p < 0.001$ and $p < 0.01$ during normoxic and anoxic conditions, respectively). Although radiation induced similar effects on cell viability during normoxic and anoxic conditions, the efficacy of derivative **12** did not increase further during anoxic conditions as compared to normoxic conditions, even though CAIX expression is upregulated under hypoxic conditions (Supplementary Fig. S1) and these conditions are essential for CAIXi binding [43] and [45]. Although derivative **12** indeed proved to be more effective in CAIX$^+$ than in CAIX$^-$ cells in combination with radiation, its efficacy might however not be solely dependent on binding to CAIX, which is consistent with unfavorable K_i values of the compound (Table 1). Exposing both cell lines to ATRi without irradiation decreased cell viability of both cell lines during normoxic and anoxic conditions, although this effect appeared to be slightly more pronounced in the CAIX$^-$ cells (Supplementary Fig. S5). Differences in ATRi response between the cell lines when combined with radiation might be explained by a lower number of cells in the resistant S-phase of the cell cycle [51], or by a decreased DNA repair capacity in cells with lower intracellular pH [52] [53], and [54], *i.e.* those that do not express CAIX. This may also explain the difference in sensitivity to cytotoxic drugs between both cell lines, although further investigations are required to prove this

causal relationship.

2.7. Phenformin derivatives

Phenformin (1-(diaminomethylidene)-2-(2-phenylethyl)guanidine) is a drug used to treat diabetes, but was withdrawn from the North-American market in the 1970s by the Food and Drug Administration (FDA) due to a high risk of developing lactic acidosis [55]. Treating patients with a similar but less potent drug metformin was found to be associated with a decrease in cancer incidence and an increased life span of cancer patients [56]. The repurposing of these compounds as anti-cancer agents is therefore being investigated where phenformin is found to be more lipophilic, thereby requiring less active transport than metformin [57]. The proposed mechanism of action of phenformin is its ability to inhibit mitochondrial respiration, which will consequently result in a decreased ATP production, thereby reducing tumor cell growth and improving tumor oxygenation as a result of decreased oxygen consumption [58] and [59]. Conjugating phenformin with CAIXi might make the drug more tumor-specific leading to reduced normal tissue toxicity. Since tumor cells are more sensitive to phenformin treatment due to their altered energy metabolism, human colorectal HCT116 cells, with or without CAIX knockdown [24] and [28] were used to study the effect of phenformin and its CAIXi conjugated derivative **18** on mitochondrial respiration. Western blotting confirms low expression of CAIX in CAIX KD cells under hypoxic conditions as compared with control cells (Supplementary Fig. S1B). As expected, CAIX levels were low in both cell lines under normoxic conditions. Phenformin significantly reduced Oxygen Consumption Rate (OCR) in both cell lines ($p < 0.05$), independent of CAIX expression levels (Fig. 3). In contrast, the CAIXi conjugated derivative **18** was ineffective in reducing OCR, even when a fourfold higher concentration was used.

3. Conclusion

Overall our hypothesis that newly designed dual-target drugs are more selective for CAIX expressing cells and are able to modulate their own efficacy by inhibiting CAIX function was not confirmed. Of all derivatives included, only one (*i.e.* the ATRi derivative **12**) proved more effective than its parental compound when combined with irradiation in $CAIX^+$ cells versus $CAIX^-$ cells. Nevertheless, the effect of this compound may not only be related to binding of the compound to CAIX due to limited binding affinity and the lack of further increase in its efficacy under hypoxic conditions. The rest of the derivatives included in this study did not show an increased efficacy in $CAIX^+$ versus $CAIX^-$ cells, or an efficacy that depended on oxygen levels, *i.e.* hypoxia versus normoxia. Nevertheless, since the parental compounds proved effective in these experiments the conjugation of the CAIXi moiety with the cytotoxic compounds may have caused conformational changes, thereby altering the compounds efficacy. In addition, these conformational changes may have also limited the binding of the CAIXi moiety (sulfonamide, sulfamide or aminoxysulfonamide) into the Zn^{2+} containing active pocket of CAIX, which explains the lack of CAIX specificity and binding affinity for human CAs (Table 1) of the compounds. Alternative strategies to target the CAIX expressing cells in a tumor, *e.g.* antibody targeting or an increased number of CAIXi conjugated molecules [60], might therefore be more promising options to pursue in the future.

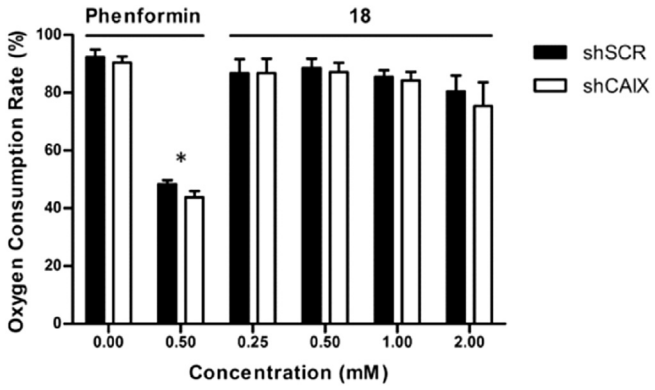


Fig. 3. Oxygen Consumption Rate (OCR) of HCT116 cells with CAIX (shSCR) or without CAIX expression (shCAIX) exposed to phenformin or the CAIXi conjugated derivative **18**. OCR was normalized to baseline OCR levels before compound injection. Average ± SEM of four independent biological repeats is shown. Asterisks indicate statistical significance (*$p < 0.05$).

4. Experimental section

4.1. Chemistry

4.1.1. General

Unless otherwise specified, reagents and solvents were of commercial quality and were used without further purification. All reactions were carried out under an inert atmosphere of nitrogen. TLC analyses were performed on silica gel 60 F_{254} plates (Merck Art.1.05554). Spots were visualized under 254 nm UV illumination, or by ninhydrin solution spraying. Melting points (mp) were determined on a Büchi Melting Point 510 and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on Bruker DRX-400 spectrometer using DMSO-d_6 as a solvent and tetramethylsilane as an internal standard. For ^1H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in Hertz. Electron Ionization mass spectra were recorded in positive or negative mode on a Waters MicroMass ZQ. All compounds that were tested in the biological assays were analyzed by High-resolution ESI mass spectra (HRMS) using on a Q-ToF I mass spectrometer fitted with an electrospray ion source in order to confirm the purity of >95%.

4.1.2. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butanoyl chloride (1)

Oxalyl chloride (32.8 mmol, 2.0 equiv.) was added slowly over a period of 1.0 h at 5–10 °C to a stirred solution of chlorambucil (16.4 mmol, 1.0 equiv.) in DCM (25.0 mL, 5.0 vol) and a catalytic amount of *N, N*-dimethylformamide. The reaction mixture was stirred at ambient temperature for 2–3 h, after which excess oxalyl chloride and DCM were removed under reduced pressure. The chlorambucil acid chloride that was obtained was a pale green solid in quantitative yield, which was used as such for the synthesis of Compounds **2a**–**c**.

4.1.3. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butan-1-ol (3)

Compound **1** (15.5 mmol, 1.0 equiv.) was dissolved in DCM (125 mL) and methanol (75 mL, 3 vol) was slowly added over a period of 1 h at 15–20 °C. The reaction mixture was stirred at ambient temperature for 2 h. The reaction mixture was concentrated and the residue was dissolved in ethyl acetate (125 mL, 5 vol) and washed successively with a 5% aq. $NaHCO_3$. Evaporation of the solvent under reduced pressure resulted in the chlorambucil methyl ester (16.4 mmol, 1.0 equiv.) in 95% yield as a light brown oil, which was added to a suspension of lithium aluminium hydride (32.8 mmol, 2 equiv.) in anhydrous THF (100 mL, 4 vol) at 0–5 °C for a period of 1 h. The reaction mixture was thereafter allowed to stir at ambient temperature for 2–3 h. Next, the reaction mixture was cooled to 0–5 °C and quenched slowly with ethyl acetate (250 mL,

10 vol) followed by water (100 mL, 4 vol). The reaction mixture was filtered through celite and ethyl acetate (50 mL, 2 vol) was used to wash the celite bed. The organic layer was washed with water (100 mL, 4 vol), dried over anhydrous Na_2SO_4, and filtered. Evaporation of the solvent under reduced pressure resulted in 99% yield of the crude alcohol as a pale yellow oil, which was used in the next step.

4.1.4. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl carbonochloridate (4)

DMF (1.4 g) and sodium carbonate (75.79 mmol, 1.1 equiv.) were added to a solution of triphosgene (37.89 mmol, 0.55 equiv.) in toluene (300 mL, 15 vol) at ambient temperature. The reaction mixture was cooled to 0–5 °C and maintained at the same temperature for 30 min. Next, a solution of **3** (68.9 mmol, 1.0 equiv.) in toluene (100 mL, 5 vol) was added to the stirred reaction mixture at 0–5 °C during 30 min. This reaction mixture was stirred for 4–5 h at room temperature. The reaction mixture was filtered thereafter and the solid was washed with toluene (100 mL, 5 vol). Evaporation of the solvent under reduced pressure resulted in the chloroformate **4** with a 64% yield as a yellow viscous liquid, which was used for the synthesis of carbamates (compounds **5a**–**c**).

4.1.5. General procedure for the preparation of compounds (2a–c and 5a–c)

To a solution of aminoalkylbenzene sulfonamide (1.0 equiv.) in acetonitrile (225 mL, 15 vol) and *N*,*N*-diisopropylethylamine (2.5 equiv.) a solution of compound **1 (2a–c)** or compound **4 (5a–c)** (1.0 equiv.) in acetonitrile (75 mL, 5 vol) was added over a period of 1 h and stirred overnight at ambient temperature. After completion, the reaction mixture was concentrated and the residue obtained was dissolved in ethyl acetate (150 mL, 10 vol). The organic layer was successively washed with 2 N HCl solution (100 mL × 2) in water, dried over anhydrous Na_2SO_4, and filtered. After evaporation of the solvent under reduced pressure a pale yellow solid was obtained as a crude product. This crude product was purified with column chromatography using a silica gel (40% ethyl acetate in hexane) to obtain compound **2a**–**c** and **5a**–**c** in a 51–87% yield.

4.1.6. General procedure for amination of 3-Chlorobenzotriazine-1,4-di-N-oxides (7 and 10)

4-(2-Aminoethyl) benzene sulfonamide (8.25 mmol, 3.0 equiv.) was added to a stirring solution of 3 chlorobenzotriazine-1,4-di-*N*-oxide (2.75 mmol, 1.0 equiv.) in dimethoxyethane (30 mL) and the mixture was stirred overnight at reflux temperature. The next day mixture was cooled to room temperature and concentrated under vacuum, after which the residue was dissolved in ammonium hydroxide solution and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by chromatography using a silica gel with methylene chloride-methanol 98:2 v-v as an eluent to obtain the expected compound as a yellow powder with an 85–94% yield.

4.1.7. General procedure for oxidation (8 and 11)

Hydrogen peroxide (12.9 mmol, 10 equiv.) was added dropwise to a stirred solution of trifluoroacetic anhydride (12.9 mmol, 10 equiv.) in DCM at 0 °C. This reaction mixture was stirred at 0 °C for 5 min, warmed to room temperature for 10 min, and cooled to 5 °C. Next, the mixture was added to a stirred solution of mono oxide (1.29 mmol, 1.0 equiv.) in DCM at 0 °C and stirred at room temperature for 2–3 days. The reaction mixture was carefully diluted with water and basified with aqueous NH_4OH and extracted with $CHCl_3$. The organic fraction was dried over anhydrous Na_2SO_4, filtered and evaporated to obtain the residue. This residue was purified by chromatography using a silica gel with methylene chloride-methanol 98:2 v-v as an eluent to obtain the expected compound as an orange red powder with a 46–96% yield.

4.1.8. General procedure for synthesis of ATRi derivatives (12 and 13)

A solution of VE-821 or VE-822 (0.54 mmol, 1.0 equiv.) and triethylamine (1.62 mmol, 3.0 equiv.) in 10 mL of methylene chloride was added to a mixture of chlorosulfonyl isocyanate (0.68 mmol, 1.2 equiv.) and *tert*-butanol (0.648 mmol, 1.2 equiv.) in 2 mL of methylene chloride. The mixture was stirred at room temperature for 1.0 h, diluted with ethyl acetate, and washed with water. The organic layer was then dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum. The residue was purified by chromatography with a silica gel and methylene chloride-methanol 98:2 as an eluent. This intermediate was thereafter diluted in a solution of trifluoro acetic acid in methylene chloride (20% vol.) and stirred at room temperature for 6 h. Next, the mixture was concentrated under vacuum and co-evaporated with diethyl ether multiple times to obtain the expected compound with a 58–65% yield.

4.1.9. General procedure for synthesis of temozolomide derivatives

To a slurry of 3-methyl-4-oxo-3,4-dihydroimidazo[5,1-d][1,2,3,5] tetrazine-8-carboxylic acid (1.0 mmol, 1.0 equiv.) in DCM, BOP (1.0 mmol, 1.0 equiv.), amine (1.1 mmol, 1.1 equiv.) and triethylamine (2.5 mmol, 2.5 equiv.) were added. This reaction mixture was stirred overnight at room temperature and filtered to obtain the expected compounds with a 41–95% yield.

4.1.10. 4-(4-(Bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenyl)butanamide (2a)

mp: 155–157 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 10.22 (s, 1H), 7.73 (d, J = 4.4, 4H), 7.23 (s, 2H), 7.05 (d, J = 8.7, 2H), 6.67 (d, J = 8.7, 2H), 3.70 (d, J = 8.6, 8H), 2.54–2.50 (m, 2H), 2.34 (t, J = 8.6, 2H), 1.90–1.78 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 171.68, 144.46, 142.23, 138.03, 129.53, 126.65, 118.51, 111.90, 52.22, 41.17, 35.84, 33.54, 26.87; MS (ESI$^+$) m/z 458.11 [M+H]+, 460.10 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{20}H_{26}N_3O_3SCl_2]^+$: 458.1072, found: 458.1075.

4.1.11. 4 -(4-(bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylbenzyl)butanamide (2b)

mp: 130–132 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.41 (t, J = 5.9, 1H), 7.76 (d, J = 8.3, 2H), 7.41 (d, J = 8.3, 2H), 7.31 (s, 2H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 4.31 (d, J = 5.9, 2H), 3.69 (s, 8H), 2.45 (t, J = 7.5, 2H), 2.15 (t, J = 7.5, 2H), 1.82–0.72 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 172.15, 144.42, 143.92, 142.54, 129.86, 129.33, 127.46, 125.68, 111.89, 52.22, 41.67, 41.17, 34.84, 33.66, 27.39; MS (ESI$^+$) m/z 472.12 [M+H]$^+$, 474.12 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{21}H_{28}N_3O_3SCl_2]^+$: 472.1228, found: 472.1236.

4.1.12. 4-(4-(Bis(2-chloroethyl)amino)phenyl)-N-(4-sulfamoylphenethyl)butanamide(2c)

mp: 108–110 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.89 (t, J = 5.6, 1H), 7.74 (d, J = 8.3, 2H), 7.38 (d, J = 8.3, 2H), 7.30 (s, 2H), 7.00 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 3.70 (d, J = 8.9, 8H), 3.29 (dd, J = 13.0, 5.6, 2H), 2.78 (t, J = 7.5, 2H), 2.41 (t, J = 7.5, 2H), 2.08–1.98 (m, 2H), 1.76–1.65 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 171.95, 144.39, 143.80, 142.01, 129.89, 129.21, 125.67, 111.87, 52.23, 41.17, 34.87, 33.61, 27.32; MS (ESI$^+$) m/z 486.14 [M+H]$^+$, 488.14 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{22}H_{30}N_3O_3SCl_2]^+$: 486.1385, found 486.1387.

4.1.13. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenyl)carbamate(5a)

mp: 156–158 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 10.01 (s, 1H), 7.76–7.69 (m, 2H), 7.64–7.56 (m, 2H), 7.22 (s, 2H), 7.04 (d, J = 8.7, 2H), 6.66 (d, J = 8.7, 2H), 4.12 (t, J = 6.0, 2H), 3.77–3.63 (m, 8H), 1.70–1.54 (m, 4H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 153.50, 144.40, 142.34, 137.48, 130.06, 129.30, 126.77, 117.50, 111.89, 64.43, 52.22, 41.17, 33.68, 28.09, 27.62; MS (ESI$^+$) m/z 488.12 [M+H]$^+$, 490.12 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{21}H_{28}N_3O_4SCl_2]^+$: 488.1178, found: 488.1184.

4.1.14. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylbenzyl)carbamate(5b)

mp: 98–100 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.76 (t, J = 8.5, 3H), 7.41 (d, J = 8.5, 2H), 7.31 (s, 2H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 4.23 (d, J = 6.1, 2H), 3.98 (s, 2H), 3.75–3.63 (m, 8H), 2.47 (s, 2H), 1.55 (m, 4H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 156.63, 144.37, 143.97, 142.61, 130.13, 129.26, 127.27, 125.70, 111.88, 63.84, 52.23, 43.37, 41.18, 33.68, 28.31, 27.64; MS (ESI$^+$) m/z 502.13 [M+H]$^+$, 504.13 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{22}H_{30}N_3O_4SCl_2]^+$: 502.1334, found: 502.1338.

4.1.15. 4-(4-(Bis(2-chloroethyl)amino)phenyl)butyl (4-sulfamoylphenethyl)carbamate(5c)

mp: 100–102 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.77–7.70 (m, 2H), 7.37 (d, J = 8.2, 2H), 7.30 (s, 2H), 7.19 (t, J = 5.5, 1H), 7.02 (d, J = 8.6, 2H), 6.66 (d, J = 8.6, 2H), 3.94 (d, J = 5.5, 2H), 3.69 (s, 8H), 3.21 (dd, J = 13.3, 6.6, 2H), 2.76 (dd, J = 16.9, 9.8, 2H), 2.46 (s, 2H), 1.52 (s, 4H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 156.30, 144.37, 143.58, 142.03, 130.15, 129.19, 125.67, 111.88, 63.51, 52.23, 41.28, 35.09, 33.67, 28.32, 27.63; MS (ESI$^+$) m/z 516.15 [M+H]$^+$, 518.15 [M+2]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{23}H_{32}N_3O_4SCl_2]^+$: 516.1491, found: 516.1490.

4.1.16. 3-(4-Sulfamoylphenethylamino) benzo [e][1,2,4] triazine 1-oxide (7)

Compound **7** was synthesized from **6** by a general amination method and resulted in a yellow solid with a yield of 94%. mp: 250–252 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.15 (s, 1H), 8.13 (s, 1H), 8.03 (s, 1H), 7.82–7.71 (m, 3H), 7.60 (d, J = 8.0, 1H), 7.47 (d, J = 8.0, 2H), 7.38–7.30 (m, 1H), 7.28 (s, 2H), 3.60 (d, J = 6.2, 2H), 3.01 (dd, J = 6.2, 2H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 158.80, 143.70, 142.07, 135.76, 129.25, 125.92, 124.66, 119.93, 41.92, 34.09; MS (ESI$^+$) m/z 346.10 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{15}H_{16}N_5O_3S]^+$: 346.0974, found: 346.0973.

4.1.17. 3-((4-Sulfamoylphenethyl)amino)benzo[e][1,2,4]triazine 1,4-dioxide (8)

Compound **8** was synthesized from **7** by a general oxidation method, resulting in an orange red solid with a yield of 96%. mp: 210–212 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.31 (t, J = 6.1, 1H), 8.22 (d, J = 8.1, 1H), 8.13 (d, J = 8.1, 1H), 7.97–7.89 (m, 1H), 7.75 (d, J = 8.3, 2H), 7.61–7.53 (m, 1H), 7.47 (d, J = 8.3, 2H), 7.29 (s, 2H), 3.67 (dd, J = 7.2, 6.1, 2H), 3.03 (t, J = 7.2, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 149.67, 143.19, 142.16, 138.19, 135.48, 130.07, 129.26, 127.04, 125.71, 121.13, 116.89, 41.76, 34.18; MS (ESI$^+$) m/z 362.09 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{15}H_{16}N_5O_4S]^+$: 362.0923, found: 362.0928.

4.1.18. 3-(4-Sulfamoylphenethylamino)-7,8-dihydro-6H-indeno [5,6-e][1,2,4] triazine 1-oxide (10)

Compound **10** was synthesized from **9** by using a general amination method, which resulted in a yellow solid with a 85% yield. mp: 238–240 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.95 (s, 1H), 7.82 (s, 1H), 7.74 (t, J = 10.0, 2H), 7.49–7.39 (m, 3H), 7.28 (s, 2H), 3.57 (dd, J = 13.0, 6.8, 2H), 3.02–2.90 (m, 6H), 2.11–1.99 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 157.56, 153.64, 142.79, 141.97, 130.10–127.99, 125.35, 112.78, 41.77, 32.35, 31.60, 25.25; MS (ESI$^+$) m/z 386.13 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{18}H_{20}N_5O_3S]^+$: 386.1287, found: 386.1291.

4.1.19. 3-((4-Sulfamoylphenethyl) amino)-7,8-dihydro-6H-indeno [5,6-e][1,2,4] triazine 1,4-dioxide (11)

Compound **11** was synthesized from **10** by using a general oxidation method resulting in an orange red solid with a yield of 46%. mp: 218–220 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.19 (s, 1H), 7.98 (d, J = 24.2, 2H), 7.75 (d, J = 7.8, 2H), 7.46 (d, J = 7.7, 2H), 7.29 (s, 2H), 3.65 (d, J = 6.2, 2H), 3.12–2.92 (m, 6H), 2.17–1.99 (m, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 154.56, 149.25, 145.07, 143.22, 142.15, 129.23, 125.71, 41.76, 32.74, 31.80, 25.24; MS (ESI$^+$) m/z 402.12 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{18}H_{20}N_5O_4S]^+$: 402.1236, found: 402.1234.

4.1.20. 6-(4-(Methylsulfonyl)phenyl)-N-phenyl-3-(sulfamoylamino)pyrazine-2-carboxamide (12)

Compound **12** was synthesized from commercially purchased VE-821 by using the general procedure described above, which resulted in a yellow solid with an overall yield of 58%. mp: 233–235 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 11.27 (s, 1H), 10.82 (s, 1H), 9.28 (s, 1H), 8.64 (d, J = 8.6, 2H), 8.08 (d, J = 8.6, 2H), 7.83–7.76 (m, 2H), 7.68 (s, 2H), 7.48–7.39 (m, 2H), 7.23 (dd, J = 14.0, 6.6, 1H), 3.30 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 164.59–163.27, 149.32–147.73, 144.46–143.38, 141.16, 139.57, 137.17–136.45, 128.69, 127.46, 125.15, 122.08, 43.46; MS (ESI$^+$) m/z 448.07 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{18}H_{18}N_5O_2S_2]^+$: 448.0749, found: 448.0748.

4.1.21. 5-(4-(Isopropylsulfonyl) phenyl)-3-(3-(4-((methylamino) methyl) phenyl) isoxazol-5-yl) pyrazin-2-carboxamide (13)

Compound **13** was synthesized from commercially purchased VE-822 by using the general procedure described above, which resulted in a yellow solid with an overall yield of 65%. mp: 242–244 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.94 (s, 1H), 8.38 (d, J = 8.5, 2H), 8.02 (d, J = 8.2, 2H), 7.93 (d, J = 8.5, 2H), 7.79 (s, 1H), 7.54 (d, J = 8.2, 2H), 7.20 (s, 2H), 6.96 (s, 2H), 4.17 (s, 2H), 3.54–3.38 (m, 1H), 2.58 (s, 3H), 1.17 (t, J = 14.1, 6H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 167.67, 162.00, 151.75, 142.47, 141.04, 139.53, 137.62, 135.78, 129.00, 127.17, 125.69, 124.47, 102.16, 54.22, 53.49, 34.94, 15.19; MS (ESI$^+$) m/z 543.15 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{24}H_{27}N_6O_5S_2]^+$: 543.1484, found: 543.1484.

4.1.22. 3-Methyl-4-oxo-N-(4-sulfamoylphenethyl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (15a)

Compound **15a** was synthesized from **14** by reacting it with 4-(2-aminoethyl) benzene sulfonamide using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a white solid with a yield of 50%. mp: 195–197 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.83 (s, 1H), 8.58 (t, J = 5.9, 1H), 7.74 (d, J = 8.3, 2H), 7.44 (d, J = 8.3, 2H), 7.30 (s, 2H), 3.86 (s, 3H), 3.58 (dd, J = 13.4, 6.8, 2H), 2.96 (t, J = 7.1, 2H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 159.67, 143.64, 142.05, 139.20, 134.45, 130.30, 129.14, 128.46, 125.73, 36.16, 34.78; MS (ESI$^+$) m/z 378.10 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for $[C_{14}H_{16}N_7O_4S]^+$: 378.0984, found: 378.0986.

4.1.23. 3-Methyl-4-oxo-N-(sulfamoyloxy)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (15b)

Compound **15b** was synthesized from **14** by reacting it with aminoxysulfonamide using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted

in a white solid with a yield of 41%. mp: 195–197 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.81 (s, 1H), 7.80 (s, 1H), 7.67 (s, 1H), 7.30 (s, 2H), 3.86 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 161.51, 139.16, 134.57, 130.51, 128.37, 45.72.

4.1.24. 3-Methyl-4-oxo-N-(4-sulfamoylbenzyl)-3,4-dihydroimidazo [5,1-d][1,2,3,5]tetrazine-8-carboxamide (**15c**)

Compound **15c** was synthesized from **14** by reacting it with 4-(aminomethyl) benzene sulfonamide hydrochloride using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a white solid with a yield of 88%. mp: 185–187 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.20 (t, J = 6.2, 1H), 8.87 (s, 1H), 7.77 (d, J = 8.3, 2H), 7.50 (d, J = 8.3, 2H), 7.31 (s, 2H), 4.55 (d, J = 6.3, 2H), 3.87 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 159.96, 143.85, 142.63, 142.63, 138.96, 134.69, 130.11, 129.28, 128.62, 127.68, 125.77, 41.94, 36.23; MS (ESI$^+$) m/z 364.08 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for [$C_{13}H_{14}N_7O_4S$]+: 364.0828, found: 364.0826.

4.1.25. 3-Methyl-4-oxo-N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl)-3,4-dihydroimidazo[5,1-d][1,2,3,5]tetrazine-8-carboxamide (15d)

Compound **15d** was synthesized from **14** by reacting it with 5-amino-1, 3,4-thiadiazole-2-sulfonamide hydrochloride using the general procedure for synthesizing temozolomide derivatives described above. This reaction resulted in a light yellow solid with a yield of 95%. mp: 128–130 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 8.63 (s, 1H), 8.06 (s, 1H), 7.81 (s, 1H), 7.35 (s, 2H), 3.83 (s, 3H); ^{13}C NMR (101 MHz, DMSO-d_6), δ 171.73, 170.89, 165.39, 161.04, 157.91, 139.53, 134.24, 127.88; MS (ESI$^+$) m/z 358.01 [M+H]$^+$. HRMS (ESI$^+$) [M+H]$^+$ calculated for [$C_8H_8N_9O_4S_2$]$^+$: 358.0141, found: 358.0140.

4.1.26. Synthesis of 4-(2-(3-carbamimidoylguanidino)ethyl) benzenesulfonamide hydrochloride salt (**18**)

4-(2-Aminoethyl)benzenesulfonamide **16** (0.5g, 1.0 equiv.) and cyanoguanidine **17** (0.21g, 1.0 equiv.) were suspended in *n*-butanol (5.0 mL) and treated with a 6.0 M aqueous hydrochloric acid solution (1.0 equiv., 0.4 mL). The mixture was treated at 100 °C overnight and the solvents were removed under vacuum. The residue was thereafter crystallized from isopropyl alcohol (IPA) to obtain compound **18** as a white solid with a 75% yield. mp: 154–159 °C; ^{1}H NMR (400 MHz, DMSO-d_6), δ 7.87 (d, 2H, J = 8.4, Ar-H), 7.82 (brs, 2H, exchangeable with D_2O), 7.50 (d, 2H, J = 8.4, Ar-H), 7.38 (brs, 1H, exchangeable with D_2O), 6.62 (brs, 2H, exchangeable with D_2O), 3.15 (t, 2H, J = 6.7), 2.98 (t, 2H, J = 6.7); ^{13}C NMR (101 MHz, DMSO-d_6), δ 164.0, 143.7, 142.0, 130.3, 127.0, 119.3, 61.4, 34.1; MS (ESI$^+$) m/z 285.11 [M+H]$^+$.

4.2. CA inhibition assays

To measure the CA-catalyzed CO_2 hydration activity an Applied Photophysics stopped-flow instrument was used [61]. To maintain ionic strength Na_2SO_4 (20 mM) was used with HEPES (20 mM, pH 7.5) as a buffer and Phenol red (0.2 mM) as an indicator working at the maximum absorbance of 557 nm, which was used to follow the initial rates of the CA-catalyzed CO_2 hydration for a duration of 10–100 s. To determine the kinetic parameters and inhibition constants varying CO_2 concentrations were included (1.7–17 mM). Initial velocity was assayed with at least six traces of the initial 5–10% of the reaction for each compound. Compounds were dissolved in distilled-deionized water (0.01 nM). The combined enzyme solutions and compounds were incubated for 15 min at room temperature to allow for the E-I complex formation prior to measurements. The nonlinear least-squares method of PRISM 3 was used to estimate the inhibition constants and the mean of three independent estimations is reported. The CA isoforms included are recombinant proteins obtained in house.

4.3. Biological assays

4.3.1. Cells

All cell lines used were cultured in DMEM supplemented with 10% fetal bovine serum. Canine kidney epithelial MDCK cells over-expressing human CAIX (CAIX$^+$) or a scrambled control vector (CAIX$^-$) have been described before [37] and [44]. The HCT116 constitutive CAIX knockdown cell line and its scrambled control have also been described before and were kindly provided by Professor Adrien Harris (Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe hospital, Oxford, UK) [24] [28], and [62]. Cells were exposed to hypoxic or anoxic conditions in a hypoxic chamber (MACS VA500 microaerophilic workstation, Don Whitley Scientific, UK) with 0.2 or $\leq$0.02% O_2, respectively, and 5% CO_2 and residual N_2 to upregulate and activate CAIX. Normoxic cells were grown in normal incubators with 21% O_2, 5% CO_2 at 37 °C.

4.3.2. Cell viability assays

The efficacy of the cytotoxic derivatives was compared to their respective parental compounds in cell viability assays using alamarBlue® (Invitrogen). In short, MDCK cells were seeded in 96-well plates and allowed to attach overnight. The next day plates were exposed to hypoxia and DMEM was replaced with pre-incubated hypoxic DMEM. In contrast, testing the ATRi was performed in anoxic conditions to decrease the radiosensitivity of the cells. In parallel normoxic 96-well plates were incubated in normal incubators with 21% O_2 and 5% CO_2. Compounds were dissolved in DMSO (0.5%, Sigma-Aldrich) and final concentrations were made with pre-incubated hypoxic or normoxic DMEM and added to the wells after 24 h of exposure. To test the ATR inhibitors cells were exposed to the compounds 1 h prior to irradiation and the 96-well plates were irradiated (225 kV Philips X-ray tube) with 2 Gy (normoxia) or 4 Gy (anoxia). Cells were exposed to compounds for a total of 2 h for chlorambucil and tirapazamine, or 72 h for temozolomide and the ATR inhibitors, after which medium was washed off and replaced with fresh medium. For chlorambucil, tirapazamine, and ATR inhibitor derivatives cells were allowed to grow for an additional 72 h under normoxic conditions prior to measurement, whereas cells exposed to temozolomide derivatives remained in hypoxic conditions prior to measurement. Cells were allowed to convert alamarBlue® for 2 h during normoxic conditions, which corresponds with their metabolic function and is a measure for cell viability.

4.3.3. Clonogenic assays

Clonogenic survival of MDCK cells was determined with high cell numbers to allow for CAIX-dependent extracellular acidification [44]. These cells were exposed to temozolomide or **15b** for 24 h during normoxic or hypoxic conditions after which cells were trypsinized and reseeded in triplicate with known cell numbers. Cells were allowed to grow for 7 days to form colonies that were quantified after staining and fixation with 0.4% methylene blue in 70% ethanol. Surviving fraction was normalized to vehicle (0.5% DMSO).

4.3.4. Basal respiration measurements

Oxygen Consumption Rates (OCR) were determined using the Seahorse XF96 extracellular Flux analyzer (Agilent Technologies). Cells were seeded in a XF96 cell plate with normal growth medium at an optimized cell density of 1.5 $\times$ 10^4 cells/well. Plates were placed in a 5% CO_2 incubator at 37 °C in order to let the cells attach. Subsequently cells were incubated for 18 h under hypoxic conditions (0.2% O_2). Culture medium was exchanged with DMEM

containing 25 mM D-glucose, 4 mM L-glutamine and 1 mM sodiumpyruvate (GIBCO, Thermo Fisher) 60 min prior to the assay and plates were placed in a CO_2-free incubator at 37 °C. Prior to the first injection, baseline OCR was determined using a mixing period of 5 min and a measurement period of 3 min followed by 3 loops of mixing and measuring for 3 min each. Medium containing vehicle (PBS, Lonza), Phenformin Hydrochloride (Sigma-Aldrich), or the CAIXi conjugated phenformin derivative **18** were injected followed by several mixing and measurements cycles. Subsequently cells were washed with PBS and lysed in a 0.05% SDS (Sigma-Aldrich) solution. Protein quantification for normalization purposes was performed using Pierce™ BCA Protein Assay Kit (Thermo Fisher).

4.3.5. Western blot

To validate CAIX expression in the genetically modified cell lines protein immunoblotting was performed after 24 h of hypoxia exposure (0.2% O_2) as described previously [43]. Primary antibodies used included the anti-CAIX M75 antibody (kindly provided by Professor Silvia Pastorekova, Institute of Virology, Slovak Academy of Science, Slovak Republic), and anti-β-actin (MP Biomedicals, #691001) as a reference protein.

4.3.6. Statistical analyses

GraphPad Prism (version 5.03) was used for all statistical analyses. For the cytotoxic compounds IC_{50} values were estimated with the curve of the log(inhibitor) vs. normalized response (Variable slope). Means between groups were compared using unpaired t-tests, where $p < 0.05$ indicated statistical significance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2016.10.037.

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