Selective inhibition of carbonic anhydrase IX decreases cell proliferation and induces ceramide-mediated apoptosis in human cancer cells.


Source

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Abstract

Recently, carbonic anhydrase (CA) inhibitors have been proposed as a potential new class of antitumor agents. The aim of this study was to evaluate the antitumor activity of three CA inhibitors, namely acetazolamide (AZ) and two newly synthesized aromatic sulfonamides with high affinity for CA IX, 2-(4-sulfamoylphenyl-amino)-4,6-dichloro-1,3,5-triazine (TR1) and 4-[3-(N,N-dimethylaminopropyl) thioiiodophenyl sulfonyl aminoethyl] benzenesulfonamide (GA15), against human tumor cells. The effects of AZ, TR1, and GA15 on cell proliferation and apoptosis were evaluated in CA IX-positive HeLa and 786-O cells and CA IX-negative 786-O/von Hippel-Lindau (VHL) cells. We also investigated whether the potential antitumor activity of these molecules might be mediated by an increase in ceramide production. AZ, TR1, and GA15 could significantly reduce cell proliferation and induce apoptosis in HeLa and 786-O cells. Moreover, all three inhibitors could decrease intracellular pH (pH(i)) and increase ceramide production in the same cells. Treatment with the ceramide synthase inhibitor fumonisin B1 prevented the apoptotic effects of the three CA inhibitors. In all experiments, the effects of aromatic sulfonamides were more pronounced than those of AZ. The three inhibitors did not show any antitumor activity in CA IX-negative 786-O/VHL cells and failed to lower pH(i) or increase intracellular ceramide levels in the same cells. In conclusion, CA inhibition can decrease cell proliferation and induce apoptosis in human tumor cells. The ability of CA inhibitors to decrease pH(i) might trigger cell apoptosis through mediation of ceramide synthesis. Activation of this apoptotic cascade probably is mediated by inhibition of the CA IX isoform.

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Selective Inhibition of Carbonic Anhydrase IX Decreases Cell Proliferation and Induces Ceramide-Mediated Apoptosis in Human Cancer Cells

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Abstract

Recently, carbonic anhydrase (CA) inhibitors have been proposed as a potential new class of antitumor agents. The aim of this study was to evaluate the antitumor activity of three CA inhibitors, namely acetazolamide (AZ) and two newly synthesized aromatic sulfonamides with high affinity for CA IX, 2–(4-sulfamoylphenyl–amino)–4,6-dichloro–1,3,5–triazine (TR1) and 4–[3–(N,N-dimethylaminopropyl)thioreidophenylsulfonylaminoethyl]benzenesulfonamide (GA15), against human tumor cells. The effects of AZ, TR1, and GA15 on cell proliferation and apoptosis were evaluated in CA IX–positive HeLa and 786-O cells and CA IX–negative 786-O/von Hippel–Lindau (VHL) cells. We also investigated whether the potential antitumor activity of these molecules might be mediated by an increase in ceramide production. AZ,
TR1, and GA15 could significantly reduce cell proliferation and induce apoptosis in HeLa and 786-O cells. Moreover, all three inhibitors could decrease intracellular pH (pHi) and increase ceramide production in the same cells. Treatment with the ceramide synthase inhibitor fumonisin B1 prevented the apoptotic effects of the three CA inhibitors. In all experiments, the effects of aromatic sulfonamides were more pronounced than those of AZ. The three inhibitors did not show any antitumor activity in CA IX–negative 786–O/VHL cells and failed to lower pHi or increase intracellular ceramide levels in the same cells. In conclusion, CA inhibition can decrease cell proliferation and induce apoptosis in human tumor cells. The ability of CA inhibitors to decrease pHi might trigger cell apoptosis through mediation of ceramide synthesis. Activation of this apoptotic cascade probably is mediated by inhibition of the CA IX isoform.

**Introduction**

The carbonic anhydrase (CA) family includes 16 catalytically active zinc metalloenzymes involved in the reversible hydration of carbon dioxide to bicarbonate and a proton (Supuran, 2004). These isoforms differ mainly in their catalytic activity, tissue distribution, and subcellular localization. Indeed, there are cytosolic, mitochondrial, secreted, and membrane–bound isoforms (Pastorekova et al., 2004). Among the latter, CA IX and CA XII have been found to be overexpressed in a wide variety of human tumors and involved in cancer aggressiveness and progression (Robertson et al., 2004; Pastorekova et al., 2007). Recent studies have revealed that CA IX is a downstream gene either induced by hypoxia through activation of hypoxia–inducible factor–1 (Wykoff et al., 2000) or constitutively expressed in von Hippel–Lindau (VHL)–defective cells (Gnarra et al., 1994). Several clinical studies have shown a clear relationship between high CA IX levels and poor prognosis (Chia et al., 2001; Giatromanolaki et al., 2001). A possible role for this enzyme has been suggested in the adaptation of tumor cells to hypoxic conditions and in tumor cell progression (Dorai et al., 2006; Chiche et al., 2010). In particular, CA IX has been proposed to promote tumor cell growth by countereacting hypoxia–induced acidosis through the alkalinization of the intracellular pH (pHi) (Chiche et al., 2009). Taken together, these findings point to CA IX as a useful therapeutic target against cancer (Thiry et al., 2006).

A possible targeted approach directed at CA IX would be to inhibit its enzymatic activity with chemical inhibitors (Supuran, 2008). Recent studies showed that CA inhibitors, such as acetazolamide (AZ) (Parkkila et al., 2000) or AZ–based new compounds (Ahlskog et al., 2009; Morsy et al., 2009), were able to inhibit the invasive capacity of renal cancer cells in vitro or the growth of various tumor types, alone or in combination with other anticancer agents. However, the molecular mechanisms underlying this antitumor activity and the precise role of the CA IX isoform are still not well understood.

The aim of this study was to evaluate the effects of CA inhibitors, namely AZ and two newly synthesized aromatic sulfonamides with high affinity for CA IX, 2–(4–sulfamoylphenylamino)–4,6–dichloro–1,3,5–triazine (TR1) and 4–[3–(N,N–dimethylaminopropyl)thioeiridophenylsulfonylaminoethyl] benzenesulfonamide (GA15), on cell proliferation and apoptosis in two human cancer cell lines and correlate these findings with CA IX expression. Moreover, we sought to investigate the molecular mechanisms underlying the potential antitumor effect of CA inhibitors.

**Previous Section**

**Next Section**
Materials and Methods

Cell Culture and Drugs.

Experiments were performed on the human renal carcinoma cell lines 786–O and 786–O/VHL and the cervical cancer cell line HeLa. 786–O cells possess a germ-line VHL frameshift mutation at codon 104, whereas 786–O/VHL cells were permanently transfected by using the pQVHL−II retroviral expression plasmid encoding the wild-type human VHL gene. Both 786–O cell lines were a kind gift from Dr. Donald Bottaro (National Cancer Institute, Bethesda, MD). The HeLa cells were purchased from Interlab Cell Line Collection, Genoa, Italy. Acetazolamide (5-acetamido-1,3,4-thiadiazole-2-sulfonamide) and the newly synthesized aromatic sulfonamides TR1 and GA15 were supplied by C.T.S. These CA inhibitors have been shown to induce a very potent, reversible inhibition of the membrane-bound isozyme CA IX compared with traditional inhibitors. The $K_i$s of AZ, TR1, and GA15 for CA IX are 25, 0.15, and 6.1 nM, respectively (Garaj et al., 2004; Puccetti et al., 2005; Supuran, 2008). The chemical structures of the three inhibitors tested are shown in Fig. 1.

The ceramide synthase inhibitor fumonisin B1 (FB1) was purchased from Cayman Chemical (Ann Arbor, MI). 786–O, 786–O/VHL, and Hela cells were maintained in a humidified 5% CO$_2$ atmosphere chamber at 37°C in Dulbecco's minimum essential medium supplemented with 10% fetal calf serum (FCS), L-glutamine, and a penicillin-streptomycin mixture. For 786–O/VHL cells, culture medium was supplemented with Geneticin (G418) (200 μg/ml). For all experiments, HeLa cells were seeded at high density ($1.5 \times 10^6$ cells per 3.5-cm dish) to induce pericellular hypoxia and thus stimulate CA IX expression.

Western Blot Analysis.

Representative Western blots were carried out to show either the expression of CA IX in tumor cells cultured in complete medium or phosphorylation of the p38 mitogen-activated protein kinase (MAPK) after cell treatment with TR1 for 24 h. HeLa, 786–O, and 786–O/VHL cells were grown at confluence, washed in PBS, and lysed with radioimmunoprecipitation assay buffer. Seventy micrograms of total proteins from cultured cells was separated on 10% sodium and 12.5% SDS-polyacrylamide gel electrophoresis in 1× Tris–glycine–SDS running buffer (MP Biomedicals, Solon, OH) and transferred electrophoretically to polyvinylidene difluoride membranes (GE Healthcare Bio–Sciences (Little Chalfont, UK)).
Buckinghamshire, UK). The membranes were incubated in 10% nonfat dried milk for 10 min and then probed with CA IX primary antibody (1:50) (supplied by S.P.) or polyclonal phospho-p38 MAPK antibody (1:1000) (Cell Signaling Technology, Danvers, MA) for 1 h at 37°C. After rinsing with PBS, the membranes were incubated for 1 h at 37°C with horseradish peroxidase–linked secondary anti-mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA) (1:400). The membranes were washed again, and the blots were visualized with enhanced chemiluminescence reagents (GE Healthcare) and immediately photographed with a charge–coupled device camera. The membrane was also blotted with anti–human α–tubulin monoclonal antibody (1:5000) (Sigma–Aldrich, St. Louis, MO).

Reverse Transcription-Polymerase Chain Reaction.

HeLa, 786-O, and 786-O/VHL cells were grown at confluence in 10-cm Petri dishes. Total RNA was isolated according to the manufacturer’s protocol (NucleoSpin RNA II; Macherey–Nagel, Bethlehem, PA) and reverse–transcribed (OmniScript; QIAGEN, Milan, Italy) by using random primers. The RNA purity was validated by PCR and gel electrophoresis by using primers for the GAPDH gene. A typical PCR (QIAGEN) was prepared for amplification of CA IX mRNA, and calibration was performed by amplification of the same cDNA sample with primers for GAPDH mRNA. Primer sequences were as follows: CA IX, 5′–TAAGCAGCTCCACACCCCTCT–3′ (sense) and 5′–TCTCATCTGCACAAAGGAACG–3′ (antisense), product size 250 base pairs; GAPDH, 5′–GAGTCAACGGATTTGGTCGT–3′ (sense) and 5′–TTGATTTTGGAGGATCTCG–3′ (antisense), product size 238 base pairs. Amplification was performed as follows: 30 s of denaturation at 94°C, 30 s of annealing at 56°C, and 1 min of extension at 72°C for 30 cycles. Amplification products were highlighted with ethidium bromide on 1.5% agarose gel. The intensities of the bands corresponding to the amplificates were quantified by densitometric analysis.

Cell Viability Assay.

The cytotoxic effects of different concentrations of the CA inhibitors AZ, TR1, and GA15 were evaluated by the 3-(4,5–dimethylthiazol–2-yl)–2,5–diphenyltetrazolium bromide (MTT) test as described previously (Cianchi et al., 2004). Experiments were performed in triplicate, and data were expressed as the percentage of treated cells compared with control cells.

Cell Proliferation Assays.

Cell counts were performed by plating subconfluent cells in 24–well plates and allowing them to adhere overnight. After starvation, 50 μM AZ, 25 μM TR1, and 25 μM GA15 were added in medium containing 10% FCS. At the indicated time point, cells were trypsinized and stained with Trypan blue. Only the cells that excluded Trypan blue were counted under the microscope. Determinations were performed in triplicate, and data were expressed as cell number per milliliter and compared with control cells.

DNA synthesis was estimated by [H]thymidine incorporation into cellular DNA as described previously (Cianchi et al., 2004). Experiments were performed in triplicate, and data were expressed as dpm per well in treated cells compared with control cells.

Caspase-3 Activity Determination.
The activity of caspase-3 was determined by using a fluorescent substrate as described previously (Cianchi et al., 2006). Determinations were done in triplicate, and data were expressed as arbitrary units per milligram of protein.

**Flow Cytometry.**

Cells under confluent conditions were treated with serum-free medium containing test drugs. For determination of apoptosis, after 24 h culture cells were harvested by Ca\(^{2+}\)-Mg\(^{2+}\)-free PBS-EDTA, washed twice in saline solution (0.9% NaCl), and then stained with a combination of AnnexinV-fluorescein isothiocyanate (FITC) and 7-amino-actinomycin D (7-AAD) (Apoptosis Detection Kit; Instrumentation Laboratory, Milan, Italy) according to the manufacturer's instructions. In brief, cells were suspended in the supplied binding buffer at a concentration of 1 × 10\(^6\)/ml, and 100 μl (1 × 10\(^4\) cells) was transferred to the test tube. Cells of each group were incubated with saturating concentrations of AnnexinV-FITC and 7-AAD for 10 min at 4°C in the dark. After the addition of 400 μl of the binding buffer to each sample, cells were immediately analyzed by a flow cytometer (Coulter XL; Beckman Coulter, Fullerton, CA). This method allows the distinction among early apoptotic cells (FITC\(^+\)/7-AAD\(^-\)), late apoptotic cells (FITC\(^+\)/7-AAD\(^+\)), and necrotic cells (FITC\(^-\)/7-AAD\(^+\)). In this study both apoptotic subpopulations were considered to represent the total fraction of apoptotic cells. Determinations were performed in triplicate, and data on apoptotic cells were expressed as percentage of total cells counted.

For determination of ceramide, cells were stained for 1 h at 4°C with 1 μg/ml anticeramide antibody (Alexis Laboratories, San Diego, CA) in PBS containing 1% FCS at a dilution of 1:5 after permeabilization with 0.1% saponin. After three washes with PBS/1% FCS, cells were stained with polyclonal phycoerythrin-conjugated goat anti-mouse Ig-specific antibody (BD Biosciences Pharmingen, Hamburg, Germany) in PBS/1% FCS at a dilution of 1:50 for 30 min. Unbound secondary antibody was removed by washing the cells twice with PBS/1% FCS, and samples were analyzed by flow cytometric analysis on a Coulter XL flow cytometer (Beckman Coulter). Phycoerythrin-fluorescence intensity was measured in a FL-2 fluorescence detector. Determinations were performed in triplicate, and data were expressed as mean fluorescence intensity of positively stained cells.

**Immunocytochemistry.**

Cell expression of ceramide and nitrotyrosine (NT) was determined by immunocytochemistry. Preconfluent Hela, 786-O, and 786-VHL cells were grown on glass coverslips, and 24 h after they were seeded 25 μM TR1 was added to the cells' medium. Twenty–four hours after treatment the cells were fixed in 3% formaldehyde for 2 h, rinsed in PBS, and incubated for 5 min with 0.3% H\(_2\)O\(_2\) (v/v) in 60% methanol (v/v) to quench endogenous peroxidase. The samples were immunolabeled overnight at 4°C with either mouse monoclonal anticeramide (Sigma-Aldrich; 1:50) or rabbit polyclonal anti-NT antiserum (Millipore, Billerica, MA; 1:100). The immune reaction was revealed by biotin–conjugated rabbit anti–mouse or goat anti–rabbit IgG (Vector Laboratories, Burlingame, CA; 1:200) followed by incubation with avidin–biotin complex (Vector Laboratories; 1:200). Negative controls were carried out by substituting the primary antiserum with nonimmune mouse or rabbit serum as appropriate. Cells were counterstained with hematoxylin before mounting. Photomicrographs were randomly taken with a digital camera applied to the light microscope. Intensity of immunocytochemical staining was classified as absent/weak or marked by optical examination.
**pH Determination.**

Measurement of pH for HeLa, 786-O, and 786-O/VHL cells was assessed as described previously (Bond and Varley, 2005). In brief, pH was measured by flow cytometry by incubating 1 × 10^6 cells with a final concentration of 10 μM carboxy–semaphorhodfluor–1–acetoxy methyl ester (SNARF) (Invitrogen, Carlsbad, CA) for 15 min at 37°C. After incubation, the cells were washed twice with PBS. Because the addition of bicarbonate can affect pH, all incubations and cell washes were performed in bicarbonate–free buffer. SNARF was excited at 488 nm and emitted at 580 and 640 nm. The ratio of the emission wavelengths at 640/580 nm was used to estimate the pH from a calibration curve. The calibration curve was produced by using the nigericin clamp technique. After SNARF labeling, aliquots of cell suspensions were resuspended in a high K⁺–containing buffer at a specific pH (usually 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8). The cell suspensions were then clamped at the specific pH value by the addition of 0.03 μM nigericin (Invitrogen). Nigericin is an ionophore that allows the exchange of H⁺ for K⁺ ions by abolishing the pH gradient across the cell membrane. When the internal and external K⁺ concentrations are approximately the same, the pH rapidly equilibrates to the pH of the bathing solution. A total of 50,000 events were acquired on a Coulter XL flow cytometer (Beckman Coulter). The fluorescent ratio values obtained for each pH point were used for the calibration curve obtained with Prism software (GraphPad Software, Inc., San Diego, CA), from which pH values of the samples were determined. Determinations were performed in quintuplicate.

**Statistical Analysis.**

Cell viability and proliferation, caspase–3 activity, flow cytometric determination of cell apoptosis and ceramide synthesis, and pH determination are expressed as means ± S.E. The differences in these parameters were compared through the paired–value Wilcoxon test or the Mann–Whitney test as appropriate. All the probabilities (P) resulted from two–sided statistical tests; P < 0.05 is considered statistically significant.

**Results**

**CA IX Expression in Tumor Cells.**

We determined the expression of CA IX in HeLa, 786–O, and 786–O/VHL cells by reverse transcription–PCR and Western blot analysis. As expected on the basis of VHL gene status, CA IX mRNA and protein were expressed in 786–O cells, whereas they were absent in 786–O/VHL cells (Fig. 2). We also confirmed CA IX expression in dense HeLa cell cultures (Fig. 2) in accordance with previously published data (Chrastina, 2003).
Effects of AZ, TR1, and GA15 on Cell Viability.

Cell viability was determined by the MTT assay. Treatment with AZ, TR1, or GA15 could decrease cell viability in a dose-dependent manner in CA IX-positive HeLa and 786-O cells (Fig. 3, A and B). AZ significantly inhibited cell viability in the two cell lines at concentrations ≥50 μM, whereas both TR1 and GA15 had significant effects at concentrations ≥25 μM. In CA IX-negative 786-O/VHL cells, administration of AZ, TR1, or GA15 failed to reduce cell viability (Fig. 3C). On this basis, all subsequent experiments were performed with 50 μM AZ and 25 μM TR1 and GA15. These drug concentrations were within the same range of CA inhibitor concentrations that have been shown to reduce tumor cell migration and invasion (Parkkila et al., 2000; Ahlskog et al., 2009; Chiche et al., 2010).

Effects of AZ, TR1, and GA15 on Cell Proliferation.

Cell proliferation was assessed by both cell count and [3H]thymidine uptake. Tumor cell proliferation was significantly inhibited by AZ, TR1, or GA15 administration in HeLa and 786-O cells (Fig. 4, A–D). The antiproliferative activity of either TR1 or GA15 was higher than that of AZ (Fig. 4, A–D). In 786-O/VHL cells, the three inhibitors could not decrease cell proliferation (Fig. 4, E and F).
Fig. 4.

Cell count and [3H]thymidine incorporation. Effects of 50 μM AZ, 25 μM TR1, and 25 μM GA15 on cell proliferation in HeLa (A and B), 786-O (C and D), and 786-O/VHL (E and F) cells. Means of three different experiments are shown. Bars indicate ± S.E. *, $P < 0.05$ and **, $P < 0.01$, significant decrease compared with control.

Effects of AZ, TR1, and GA15 on Cell Apoptosis.

We further questioned whether CA inhibitors could influence the apoptotic process in cancer cells. We first evaluated the involvement of these molecules in inducing the early events of the apoptotic process, i.e., caspase-3 activation. AZ and, more efficiently, TR1 and GA15 significantly increased caspase-3 activity in HeLa and 786-O cells (Fig. 5, A and C). Flow cytometric detection of cell apoptosis confirmed our findings on caspase-3 activation. We found a significant increase in the number of apoptotic cells after treatment with AZ, TR1, and GA15 in HeLa and 786-O cells (Fig. 5, A and C). Both TR1 and GA15 determined a more pronounced apoptotic effect than did AZ (Fig. 5, A-D). In 786-O/VHL cells, AZ, TR1, or GA15 administration did not influence apoptotic activity (Fig. 5, E and F).
Caspase-3 activity and flow cytometric detection of cell apoptosis. Effects of 50 μM AZ, 25 μM TR1, and 25 μM GA15 on cell apoptosis in HeLa (A and B), 786-O (C and D), and 786-O/VHL (E and F) cells. Means of three different experiments are shown. Bars indicate ± S.E. *, P < 0.05 and **; P < 0.01, significant increase compared with control.

**Effects of AZ, TR1, and GA15 on pH.**

CAs have been shown to have an important role in protecting cytosol from acidification, thus allowing cell survival and proliferation even in hypoxic conditions (Swietach et al., 2007). Therefore, we investigated whether CA inhibition might interfere with pH in cancer cells. Treatment of HeLa and 786-O cells with CA inhibitors determined a significant decrease in pH (Fig. 6, A and B). The highest effect was obtained by either TR1 or GA15 administration (Fig. 6, A and B). None of the CA inhibitors tested significantly decreased pH in 786-O/VHL cells (Fig. 6C).

**Fig. 6.**

Flow cytometric measurement of pH. Effects of 50 μM AZ, 25 μM TR1, and 25 μM GA15 on pH in HeLa (A), 786-O (B), and 786-O/VHL (C) cells. Data are expressed as means of five independent experiments. Bars indicate ± S.E. *, P < 0.05 and **, P < 0.01, significant decrease compared with control.

**Effects of AZ, TR1, and GA15 on Ceramide Production.**

Ceramide is a ubiquitous sphingolipid messenger that plays an important role in the control of tumor cell fate (Ogretmen and Hannun, 2004). Moreover, caspase-3 activation is known to be a downstream target of ceramide accumulation (Cianchi et al., 2009). Therefore, we investigated whether treatment with CA inhibitors could increase intracellular ceramide production. In agreement with apoptosis results, treatment with AZ and, more efficiently, with TR1 and GA15 significantly increased ceramide production in HeLa and 786-O cells (Fig. 7, A and C). These results were confirmed by immunocytochemical detection of ceramide in the two cell lines. We found that treatment of HeLa and 786-O cells with TR1, the most effective compound in reducing pH, determined an increase in ceramide cell expression compared with controls (Fig. 8, A–D). The apoptotic effect of the three inhibitors was significantly prevented by the administration of the ceramide synthase inhibitor FB1 in the two cell lines (Fig. 7, B and D). AZ, TR1, and GA15 did not increase ceramide production...
in 786–O/VHL cells (Figs. 7E and 8E and F). FB1 administration in the same cells did not have any effect on apoptotic activity after CA inhibitor administration (Fig. 7F).

**Fig. 7.**
Flow cytometric detection of ceramide and cell apoptosis. A, C, and E, effects of 50 μM AZ, 25 μM TR1, and 25 μM GA15 on ceramide production in HeLa (A), 786–O (C), and 786–O/VHL (E) cells. B, D, and F, effects of 50 μM AZ, 25 μM TR1, and 25 μM GA15 (solid bars) and 10 μM ceramide synthase inhibitor FB1 (lined bars) on cell apoptosis in HeLa (B), 786–O (D), and 786–O/VHL (F) cells. Means of three different experiments are shown. Bars indicate ± S.E. *, \( P < 0.05 \) and **, \( P < 0.01 \), significant increase compared with control; §, \( P < 0.05 \), significant decrease compared with AZ, TR1, or GA15 treatment.

**Fig. 8.**
Immunocytochemical detection of ceramide. Effects of 25 μM TR1 on ceramide production in HeLa (A and B), 786–O (C and D), and 786–O/VHL (E and F) cells. Brownish immunoreactivity in the cytoplasm of the tumor cells was classified as absent/weak in A, C, E, and F and marked in B and D. Results are representative of at least three experiments. Hematoxylin counterstain was used. Original magnification: \( \times 25 \).
Effects of TR1 on p38 MAPK Activation and NT Production.

p38 MAPK is known as a downstream mediator of ceramide-induced apoptosis (Chen et al., 2008). We found that treatment of HeLa and 786–O cells with TR1 resulted in a significant increase in the phosphorylation of p38 MAPK (Fig. 9). TR1 did not influence phosphorylation of p38 MAPK in 786–O/VHL cells (Fig. 9). It is also known that ceramide stimulates formation of nitrooxidative species, including peroxynitrite, that can induce cell apoptosis in several pathophysiological conditions (Cuzzocrea et al., 2008). We found that treatment of HeLa and 786–O cells with TR1 increases cell expression of NT, the stable product of the action of peroxynitrite on tyrosine–containing proteins (Fig. 10, A–D). No significant effect on NT expression was found in 786–O/VHL cells (Fig. 10, E and F).

![Figure 9](image_url)

**Fig. 9.**
Western blot analysis. Effect of 25 μM TR1 on p38 phosphorylation (phospho–p38) in HeLa, 786–O, and 786–O/VHL cells.

![Figure 10](image_url)

**Fig. 10.**
Immunocytochemical detection of NT. Effects of 25 μM TR1 on NT production in HeLa (A and B), 786–O (C and D), and 786–O/VHL (E and F) cells. Brownish immunoreactivity in the cytoplasm of the tumor cells was classified as absent/weak in A, C, E, and F and marked in B and D. Results are representative of at least three experiments. Hematoxylin counterstain was used. Original magnification: ×25.
Recently, CA inhibitors have been proposed as a potential new class of antitumor agents (Supuran and Scozzafava, 2002; Supuran, 2008). CA IX has been shown to be up–regulated in a number of human cancer tissues as a consequence of either hypoxia–induced or constitutive hypoxia–inducible factor–1 activation, whereas it is not expressed in their normal counterparts, except for gastric mucosa (Robertson et al., 2004; Pastorekova et al., 2007). Although these characteristics make CA IX an interesting target for novel approaches in anticancer therapy, the exact role of CA IX in tumor growth and progression is still unknown. It has been hypothesized that CA IX activity contributes to the environmental acidification of hypoxic tumors through the decrease in extracellular pH (Swietach et al., 2007, 2009), and low pH has been associated with tumorigenic transformation, chromosomal rearrangements, extracellular matrix breakdown, tumor cell migration, and invasion (Swietach et al., 2009). Some new CA inhibitors with a high affinity for the CA IX isoform have been shown to have potential antitumor activity in several tumor cell lines (Morsy et al., 2009). However, the antiproliferative effect of CA inhibitors might be caused by their effect not only on CA IX but on other CA isozymes, in particular the other tumor–associated isoforms, CA XII and CA II (Thiry et al., 2006).

In this study, we evaluated for the first time the antitumor effect of two newly synthesized aromatic sulfonamides, TR1 and GA15, in two CA IX–positive cancer cell lines. These new CA inhibitors have been previously shown to have higher selectivity toward the membrane–bound, tumor–associated isoform CA IX compared with traditional CA inhibitors such as AZ (Garaj et al., 2004; Puccetti et al., 2005). We found that AZ, TR1, and GA15 can significantly reduce cell proliferation and increase apoptosis in HeLa and 786–O cells. It is noteworthy that we demonstrated that both TR1 and GA15 are more effective than AZ. These findings clearly show that inhibition of CA activity can induce a significant antitumor effect and that this effect is augmented by selective inhibition of CA IX. Restoration of active VHL gene in 786–O cells and, consequently, down–regulation of CA IX expression completely abolish the antitumor activity of AZ, TR1, and GA15 in 786–O/VHL cells. These results strongly suggest that inhibition of the CA IX isoform may have a pivotal role in mediating the antitumor effect of CA inhibitors. Parkkila et al. (2000) have previously shown that AZ can inhibit the invasive capacity of renal cancer cells in vitro. This effect was highest in the cell lines expressing high levels of CAII and CAXII, whereas the only CAIX–positive cell line, A–498, showed the least response to AZ. On this basis, Parkkila et al. (2000) concluded that this effect may be attributable to the inhibition of the CAII and CAXII isoforms. The difference between their findings and ours may be explained by the different cell lines used, the different antitumor effects tested (invasion versus proliferation/apoptosis), and the low affinity of AZ for CA IX.

We sought to investigate the possible molecular mechanisms underlying the antitumor activity of CA inhibitors. It is known that tumors, in contrast to normal tissues, have a very high capacity to produce lactic acid, which, when combined with inadequate vasculature, causes acidosis (Gatenby and Gillies, 2004; Swietach et al., 2007). However, the pH lies between 7.0 and 7.4 in most tumors, similar to nontumor cells, whereas extracellular pH is typically lower, between 6.9 and 7.0 (Vaupel et al., 1990). Low extracellular pH seems to provide a selective advantage for tumor growth and development in human tumors (Vaupel et al., 1990). On the contrary, intracellular acidosis poses a threat to cell survival; therefore, maintenance of a normal pH is a key cellular strategy to protect against apoptotic death and permit tumor cell proliferation (Pastorekova et al., 2007; Swietach et al., 2007). Recent
Experimental evidence has demonstrated the pivotal role of CAs in keeping pH alkaline (Swietach et al., 2009). Therefore, it has been suggested that CA inhibitors can exert their antitumor effect by altering pH (Supuran and Scozzafava, 2002). Here, we demonstrate that AZ, TR1, and GA15 can significantly decrease pH in the two CA IX-positive cancer cells. Because Barrier et al. (2005) have shown that acidosis can stimulate de novo ceramide synthesis in rat cortical astrocytes, we investigated whether CA inhibition could interfere with the production of this important apoptosis-associated sphingolipid. Now, we show for the first time that AZ and, more efficiently, TR1 and GA15 can stimulate ceramide production in HeLa and 786-O cells and that the apoptotic effect of these inhibitors was reversed by the administration of the ceramide synthase inhibitor FB1. We also show that the administration of TR1 can both activate the p38 MAPK signaling pathway and increase the synthesis of peroxynitrite, two well known downstream mediators of ceramide-induced apoptosis (Chen et al., 2008; Cuzzocrea et al., 2008). It is noteworthy that administration of the three CA inhibitors did not interfere with both pH and ceramide production in the CA IX-negative 786-O/VHL cells. Moreover, TR1 administration did not increase phosphorylation of p38 MAPK and peroxynitrite synthesis in 786-O/VHL cells. Altogether, these findings suggest that the potential antitumor effect of CA inhibition may be linked to intracellular acidification, which eventually leads to ceramide de novo synthesis, up-regulation of p38 MAPK activity, stimulation of nitro-oxidative stress, and tumor cell apoptosis. In particular, we show for the first time that activation of this apoptotic cascade is mediated mainly by inhibition of the CA IX isoform in those cells that express this enzyme.

In conclusion, our study demonstrates that CA inhibition can decrease cell proliferation and induce apoptosis in cancer cells. One of the most relevant mechanisms potentially involved in this antitumor effect is the ability of CA inhibitors to induce intracellular acidosis by suppressing CA IX activity. Indeed, lowering the pH might trigger tumor cell apoptosis through activation of ceramide-controlled signaling pathways. These findings point to CA IX inhibition as a useful tool in the clinical management of those human tumors that highly express this enzyme.

References


2. CrossRefMedline
1. Barrier L,
2. Ingrand S,
3. Piriou A,
4. Touzalin A,
5. Fauconneau B


 CrossRef Medline

3. 
1. Bond J,
2. Varley J


 Medline

4. 
1. Chen CL,
2. Lin CF,
3. Chang WT,
4. Huang WC,
5. Teng CF,
6. Lin YS


 Abstract/FREE Full Text

5. 
1. Chia SK,
2. Wykoff CC,
3. Watson PH,
4. Han C,
5. Leek RD,
6. Pastorek J,
7. Gatter KC,
8. Ratcliffe P,
9. Harris AL


Abstract/FREE Full Text

11. Cianchi F, Cortesini C, Magnelli L, Fanti E, Papucci L, Schiavone N, Messerini L, 
Vannacci A, Capaccioli S, Perna F, et al. (2006) Inhibition of 5-lipoxygenase by MK886 
augments the antitumor activity of celecoxib in human colon cancer cells. Mol Cancer Ther 5:2716–
2726.

Abstract/FREE Full Text

12. Cuzzocrea S,
2. Di Paola R,
3. Genovese T,
4. Mazzon E,
5. Esposito E,
6. Crisafulli C,
7. Bramanti P,
8. Salvemini D


Abstract/FREE Full Text

13. —
1. Dorai T,
2. Sawczuk I,
3. Pastorek J,
4. Wiernik PH,
5. Dutcher JP


CrossRefMedline

14. —
1. Garaj V,
2. Puccetti L,
3. Fasolis G,
4. Winum JY,
5. Montero JL,
6. Scozzafava A,
7. Vullo D,
8. Innocenti A,
9. Supuran CT


CrossRefMedline

15. —
1. Gatenby RA,
2. Gillies RJ

CrossRefMedline

16. 
   1. Giatromanolaki A,
   2. Koukourakis MI,
   3. Sivridis E,
   4. Pastorek J,
   5. Wykoff CC,
   6. Gatter KC,
   7. Harris AL


Abstract/FREE Full Text

17. 
   1. Gnarra JR,
   2. Tory K,
   3. Weng Y,
   4. Schmidt L,
   5. Wei MH,
   6. Li H,
   7. Latif F,
   8. Liu S,
   9. Chen F,
   10. Duh FM,
   11. et al


CrossRefMedline

18. 
   1. Morsy SM,
   2. Badawi AM,
   3. Cecchi A,
   4. Scozzafava A,
   5. Supuran CT

(2009) *Carbonic anhydrase inhibitors. Biphenylsulfonamides with inhibitory action towards the transmembrane, tumor-associated isozymes IX possess*

CrossRef Medline


CrossRef Medline


Abstract/FREE Full Text


CrossRef Medline


1. Supuran CT,
2. Scozzafava A


CrossRef

28. Swietach P, Patiar S, Supuran CT, Harris AL, Vaughan-Jones RD


Abstract/FREE Full Text

29. Swietach P, Vaughan-Jones RD, Harris AL


CrossRefMedline

30. Thiry A, Dogné JM, Masereel B, Supuran CT


CrossRefMedline

31. Vaupel P, Kallinowski F, Okunieff P

Medline

32. 1
1. Wykoff CC,
2. Beasley NJ,
3. Watson PH,
4. Turner KJ,
5. Pastorek J,
6. Sibtain A,
7. Wilson GD,
8. Turley H,
9. Talks KL,
10. Maxwell PH,
11. et al


Abstract/FREE Full Text