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Novel Metronidazole-Sulfonamide Derivatives as Potent and Selective Carbonic Anhydrase Inhibitors: Design, Synthesis and Biology Analysis

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Abstract

Metronidazole-sulfonamide derivatives **4a**-**4l**, a new class of human carbonic anhydrase inhibitors (hCA), were designed, synthesized, isolated**,** and evaluated for their ability to inhibit the enzymatic activity of the physiologically dominant isozymes hCA II and the tumor-associated isozyme hCA IX (h = human). Many of these compounds inhibited CA II and IX in the range of 16-137 and 38-169 nM, respectively. Among all the compounds, the most potent inhibitor against hCA II and IX were compounds **4b** (IC_{50} = 16 nM) and **4h** (IC_{50} = 38 nM). Conversely compounds **4e** and **4d** displayed the most potent growth inhibitory activity against B16-F10 and MCF-7 cancer cell line in vitro repectively, with an IC_{50} value of 150 nM for B16-F10 and 6.5 nM for MCF-7. These metronidazole-sulfonamide derivatives may prove interesting lead candidates to target tumor-associated CA isozymes, wherein the CA domain is located extracellularly. All the new compounds were evaluated for cytotoxicity against human macrophage by MTT assay.

Introduction：

In many types of solid tumors, hypoxia is an advantageous condition for the culture of pluripotent stem cells. It induces the activation of a transcription factor, aptly named hypoxia-inducible factor (HIF[a]).[1] High levels of HIF regulate a signaling cascade which adapts cellular functions to allow solid tumor cells to not only survive hypoxia but to proliferate and metastasize. Among them are the carbonic anhydrases (CAs, EC 4.2.1.1) involved in pH homeostasis, ion transport, and biosynthetic processes.[2] CAs are Zn (II) metal-loenzymes involved in pH buffering of extra- and intracellular spaces by catalyzing the reversible hydration of carbon dioxide and water to bicarbonate and a proton: $CO_2 + H_2O \leftrightarrow HCO^{3-} + H^+$.[3] This reaction is known to regulate a broad range of physiological functions by the respiration and transport of CO_2/HCO_3^-.[4] In humans, the carbonic anhydrase enzymes, specifically isozymes IX (CA IX) have recently been shown to be druggable targets for imaging and treatment of hypoxic tumors.[5] This enzyme is a multidomain protein with the CA subdomain situated outside the cell and possessing a very high CO_2 hydrase catalytic activity, which is also inhibited by the classical CA inhibitors belonging to the sulfonamide, sulfamate, and sulfamide classes of compounds.[6] There are evidences that CA IX contributes to creating a pH-regulating system suitable for cell viability and proliferation which makes for the extracellular acidification of the solid tumor microenvironment. Tumor cells prone to survive in hypoxic and acidic microenvironment attribute to these enzymes.[7]

CA IX expression is strongly increased in many types of solid tumors, such as mesotheliomas, kidneys, lungs and breast. Furthermore, such hypoxic tumors do not generally respond to the classic chemo- and radiotherapy and the strong acidification produced by CA IX overexpression also triggers the development of metastases.[8] Suggested by Svastova *et al,* the assumption that pH_e in different tumor cell cultures recovers to an extent more natural along with an remarkable increased apoptosis of the tumor cells, when CA IX is inhibited by potent and selective sulfonamide inhibitors, is being widely accepted.[9] For example, Pouyssegur's group showed recently that in hypoxic LS174Tr tumor cells either one or both CA isoforms were expressed, in response to a "CO_2 load", and both enzymes contribute to extracellular acidification and maintain a more alkaline resting intracellular pH (pH_i), an action that preserves ATP levels and cell survival in a range of acidic outside pH (6.0 - 6.8) and low bicarbonate medium. In vivo experiments showed that silencing of CA IX alone

leads to a 40% reduction in xenograft tumor volume, with up-regulation of the second gene, that encoding for CA XII, whereas invalidation of both CAIX and CAXII gives an impressive 85% reduction of tumor growth.[10] Correlated with this fact, it has been shown earlier that some CA inhibitors showed anticancer activity in vivo,[11] CA IX inhibition may compose a fascinating new approach for the management of hypoxic tumors, which generally do not respond to the classical radio- and chemotherapy. [12]

One approach aimed at improving the selectivity of tumor cell killing by antitumor drugs is the use of less toxic prodrug forms that can be selectively activated in tumor tissue (tumor-activated prodrugs; TAP), utilizing some unique aspects of tumor physiology such as selective enzyme expression or hypoxia.[13] To characterize the potential effect of inhibition of CA IX and XII in tumor hypoxia, there is an implied need to develop small molecules, which are capable of targeting tumor cells to make for experimental advances.[14] Since the discovery of E-7010 in 1992[15], sulfonamides have been proved to be an important class of anticancer agents, which interact with various cellular targets. Indicatively, indisulam (E7070,[16] HMN-214,[17] T138067[18] is in phase II clinical trials as an antitumor sulfonamide with a complex mechanism of action also involving CA inhibition of several isozymes participating in tumor genesis.

It is also known that sulfonamides are found to be an important class of drugs possessing various types of biological properties such as anti-carbonic anhydrase,[19] and antibacterial.[20] Many of structurally novel sulfonamide derivatives have recently been reported to show substantial antitumor activity and low toxicities, both in vitro and/or in vivo. Some of these derivatives are currently being evaluated in clinical trials, and there is much optimism that they may lead to novel alternative anticancer drugs, devoid of the side effects of the presently available pharmacological agents.[21]

Moreover, in a previous study, nitroimidazole derivatives have attracted considerable attention as they showed a tendency to penetrate and accumulate in regions of tumors,[22] and can undergo bioreduction to yield electrophilic substances which can damage protein and nucleic acids.[23] All these findings encouraged us to explore the synthesis of metronidazole-sulfonamide derivatives as potential CA inhibition agents.

In this paper we report the synthesis of a series of metronidazole-sulfonamide derivatives, which might exhibit synergistic effect in anticancer activity, evaluation of their CA inhibition, anticancer activity and their interaction with CA, by docking

studies.

Results and discussion

Thiry *et al*.[24] reported that from the analysis of the CA active site and the structure of inhibitors described in the literature,[25] a general pharmacophore (Figure 2) for the compounds which acted as carbonic anhydrase inhibitors was found. In order to make this pharmacophore interact with CA inhibitors, it should include the structural elements that are required to be present in the compounds. A sulfonamide moiety should be included, which coordinates with the zinc ion of the active site of the CA and is attached to a benzene ring scaffold. The side chain, which might interact with the hydrophobic and hydrophilic parts of the CA active site, can substitute an aromatic or heterocyclic sulfonamide scaffold. Therefore, different hydrophobic side chains were incorporated in the indanesulfonamide scaffold with an amide linker that can interact with the hydrophilic part of the active site, and a hydrophobic moiety which can interact with the hydrophobic part of the CA active site.

Figure 3 illustrates representative examples of the newly synthesized compounds showing compliance with the above-mentioned pharmacophore model. The new compounds were synthesized according to Scheme 1.

Chemistry:

All novel metronidazole-sulfanilamide derivatives CA inhibitors (**4a-4l**) described herein were synthesized following the synthetic pathway depicted in Scheme 1. The starting MET-OTs (2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethyl 4-methylbenzene-sulfonate, compound **2**) has been previously synthesized from metronidazole using excess of tosyl chloride in the presence of triethylamine in dichloromethane.[26] The synthesis of metronidazole-benzaldehyde **3a-3b** was performed by a nucleophilic displacement reaction of the corresponding MET-OTs in the presence of *o*- or *p*-hydroxybenzaldehyde and K_2CO_3 in DMF. Subsequently, compounds **4a-4l** were prepared from reaction of **3a**, **3b** with the corresponding sulfanilamide in ethanol at the room temperature. The reactions were monitored by thin layer chromatography (TLC) and the crude products were purified by recrystallization with ethanol, ethyl acetate and acetone (1:1:0.05). All of the target compounds gave satisfactory analytical and spectroscopic data IR, ^{1}H NMR, ^{13}C NMR, ESI MS, which are in accordance with their depicted structures.

Biological activity

Antiproliferation assay and MTT Assays

In the present work, twelve of the newly synthesized compounds **4a-4l** were evaluated for their in vitro growth inhibitory activities against two cell lines, which are mouse melanoma cells (B16-F10) and breast carcinoma cell lines (MCF-7) in comparison to the known anticancer drugs: doxorubicin and semaxanib as reference drugs. The values obtained for the twelve compounds against the B16-F10 and MCF-7 cell lines were shown in Table 1. As shown in Table 1, it was observed that metronidazole-sulfanilamide derivatives were found to inhibit the growth of two tumor cell lines with moderate IC_{50} values. For the MCF-7 cell line, which over-expressed CA, as previously shown, metronidazole- sulfanilamide derivatives, in particular, displayed more potent antiproliferative action than in the other tumor cell lines. The IC_{50} values of most metronidazole- sulfanilamide derivatives ranged from 6.5 nM to 120 nM in MCF-7. Among them, compound **4d** displayed the most potent antiproliferative activity with IC_{50} of 6.5 nM, comparable to the positive control doxorubicin (IC_{50} =65.3 nM) and semaxanib (IC_{50} =3.1 nM). Nevertheless, IC_{50} values would have a 1~10 folds in B16-F10 on average. More potent antiproliferative activity for MCF-7 together with virtual screening results both indicated that the over-expressed CA might be a potential target which these metronidazole-sulfanilamide derivatives interacted with.

CA II and IX enzyme assay

Inhibition of two physiologically relevant CA isoforms with compounds **4a-4l** AAZ and SA, tow clinically used drugs) were presented in Table 2.[27] In this study which has included hCAs II (cytosolic, widespread enzymes) and hCAs IX (transmembrane, tumor-associated CAs) account of their relevance as targets/off targets when developing CAIs. In fact, CA II is the drug target for developing antiglaucoma CAIs,[28]

CA IX is overexpressed in breast cancer, and studies have showed that CA IX and CA XII are variably expressed in breast cancer cell lines. Moreover, CA IX expression is associated with poor survival in an unselected cohort of patients with invasive breast carcinoma and it is significantly associated with distant metastasis (Lou *et al*., unpublished results).[10] Therefore, we chose breast cancer as a malignancy model for *in vivo* testing, and antimetastatic profile of some of the CA IX inhibitors. The newly described metronidazole-sulfanilamide derivatives **4a-4l** were tested in an carbonic anhydrase assay *in vitro* to evaluate their potential as antitumor drugs. The results are summarized in Table 2 as IC_{50} values.

The following structure-activity relationship (SAR) can be observed from the data of Table 2. CA activities of these compounds were tested against the standard clinically used inhibitors AAZ and SA. The physiologically dominant and highly active cytosolic isoform hCA II was inhibited by compounds **4a-4l** with IC_{50} in the range of 16-137 nM. Compound **4b** was the most active having an IC_{50} value of 16 nM, whereas compound **4a** was the least active with IC_{50} value of 137 nM. We simplified the situation by only treating steric complexities as the single factor to deal with. Most of these compounds with *p*-substituted metronidazole residues on the benzene ring and the same sulfanilamide derivatives residues were more effective as hCA II inhibitors compared to the *p*-substituted metronidazole residues on the benzene ring. The opposite effect was noticed for hCA IX. Moreover, the tumor-associated isoform hCA IX was inhibited by compounds **4a-4l** with IC_{50} in the range of 38-169 nM (Table 2). The results indicated that the sulfonamides incorporating the metronidazole lead to highly effective hCA IX inhibitors. Across the series of reported compounds, the best inhibitors were those incorporat, which the thiazole residue. Compound **4h** was the most active having IC_{50} value of 38 nM, whereas compound **4a** was the least active with IC_{50} value of 169 nM. In above, we can conclude compounds **4b** and **4h** had been identified as the most potent inhibitors.

Molecular Modeling

To gain better understanding on the potency of the 12 compounds, we examined the interaction of these compounds with hCA II by molecular docking, which was performed by simulation of the 12 compounds into the ATP binding site in hCA II. The protein structure of the hCA II was downloaded from the PDB (3N4B.pdb)[29] and was preprocessed using the Schrodinger Protein Preparation Guide, hydrogens were added to the structure, H-bonds within the protein were optimized, and the protein was minimized to an rmsd of 0.3 Å. A 9.9 Å sphere of water molecules was added around the ligand and a short (3ps) dynamics run was carried out, followed by several cycles of minimization using Quanta/CHARMm. The entire protein- ligand- water complex was allowed to move during calculations.[30]

The predicted binding interaction energy was used as the criterion for ranking. The estimated interaction energy of other synthesized compounds was ranging from –53.72 to –40.84 kcal/mol. The selected pose of **4c** and **4f** had an estimated binding free energy of –53.72 kcal/mol, –42.04 kcal/mol, respectively. The binding model of compounds **4c**, **4f** and hCA II was depicted in Figure 4. The amino acid residues

which had interaction with hCA II were labeled. In the binding mode, compound **4c** was nicely bound to the ATP binding site of hCA II hydrophobic interaction and binding was stabilized by one hydrogen bonds. the hydrogen of THR199 was formed one hydrogen bonds interaction with oxygen atom of N=O bonds of compound **4c** (angle N HD22 = 172.3°, distance = 2.42 Å), Besides, compound **4f** was also nicely bound to the ATP binding site of hCA II hydrophobic interaction and binding was stabilized by four hydrogen bonds (angle TRP5:HE1 4f:O29 = 119.7°, distance = 2.23 Å; ASN67:HD22 4f:N17 = 154.3°, distance = 2.23 Å; THR199:HN 4f:O32 = 112.5°, distance = 2.42 Å and 4f:H53 A:THR199:OG1 = 145.9°, distance = 1.97 Å), and a π-cation interaction (4f A:HIS94). This molecular docking result, along with the biological assay data, suggested that compounds **4c** and **4f** were a potential inhibitor of CA.

Cytotoxicity

All compounds were evaluated for their toxicity against human macrophage with the median cytotoxic concentration (CC_{50}) data of tested compounds by the MTT assay, as showed in Table 3. These compounds were tested at multiple doses to study the viability of macrophage.

As shown in Table 3, all compounds showed that they almost did not exhibit cytotoxicity.

Analysis of apoptosis by Annexin V-PE fluorescence-activated cell sorting (FACS)

To test whether the inhibition of cell growth of A549 was related to cell apoptosis, A549 cell apoptosis induced by compound **4d** was determined using flow cytometry. The uptake of Annexin V-PE was significantly increased, and the uptake of normal cells was significantly decreased in a time-dependent manner. Finally, the percentage of early apoptotic cells was markedly elevated in a density-dependent manner from 6.28 to 19.6% at 48 h (Figure 5).

Conclusion:

In summary, CA is an emerging target for the development of novel antitumour chemotherapeutics. We had designed and synthesized novel series of metronidazole-sulfanilamide derivatives which were tested for their inhibitory activities against B16-F10 and MCF7. These compouneds showed a very interesting profile for the inhibition of hCAs II (cytosolic, off-target isoform) and hCAs IX (transmembrane, tumor-associated enzyme). Most of them exhibited CA inhibitory

activity and did not show any toxicity. Docking simulation was performed to decipher the probable binding models and poses. The results indicate that metronidazole-sulfanilamide derivatives, which acted as potent antibacterias and as such have the potential to be novel and potent antitumour agents. Given the unforeseen structural differences within the active site of some pathogenic enzymes, the key to discovering inhibitors with broad-spectrum antitumour activity lies in a detailed understanding of the CA active sites. Further studies on the CA inhibition ability of this compound, and further modifications of the current series with the hope of improving both enzymatic inhibition and physical properties, are currently underway.

Experiments

Materials and measurements

All chemicals used were purchased from Aldrich (USA). All reagents used in the current study were of analytical grade. Thin layer chromatography (TLC) was performed on silica gel plates with fluorescent indicator. All analytical samples were homogeneous on TLC in at least two different solvent systems. Melting points (uncorrected) were determined on a X-4 MP apparatus (Taike Corp, Beijing, China). All the ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX 300 model Spectrometer in DMSO-d_6 and chemical shifts (δ) were reported as parts per million (ppm). ESI-MS spectra were recorded on a Mariner System 5304 Mass spectrometer. FT-IR spectra on KBr pellets were recorded on a Thermo Nicolet NEXUS870 model spectrometer.

General procedure for the synthesis of compounds 3a, 3b.

Anhydrous K_2CO_3 (8.0 g, 58 mmol) was added to a stirred solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethyl-4-methylbenzenesulfonate (**2)** (4.0 g, 12.1 mmol) in anhydrous DMF (150 mL) at 80 ˚C. Subsequently, *p*- or *m* -hydroxybenzaldehyde (1.9 g, 15.7 mmol) was added to the reaction mixture and stirring continued for 20 h. The mixture was poured into water (200 mL) and extracted with EtOAc (3 × 250 mL). The combined organic extracts were washed with saturated Na_2CO_3 solution, brine (200 mL), dried ($MgSO_4$), and concentrated in vacuo. The crude product was purified by column chromatography (silica gel, EtOAc/PE 1:4) to give compounds **3a**, **3b**.

3a: Yellow solid, yield 71.6%, R_f =0.44 (EtOAc/PE 1:1); m.p. 41~43 ˚C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 9.95 (s, 1H, CHO), 7.91 (s, 1H, CH), 7.47~7.05 (m, 4H, ArH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.64 (s, 3H,

CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 190.46, 151.81, 142.24, 138.63, 136.56, 133.23, 128.35, 127.38, 62.51, 44.96. ESI-MS: 292.1 $[M+H]^+$.

3b: Faint yellow solid, yield 51.3%, R_f =0.43 (EtOAc/PE 1:1); m.p. 68~70 ˚C; ^1H NMR (DMSO-d_6, 300 MHz) δ: 9.95 (s, 1H, CHO), 7.91 (s, 1H, CH), 7.96 (s, 1H, ArH), 7.47~7.05 (m, 3H, ArH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.64 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 190.44, 151.76, 142.24, 138.63, 136.56, 133.23, 127.38, 126.98, 62.51, 44.96. ESI-MS: 292.1 $[M+H]^+$.

General procedure for the Synthesis of compounds 4a-4l.

To a solution of compound **3a** or **3b** (7.6 mmoL) in ethanol (15 mL) the requisite substituted sulfonamide (7.6 mmol) and acetic acid (0.5 mL) was added. The mixture was heated at 55 ˚C for 3 h. A solid product was immediately formed, which was filtered and washed with water. The crude products were purified by recrystallization with ethanol, ethyl acetate and acetone (1:1:0.5) washed with ice-water (25 mL) three times and dried to give the title compounds **4a-4l** as yellow solid.

4-(4-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)benzene-sulfonamide (**4a**)

Yellow crystals, yield 66.5%. R_f =0.25 (EtOAc/PE 1:1); m.p. 195~197 ˚C; ^1H NMR (DMSO-d_6, 300 MHz) δ: 8.52 (s, 1H, CH), 8.03 (s, 1H, CH), 7.88~7.03 (m, 8H, ArH), 7.04 (s, 1H, NH), 7.02 (s, 1H, NH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 191.03, 157.82, 151.21, 137.54, 131.98, 129.76, 127.68, 123.58, 121.08, 113.23, 112.15, 66.31, 45.16, 30.31, 14.06. IR (KBr, v, cm^{-1}): 3334, 3222, 3200 (NH, NH_2), 1596 (C=N), 1576, 1374 (NO_2), 1343, 113 6(SO_2). ESI-MS: 430.5 $[M+H]^+$.

4-(4-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(thiazol-2-yl)benzenesulfonamide (**4b**)

Yellow crystals, yield 50.0%. R_f =0.32 (EtOAc/PE 1:1); m.p. 258~259 ˚C; ^1H NMR (DMSO-d_6, 300 MHz) δ: 8.49 (s, 1H, CH), 8.02 (s, 1H, CH), 7.85~7.77 (m, 4H, ArH), 7.29 (d, J = 5.0 Hz, 2H, ArH), 7.23 (d, J = 2.7 Hz, 1H, CH), 7.02 (d, J = 5.2 Hz, 2H, ArH), 6.81 (d, J = 2.7 Hz, 1H, CH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 168.36, 158.12, 152.01, 149.23, 131.36, 129.86, 126.46, 125.02, 121.38, 112.68, 107.36, 14.08. IR (KBr, v, cm^{-1}): 3220 (NH), 1596 (C=N), 1583, 1379 (NO_2), 1348, 1139 (SO_2). ESI-MS: 513.4 $[M+H]^+$.

N-Carbamimidoyl-4-(4-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneam

ino)benzenesulfonamide (**4c**)

Orange crystals, yield 84.3%. R_f =0.28 (EtOAc/PE 1:1); m.p. 235~237 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.43 (s, 1H, SO_2NH), 8.49 (s, 1H, CH), 7.85~7.77 (m, 4H, ArH), 7.29 (d, J = 5.0 Hz, 2H, ArH), 7.23 (d, J = 2.7 Hz, 1H, CH), 7.02 (d, J = 5.2 Hz, 2H, ArH), 6.80 (s, 3H, NH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 190.23, 162.37, 160.52, 157.77, 154.03, 150.87, 141.15, 132.49, 131.44, 130.47, 127.01, 126.57, 120.39, 114.48, 114.32, 112.41, 66.61, 45.17, 14.06. IR (KBr, v, cm^{-1}): 3396, 3236, 3110 (NH, NH_2), 1588 (C=N), 1570, 1368 (NO_2), 1341, 1144 (SO_2).

N-(4, 6-Dimethylpyrimidin-2-yl)-4-(4-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy) benzylideneamino)benzenesulfonamide (**4d**)

Yellow crystals, yield 64.3%. R_f =0.32 (EtOAc/PE 1:1); m.p. 235~237 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.02 (s, 1H, CH), 8.04 (s, 1H, CH), 8.00~6.75 (m, 8H, ArH), 6.55 (s, 1H, CH), 5.95 (s, 1H, SO_2NH), 4.74 (t, J = 4.9 Hz, 2H, -CH_2), 4.45 (t, J = 4.9 Hz, 2H, -CH_2), 2.53 (s, 3H, CH_3), 2.25 (s, 6H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 171.28, 159.01, 156.78, 153.06, 151.68, 141.96, 137.48, 132.76, 129.86, 129.56, 125.86, 124.75, 119.36, 118.43, 116.54, 95.35, 14.08, 12.02. IR (KBr, v, cm^{-1}): 3187 (NH), 1591 (C=N), 1581, 1376 (NO_2), 1345, 1140 (SO_2). ESI-MS: 536.4 $[M+H]^+$.

4-(4-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(4-methylp yrimidin-2-yl)benzenesulfonamide (**4e**)

Yellow crystals, yield 63.2%. R_f =0.26 (EtOAc/PE 1:1); m.p. 207~209 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.12 (s, 1H, SO_2NH), 9.02 (s, 1H, CH), 8.18 (s, 1H, CH), 8.04 (d, J =1.95 Hz, 1H, CH), 8.00~6.75 (m, 8H, ArH), 6.55 (d, J =2.0 Hz,1H, CH), 4.74 (t, J = 4.9 Hz, 2H, CH_2), 4.45 (t, J = 4.9 Hz, 2H, -CH_2), 2.53 (s, 3H, CH_3), 2.28 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 190.06, 167.66, 157.00, 156.77, 151.96, 132.47, 131.40, 129.91, 125.64, 114.30, 114.18, 112.16, 66.49, 45.10, 23.32, 14.06. IR (KBr, v, cm^{-1}): 3218 (NH), 1592 (C=N), 1576, 1365 (NO_2), 1344, 1139 (SO_2). ESI-MS: 522.4 $[M+H]^+$.

4-(4-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(5-methylis oxazol-3-yl)benzenesulfonamide (**4f**)

Yellow crystals, yield 61.8%. R_f =0.30 (EtOAc/PE 1:1); m.p. 285~287 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.78 (s, 1H, SO_2NH), 9.02 (s, 1H, CH), 8.18 (s, 1H, CH), 8.97~7.44 (m, 8H, ArH), 6.21 (s, 1H, CH), 4.74 (t, J = 4.9 Hz, 2H, -CH_2), 4.45 (t, J =

4.9 Hz, 2H, -CH_2), 2.53 (s, 3H, CH_3), 2.23 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 169.69, 159.64, 157.06, 152.56, 151.37, 142.42, 137.68, 132.96, 130.37, 129.93, 129.11, 125.79, 125.03, 124.70, 119.00, 118.69, 116.94, 95.15, 12.02. IR (KBr, *v,* cm^{-1}): 3221 (NH), 1588 (C=N), 1585, 1376 (NO_2), 1346, 1139 (SO_2). ESI-MS: 511.6 $[M+H]^+$.

4-(3-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)benzene-sulfonamide (**4g**)

Yellow crystals, yield 64.3%. R_f =0.29 (EtOAc/PE 1:1); m.p. 193~195 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 8.52 (s, 1H, CH), 8.03 (s, 1H, CH), 7.93~7.08 (m, 8H, ArH), 7.04 (s, 1H, NH), 7.02 (s, 1H, NH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 191.17, 157.84, 151.16, 137.79, 132.52, 129.82, 127.43, 123.50, 120.78, 113.34, 112.33, 66.36, 45.21, 30.32, 14.06. IR (KBr, *v,* cm^{-1}): 3338, 3221, 3208, 3115 (NH, NH_2), 1594 (C=N), 1577, 1374 (NO_2), 1345, 1133 (SO_2). ESI-MS: 430.3 $[M+H]^+$.

4-(3-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(thiazol-2-yl)benzenesulfonamide (**4h**)

Yellow crystals, yield 68.9%. R_f =0.23 (EtOAc/PE 1:1); m.p. 148~150 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 12.43 (s, 1H, SO_2NH), 9.95 (s, 1H, CH), 8.04 (s, 1H, CH), 7.44~6.54 (m, 7.40, 4H, ArH), 7.19 (d, J = 5.0 Hz, 2H, ArH), 7.17 (s, 1H, CH), 7.02 (d, J = 5.2 Hz, 2H, ArH), 6.58~6.54 (m, 4H, ArH),, 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 167.98, 158.32, 151.96, 149.03, 130.78, 129.32, 127.46, 124.42, 120.98, 112.48, 108.96, 14.06. IR (KBr, *v,* cm^{-1}): 3221 (NH), 1594 (C=N), 1585, 1378 (NO_2), 1348, 1140 (SO_2). ESI-MS: 513.5 $[M+H]^+$.

N-Carbamimidoyl-4-(3-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)benzenesulfonamide (**4i**)

Light yellow crystals, yield 65.9%. R_f =0.25 (EtOAc/PE 1:1); m.p. 229~231 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 11.43 (s, 1H, SO_2NH), 8.49 (s, 1H, CH), 7.85~7.77 (m, 4H, ArH), 7.29 (d, J = 5.0 Hz, 2H, ArH), 7.23 (s, 1H, CH), 7.02 (d, J = 5.2 Hz, 2H, ArH), 6.80 (s, 3H, NH), 4.75 (t, J = 4.7 Hz, 2H, -CH_2), 4.39 (t, J = 5.0 Hz, 2H, -CH_2), 2.48 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 189.26, 162.31, 160.42, 156.07, 153.95, 150.27, 141.11, 133.06, 131.61, 130.53, 127.31, 125.97, 120.29, 114.08, 114.23, 112.36, 66.58, 45.22, 14.08. IR (KBr, *v,* cm^{-1}): 3390, 3233, 3111 (NH, NH_2), 1593 (C=N), 1573, 1370 (NO_2), 1343, 1138 (SO_2). ESI-MS: 472.6 $[M+H]^+$.

N-(4,6-Dimethylpyrimidin-2-yl)-4-(3-(2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy) benzylideneamino)benzenesulfonamide (**4j**)

Yellow crystals, yield 71.2%. R_f =0.26 (EtOAc/PE 1:1); m.p. 226~228 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.02 (s, 1H, CH), 8.04 (s, 1H, CH), 8.00~6.75 (m, 8H, ArH), 6.55 (s, 1H, CH), 5.95 (s, 1H, SO_2NH), 4.74 (t, J = 4.9 Hz, 2H, CH_2), 4.45 (t, J = 4.9 Hz, 2H, CH_2), 2.53 (s, 3H, CH_3), 2.25 (s, 6H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 171.18, 159.86, 157.08, 152.86, 151.28, 141.78, 138.02, 132.56, 130.14, 129.58, 125.46, 124.70, 119.36, 118.23, 116.54, 95.35, 14.08, 12.02. IR (KBr, v, cm^{-1}): 3228 (NH), 1587 (C=N), 1585, 1368 (NO_2), 1346, 1139 (SO_2). ESI-MS: 536.7 $[M+H]^+$.

4-(3-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(4-methylp yrimidin-2-yl)benzenesulfonamide (**4k**)

Light yellow crystals, yield 62%. R_f =0.28 (EtOAc/PE 1:1); m.p. 128~130 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.95 (s, 1H, CH), 8.31 (d, J =5.1 Hz, 2H, ArH), 8.03 (s, 1H, CH), 7.66~7.51 (m, 6H, ArH), 7.34 (s, 1H, SO_2NH), 6.95 (s, 1H, CH), 6.88 (s, 1H, CH), 4.74 (t, J = 4.9 Hz, 2H, CH_2), 4.45 (t, J = 4.9 Hz, 2H, -CH_2), 2.55 (s, 3H, CH_3), 2.31 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 191.37, 157.03, 156.81, 152.08, 132.44, 129.89, 129.58, 129.15, 123.13, 120.80, 120.10, 114.20, 112.80, 112.12, 66.44, 45.18, 23.32, 14.08. IR (KBr, v, cm^{-1}): 3220 (NH), 1592 (C=N), 1585, 1380 (NO_2), 1347, 1139 (SO_2). ESI-MS: 522.5 $[M+H]^+$.

4-(3-(2-(2-Methyl-5-nitro-1*H*-imidazol-1-yl)ethoxy)benzylideneamino)-*N*-(5-methylis oxazol-3-yl)benzenesulfonamide (**4l**)

Yellow crystals, yield 65.7%. R_f =0.24 (EtOAc/PE 1:1); m.p. 88~90 ˚C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ: 9.31 (s, 1H, CH), 8.04~7.83 (m, 4H, ArH), 7.63 (s, 1H, CH), 7.33~6.74 (m, 4H, ArH), 6.55 (s, 1H, CH), 5.95 (s, 1H, SO_2NH), 4.74 (t, J = 4.92 Hz, 2H, -CH_2), 4.45 (t, J = 4.92 Hz, 2H, CH_2), 2.53 (s, 3H, CH_3), 2.23 (s, 3H, CH_3). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 168.96, 157.88, 157.69, 151.60, 137.30, 132.46, 129.84, 128.50, 127.87, 125.83, 123.40, 120.78, 113.02, 112.46, 94.91, 66.38, 45.21, 14.08, 11.97. IR (KBr, v, cm^{-1}): 3220 (NH), 1593 (C=N), 1582, 1379 (NO_2), 1350, 1139 (SO_2). ESI-MS: 511.6 $[M+H]^+$.

CA Inhibition Assay.

An Applied Photophysics stopped-flow instrument has been used for assaying the CA-catalyzed CO_2 hydration activity.[31] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10

mM Hepes (pH = 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant the ionic strength), at 25 ° C, following the CA-catalyzed CO_2 hydration reaction for a period of 10-100 s (the uncatalyzed reaction needs around 60-100 s in the assay conditions, whereas the catalyzed ones are of around 6-10 s). The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of kinetic parameters. For each inhibitor, tested in the concentration range between 0.01 nM and 100 μM, at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10-20% (*v*/*v*) DMSO (which is not inhibitory at these concentra-tions), and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E - I complex. The inhibition constants were obtained by nonlinear least-squares methods using $PRISM_3$. The curve-fitting algorithm allowed us to obtain the IC_{50} values, working at the lowest concentration of substrate of 1.7 mM, from which K_i values were calculated by using the Cheng-Prusoff equation. The catalytic activity (in the absence of inhibitors) of these enzymes were calculated from Lineweaver-Burk plots, as reported earlier, and represent the mean from at least three different determinations. 38 - 42 Enzyme concentra-tions in the assay system were 7.3 nM for hCA II, and 8.5 nM for hCA IX. Enzymes used here were recombinant ones, prepared and purified as described earlier.[32]

Cell proliferation assay

MTT is much more convenient and helpful than MTT for analyzing cell proliferation, because it can be reduced to soluble formazan by dehydrogenase in mitochondria and has little toxicity to cells. Cell proliferation was determined using MTT dye (BeyotimeInst Biotech, China) according to manufacture's instructions. Briefly, 1-5 × 10^3 cells per well were seeded in a 96-well plate, grown at 37 ℃ for 12 h,Subsequently, cells were treated with the target compounds at increasing concentrations in the presence of 10% FBS for 24 or 48 h. After 10 μL MTT dye was added to each well, cells were incubated at 37 ℃ for 1-2 h and Plates were read in a Victor-V multilabel counter (Perkin-Elmer) using the default europium detection protocol. Percent inhibition or IC_{50} values of compounds were calculated by comparison with DMSO-treated control wells.

Flow cytometry

Cells (1.3×10^5 cells/mL) were cultured in the presence or not of novobiocin analogues at 200 μM. Nvb at the same concentration served as reference inhibitor. After treatment for 48 and 72 h, cells were washed and fixed in PBS/ ethanol (30/70). For cytofluorometric examination, cells (10^4 cells/mL) were incubated for 30 min in PBS/ Triton X100, 0.2% /EDTA 1 mM, and propidium iodide (PI) (50 μg/mL) in PBS supplemented by RNase (0.5 mg/mL). The number of cells in the different phases of the cell cycle was determined, and the percentage of apoptotic cells was quantified. Analyses were performed with a FACS Calibur (Becton Dickinson, Le Pont de Claix, France). Cell Quest software was used for data acquisition and analysis.[33]

Experimental protocol of docking study

Molecular docking of compound **4c** and **4f** into the three dimensional X-ray structure of human hCA II (PDB code: 3N4B) was carried out using the Discovery Studio (version 3.5) as implemented through the graphical user interface DS-CDOCKER protocol. Thethree-dimensionalstructures of the aforementioned compounds were constructed using Chem. 3D ultra 12.0 software [Chemical Structure Drawing Standard; Cambridge Soft corporation, USA (2010)], then they were energetically minimized by using MMFF94 with 5000 iterations and minimum RMS gradient of 0.10. The crystal structuresof protein complex was retrieved from the RCSB Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). All bound waters and ligands were eliminated from the protein and the polar hydrogen was added to the proteins. Molecular docking of all twenty compounds was then carried out using the Discovery Stutio (version 3.5) as implemented through the graphical user interface CDOCKER protocol. CDOCKER is an implementation of a CHARMm based molecular docking tool using a rigid receptor.

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Keywords: Metronidazole-sulfonamide derivatives Carbonic anhydrase Antitumor activity Cytotoxicity

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Figure captions

Table1 In vitro anticancer activities (IC_{50}, nM) against two human tumor cell lines

Table 2 In vitro anticancer activities (IC_{50}, nM) against two human tumor cell lines

Table 3 The median cytotoxic concentration (CC_{50}) data of tested compounds.

Figure 1. Known human CA inhibitors including some clinically used drugs.

Figure 2. Structural elements of CA inhibitors in the CA enzymatic active site.

Figure 3. Representative examples of the synthesized compounds showing compliance to the general pharmacophore of sulfonamide compounds acting as CA inhibitors.

Figure 4 Docking of compound **4c** and **4f** in the ATP binding site of hCA II: (**a)** Left: 2D model of the interaction between compound **4c**, **4f** and the ATP binding site. Right: 3D model of the interaction between compound **4c**, **4f** and the ATP binding site, resbectively.

Figure 5. Compound **4d** induced apoptosis in A549 cells with the density of 2.5, 10, 40, 160 μg/mL. A549 cells were treated with for 48 h. Values represent the mean, n =3.P <0.05 versus control. The percentage of cells in each part was indicated.

Scheme 1[a]

Table1 In vitro anticancer activities (IC_{50}, nM) against two human tumor cell lines

Compounds	IC_{50} (nM)[a]	IC_{50} (nM)[a]
	B16-F10[b]	MCF-7[b]
4a	270	87
4b	310	6.8
4c	200	9.5
4d	190	6.5
4e	220	7.2
4f	220	120

4g	230	93
4h	150	47
4i	300	58
4j	160	30
4k	270	7.8
4l	180	90
Doxorubicin	72.3	65.3
Semaxanib	3.6	3.1

[a]Antiproliferation activity was measured using the MTT assay. Values were the average of three independent experiments run in triplicate. Variation was generally 5-10%.
[b]Cancer cells kindly supplied by State Key Laboratory of Pharmaceutical Biotechnology, Nanjing University.

Table 2 In vitro anticancer activities (IC_{50}, nM) against two human tumor cell lines

Compounds	IC_{50} (nM)[a]	IC_{50} (nM)[a]
	hCA II[b]	hCA IX[b]
4a	137	169
4b	16	93
4c	70	135
4d	48	110
4e	22	81
4f	33	112
4g	53	42
4h	89	38
4i	114	69
4j	81	61
4k	86	126
4l	52	56
AAZ	16	28

SA	256	298

[a] Errors were in the range of (5 - 10% of the reported values, from three different assays. [b] Human recombinant enzymes, by the esterase assay (4-nitrophenylacetate as substrate).

Table 3 The median cytotoxic concentration (CC_{50}) data of tested compounds.

compounds	CC_{50}, μmol [a]	compounds	CC_{50}, μmol [a]
4a	0.53	**4g**	0.63
4b	0.46	**4h**	0.49
4c	0.58	**4i**	0.52
4d	0.72	**4j**	0.62
4e	0.64	**4k**	0.56
4f	0.68	**4l**	0.68

[a] Minimum cytotoxic concentration required to cause a microscopically detectable alteration of normal cell morphology.

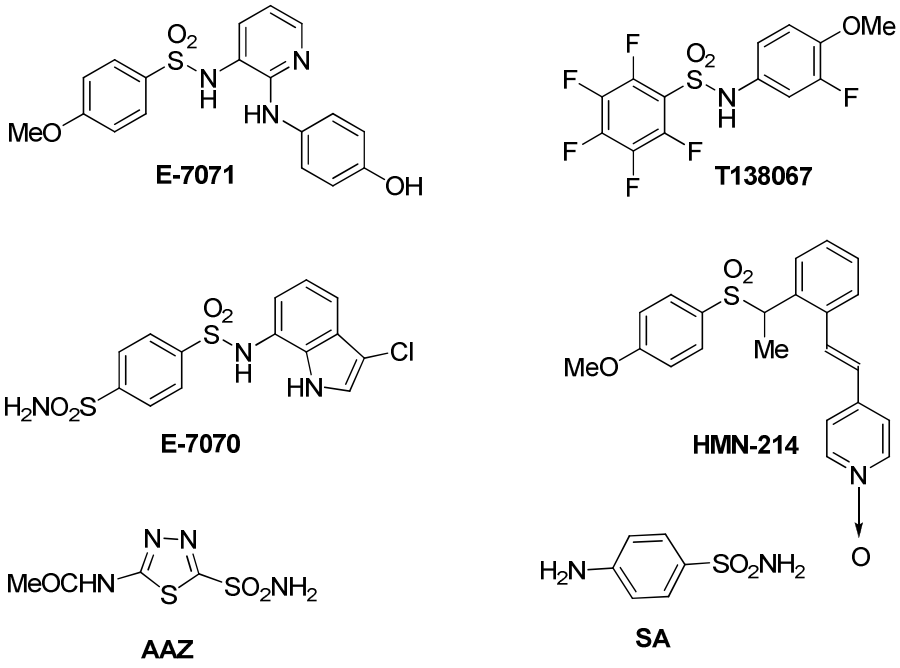


Figure 1. Known human CA inhibitors including some clinically used drugs.

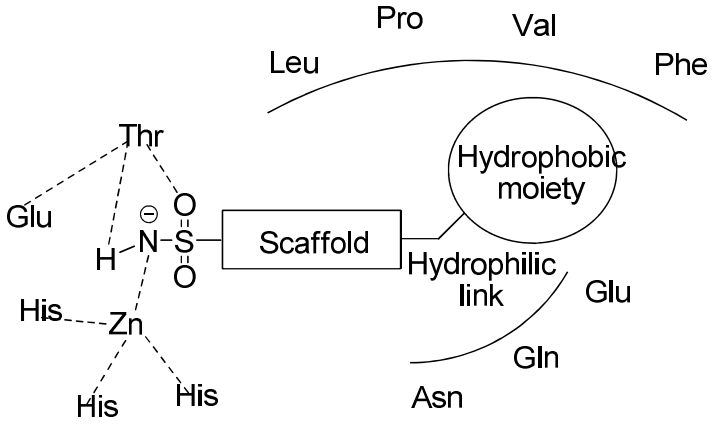


Figure 2. Structural elements of CA inhibitors in the CA enzymatic active site.

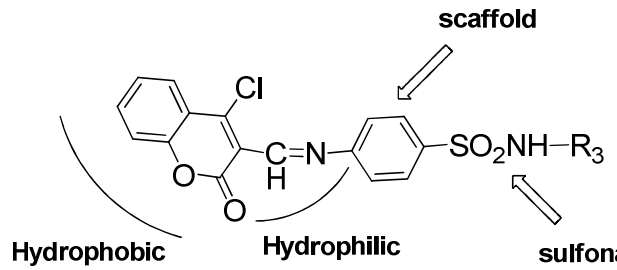
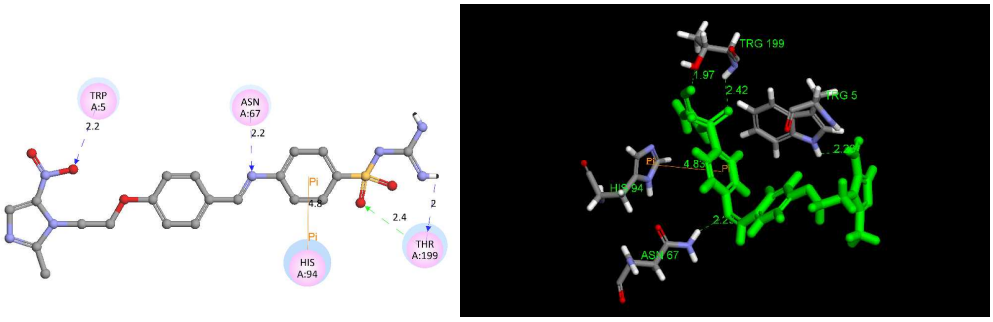


Figure 3. Representative examples of the synthesized compounds showing compliance to the general pharmacophore of sulfonamide compounds acting as CA inhibitors.

a



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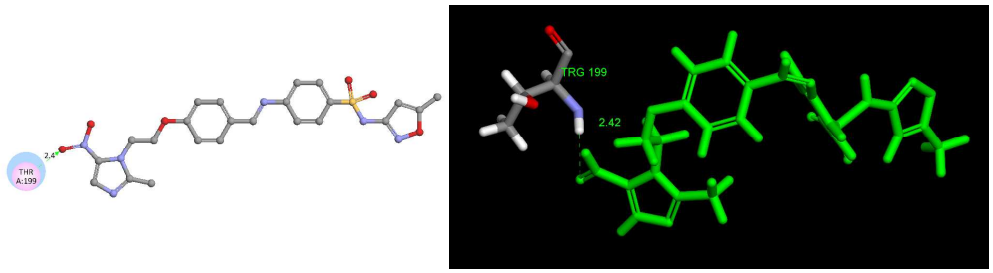

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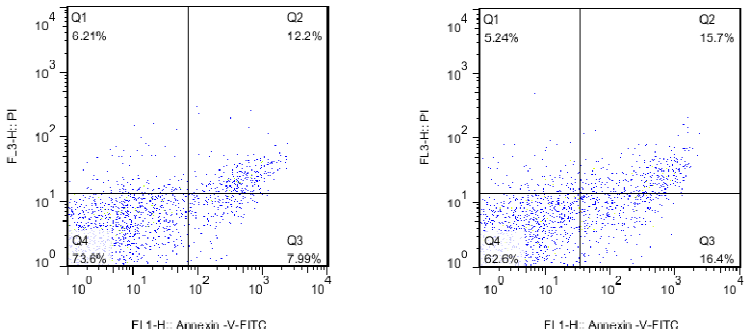
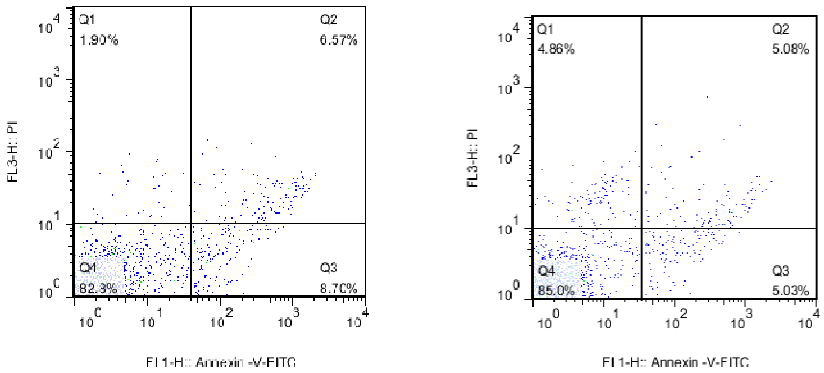
a





b

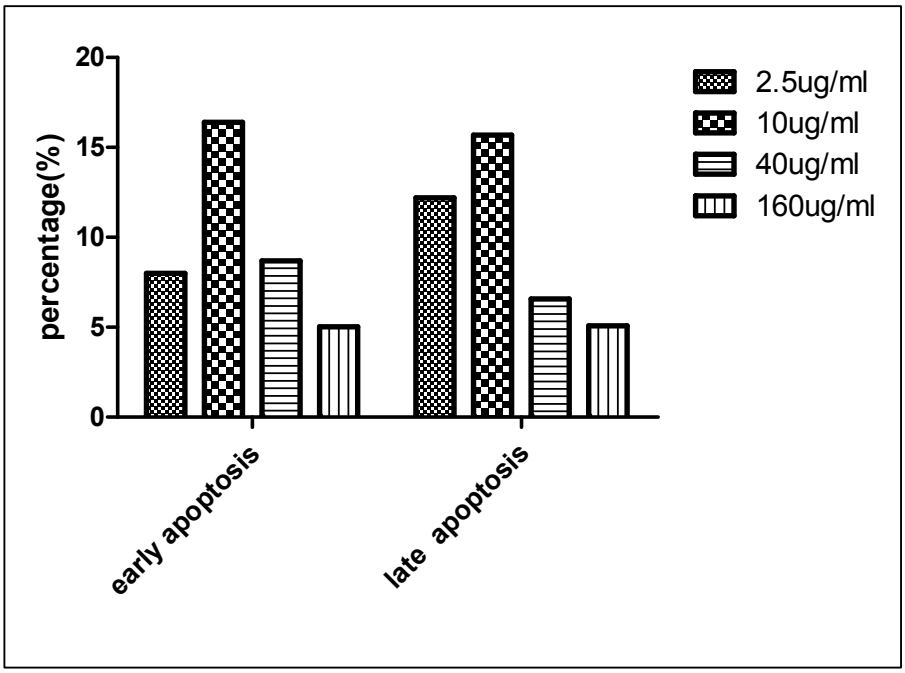
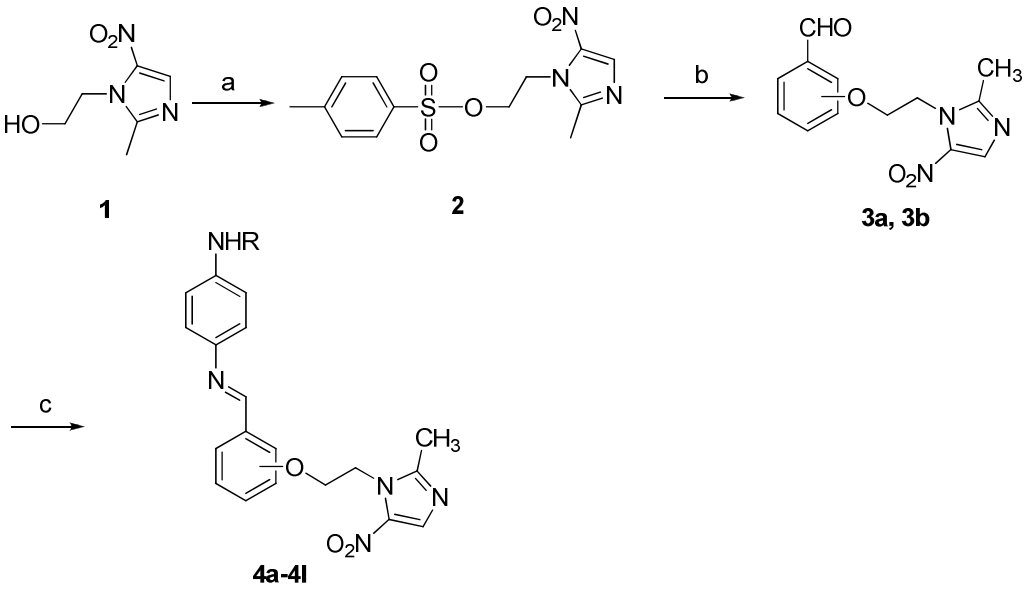


Figure 5. Compound **4d** induced apoptosis in A549 cells with the density of 2.5, 10, 40, 160 μg/mL. A549 cells were treated with for 48 h. Values represent the mean, n =3.P <0.05 versus control. The percentage of cells in each part was indicated.

Scheme 1[a]



[a] Reagents and conditions: (a) 30 equiv tosyl chloride, CH_2Cl_2, RT, 12 h, 75%; (b) 15.7 equiv **3a**, *p*-hydroxybenzal- dehyde; **3b**, *m*-hydroxybenzaldehyde, K_2CO_3, DMF, 80 °C, 20 h, **3a**, 71.6%; **3b**, 51.3%; (c) 1.0 equiv *p*-$RNHSO_2PhNH_2$, CH_3CH_2OH, 50 °C, 8 h, 50.0-84.3%;

Novel Metronidazole-Sulfonamide Derivatives as Potent and Selective Carbonic Anhydrase Inhibitors: Design, Synthesis and Biology Analysis

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