**The three-tails approach as a new strategy to improve selectivity of action of sulphonamide inhibitors against tumour-associated carbonic anhydrase IX and XII**

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**ABSTRACT**

Human (h) carbonic anhydrase (CAs, EC 4.2.1.1) isoforms IX and XII were recently confirmed as anticancer targets against solid hypoxic tumours. The “three-tails approach” has been proposed as an extension of the forerunner “tail” and “dual-tail approach” to fully exploit the amino acid differences at the medium/outer active site rims among different hCAs and to obtain more isoform-selective inhibitors. Many three-tailed inhibitors (TTIs) showed higher selectivity against the tumour-associated isoforms hCA IX and XII with respect to the off-targets hCA I and II. X-ray crystallography studies were performed to investigate the binding mode of four TTIs in complex with a hCA IX mimic. The ability of the most potent and selective TTIs to reduce in vitro the viability of colon cancer (HT29), prostate adenocarcinoma (PC3), and breast cancer (ZR75-1) cell lines was evaluated in normoxic (21% O₂) and hypoxic (3% O₂) conditions demonstrating relevant anti-proliferative effects.

**1. Introduction**

Tumour growth, malignant progression, and resistance to chemotherapy and radiotherapy appear to be strongly associated with tumour hypoxia. Hypoxia is the main cause responsible for the overexpression of the hypoxia-inducible factor (HIF-1) and the Warburg effect in tumours, an indispensable metabolic reprogramming of cancer cells from glycolytic metabolism to fermentation. In order to survive in hypoxic conditions and acidosis due to fermentative metabolism, HIF-1 triggers a signalling cascade, that upregulates the expression of several genes, coding for the lactate-proton symporters (MCT4), other proton-transporters, and the tumour-associated isoforms IX and XII of carbonic anhydrases (CAs, EC 4.2.1.1), that catalyse the reversible hydration of carbon dioxide (CO₂) into a proton (H₊) and bicarbonate (HCO₃⁻). These proton export mechanisms, in concert with poor vascular drainage, are responsible to maintain an intracellular pH of 7.2–7.4, acidifying the extracellular pH to 6.2–6.8, which is strongly associated with the propagation, malignant progression, and resistance to chemotherapy and radiotherapy of tumours. In detail, the CA IX and XII expression is strongly increased in many types of tumours and is downregulated by the wild-type von Hippel–Lindau tumour suppressor protein (pVHL). In some cancer cells, the VHL gene is mutated leading to the strong upregulation of tumour-associated CA isoforms as a consequence of constitutive HIF activation. Recent studies have shown that isoform hCA IV, prevalently anchored on the membrane of the astrocytes, is responsible for regulating interstitial pH and for regulating transmembrane lactate transport, interacting with the chaperones of the monocarboxylate transporters in the brain cells. Whereas targeting of hCA IV with inhibitors does not yet have clear antitumor therapeutic applications, in the last decades, several studies corroborated CA IX and XII as targets for the development of carbonic anhydrases inhibitors (CAIs) as novel antitumor drugs and, to date, the sulphonamide inhibitor SLC-0111 is in phase two clinical trials as an antitumor agent.

Among the large number of CAI chemotypes, the zinc binder sulphonamides led to many potent and fruitful inhibitory molecules. However, their lack of selectivity and inability to discern among the 15 human (h) CA isoforms, prevents their wider use as therapeutic agents, at least for the first and second generation of such inhibitors. In fact, the inhibition of ubiquitous and cytosolic isoforms hCA I and II is responsible for the side effect in the treatment with CAIs. To overcome their promiscuous inhibition, the “three-tails approach” was applied as an extension of the previously proposed “tail” and “dual tails approach” (Figure 1(A)). A careful 3D analysis has shown different dimensions of the 15 hCAs active site that together with diverse architecture and extension of the hydrophilic and lipophilic areas, line disparate pockets that could be targeted by specific tails. The three-tails approach consists of appending three pendants of various nature on a CA inhibitory (CAI) scaffold (e.g. benzensulphonamide) in order to interact with the most variable residues among the fifteen hCAs in the middle/outer rim of the active site, conferring to the sulphonamide inhibitors some...
important properties, such as water solubility\(^{37}\) and subsequently membrane (im)permeability\(^{40}\), improving the interactions with the hydrophilic and hydrophobic halves of the active sites and increasing the matching and fitting of the ligand-target contacts to attain the proper hCA selective inhibition\(^{35}\).

2. Material and methods

2.1. Chemistry

The synthesis and characterisation of sulphonamides 1–50 was reported earlier by our group\(^{36}\).

2.2. Carbonic anhydrase inhibition

An applied photophysics stopped-flow instrument has been used for assaying the CA catalysed CO\(_2\) hydration activity\(^{41}\) as reported earlier\(^{42}\). Enzyme concentrations were in the range 5–18 nM. All CA isoforms were recombinant ones obtained in-house as reported earlier\(^{43}\).

2.3. X-ray crystallography

2.3.1. Protein expression and purification

Competent BL21 *Escherichia coli* cells were transformed with plasmid DNA containing the hCA IX-mimic gene using standard protocols as described earlier\(^{44,45}\).

2.3.2. Crystallisation

Inhibitors were successfully cocrystallised with CAII and CAIX-mimic via the hanging-drop vapour diffusion method. 0.5 mL of mother liquor consisting of 1.6 M sodium citrate and 50 mM Tris at pH 7.8 was used in the wells for setting up crystal trays. Each cover slip contained a 1:1 ratio of 10 mg/mL protein to mother liquor. DMSO was used to dissolve inhibitors to 1 mM, with the drops final concentration ~100 μM. Cocrystals of CAII and CAIX formed within a week.

2.3.3. Data collection and processing

Diffraction data were collected via the F1 beamline at Cornell High Energy Synchrotron Source (CHESS, Ithaca, NY) at 0.977 Å wavelength and at Stanford Synchrotron Radiation Lightsource (SSRL, Menlo Park, CA). A Pilatus 6 M detector collected data sets with a crystal-to-detector distance of 270 mm, 1° oscillation, and 4 s image exposure, for a total of 180 images. Diffraction data were indexed and integrated with XDS\(^{46}\). Data were scaled in space group P2\(_1\) via AIMLESS\(^{47}\) from the CCP4 program suite\(^{48}\). Phases were determined via molecular replacement using PDB: 4ZAO\(^{49}\) as a search model. Modifications to the model, such as addition of inhibitor, ligand (glycerol), zinc, and water to the active site of CA were executed in Coot\(^{50}\) along with ligand PDB file modifications. Refinements were completed and ligand restraint files were created in Phenix\(^{51}\). Figures were generated with PyMol (Schrödinger). Protein-ligand bond lengths and active site interactions were observed with (LigPlot Plus, Hinxton, Cambridgeshire, UK)\(^{52}\).

2.4. Antiproliferative assays

2.4.1. Cell culture and treatments

Human prostate cancer cell line PC3, human breast cancer cell line ZR75-1, and human colon cancer cell line HT-29 were obtained from American Type Culture Collection (Rockville, MD). PC3, ZR75-1, and HT-29 were cultured in DMEM high glucose with 10% FBS in 5% CO\(_2\) atmosphere at 37°C. Media contained 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma, Milan, Italy). Cells were plated in 96-well cell culture (1.10⁴/well) and, 24 h after, treated with the tested compounds (0–200 μM) for 48 h. Low oxygen conditions were acquired in a hypoxic workstation (Concept 400 anaerobic incubator, Ruskinn Technology Ltd., Bridgend, UK). The atmosphere in the chamber consisted of 1% O\(_2\) (hypoxia), 5% CO\(_2\), and residual N\(_2\). In parallel, normoxic (20% O\(_2\)) dishes were incubated in air with 5% CO\(_2\).

2.4.2. Cell viability assay

PC3, ZR75-1, and HT-29 cell viability were evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as an index of mitochondrial compartment functionality. Cells were plated and treated as described. Post-treatment, after extensive washing, 1 mg/mL MTT was added into each well and incubated for 30 min at 37°C. After washing, the formazan crystals were dissolved in 150 μL of DMSO. The absorbance was measured at 550 nm. Experiments were performed in quadruplicate on at least three different cell batches.

2.4.3. Statistical analysis

Results were expressed as mean ± SEM, and the analysis of variance was performed by one-way ANOVA. A Bonferroni’s significant
inhibitor structure. A. Several drug design studies of CAIs adopted the p-substituted benzenesulfonamide as a main scaffold against heteroaromatic sulphonamides to notably simplify the synthetic procedures and allow to focus on the attachment of variable pendants on the scaffold against heteroaromatic compounds. The structure of the three-tailed inhibitors (TTIs) was selected to merge an easy and versatile chemistry with the opportunity to expand it to many different chemical groups, which is significant for generating a range of tail combinations (Figure 1B). The synthetic strategies adopted to yield the TTI derivatives here discussed were previously reported by us.

3. Results and discussion

Several drug design studies of CAIs adopted the p-substituted benzenesulfonamide as a main scaffold against heteroaromatic sulphonamides to notably simplify the synthetic procedures and allow to focus on the attachment of variable pendants on the inhibitor structure. A. P-substituted benzenesulfonamide was adopted by us as a CAI scaffold to converge efforts and attention on studying the three-tailing effects on CA inhibition. The structural changes in the TTI series were previously reported by us. The synthetic strategies adopted to yield the TTI derivatives here discussed were previously reported by us.

3.1. Carboxic anhydrase inhibition

In this first screening, mono-tailed (1–7) and three-tailed (18–50) compounds were analysed by a stopped-flow kinetic assay with: the tumour-associated isoforms CA I, II, and IV and XII. The selectivity index of mono-tailed and three-tailed compounds vs. the off-target isoforms CA I and II are reported in Table S1 (Supplementary Information).

While the structure–activity relationship (SAR) against CA I, II, and IV have already been discussed in depth, only the inhibitory action of mono-tailed inhibitors and TTIs against the tumour-
associated isoforms CA IX and XII was here investigated and compared.

Generally, the inhibition data reported in Table 1 highlighted that mono-tailed compounds 1–7 were medium to high nanomolar inhibitors of CA I ($K_i = 68.4 – 458.1 \text{ nM}$), II ($K_i = 62.8 – 153.7 \text{ nM}$), IX ($K_i = 56.5 – 108.7 \text{ nM}$) and XII ($K_i = 55.4 – 113.2 \text{ nM}$), and weak inhibitors of CA IV with inhibition constant ($K_i$) values in the low micromolar range ($1.1 – 6.2 \mu\text{M}$).

In detail, the tumour-associated isoforms CAs IX and XII were inhibited almost immediately by the single-tail compounds 1–7. Nonetheless, derivatives 3 ($R_1 = 4\text{-F-}C_6H_4$) and 5 ($R_1 = \text{Fu}$) stood out as the best inhibitors of CA IX ($K_i = 56.5 \text{ nM}$) and XII ($K_i = 55.4 \text{ nM}$), respectively, whereas the cyanoalkyl- and phenethyl-tailed compounds 6 and 7 exhibit $K_i$s above 100 nM against both isoforms.

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41 and 43 were medium nanomolar inhibitors with \( K_i \) of 11.1 and 34.3 nM, respectively. Again, derivative 40 is the most selective inhibitor against CA IX vs. the off-target CA II (CA II/CA IX = 91.8).

The tumour-associated CA XII was strongly inhibited by 42 with a subnanomolar \( K_i \) of 0.6 nM that makes it the most potent and selective compounds against this isoform (CA I/CA XII = 752.3; CA II/CA XII = 50.7), whereas 40 (\( K_i \) = 83.7 nM), 41 (\( K_i \) = 42.7 nM), and 43 (\( K_i \) = 10.5 nM) acted with a \( K_i \) in the medium nanomolar range.

(v) The fifth subset (45–48) obtained by the introduction of a potentially negatively charged tail in R3 showed a general increment of the inhibition activity against CA I, II, IV, IX, and XII compared to their analogues 34, 35, 37, and 38. Against CA IX compound 45 and 46 acted in the low nanomolar range (\( K_i \) = 7.5 and 1.2 nM, respectively), where the last one resulted to be the most potent and the third most selective inhibitor vs. CA I (CA I/CA IX = 66), while 47 (\( K_i \) = 11.1 nM) and 48 (\( K_i \) = 22.1 nM) inhibited this isoform with \( K_i \) in the medium nanomolar range.

Moreover, derivatives 46–48 were low nanomolar inhibitors of CA XII (\( K_i \) = 7.1, 8.8, and 6.8 nM, respectively), whereas 48 and 46 resulted to be the second and third most potent inhibitors of this glaucoma-associated isoform, while compound 45 acted with a \( K_i \) of 29.7 nM.

Comparing the fourth (40–43) and the fifth subset (45–48) it was detected that the presence of R3 = \((\text{CH}_2)_2\text{COOH}\) in place of amine tails shifted the activity against CA I.

Finally, the loss of the hydrophilic tail R3 in 50 decreased the activity against CA I (\( K_i \) = 737.9 nM), II (\( K_i \) = 132.0 nM), IV (\( K_i \) = 1.8 \( \mu \)M), and IX (\( K_i \) = 61.1 nM) without effects against CA XII (\( K_i \) = 5.5 nM), obtaining the second most potent and selective compound against this isoform (CA I/CA XII = 134.2).

### 3.2. X-ray crystallography

Co-crystallisation of hCA II and hCA IX-mimic with some of the new inhibitors resulted in solved crystal structures with resolutions between 1.39 and 1.56 Å (Figure 2 and Table S2, Supplementary Information). All inhibitors contain a conserved benzenesulfonamide in the same orientation that acts as a zinc-binding group that displaces zinc bound water (ZBW) to form a hydrogen bond between the amide backbone of Thr199 and oxygen of sulfonamide (2.9–3.0 Å). With these similarities in the inhibitors, any difference in observed binding affinity results from modifications to the tail regions.

In complex with hCA IX-mimic, compound 41 showed an observed omit map electron density lacking the whole entirety of T2 and T3 tails (PDB: 7SUW, Figure 3(A)). The T1 furyl moiety is accommodated within the lipophilic pocket made by residues Val135, Leu198, Pro202, and Ala204, as it did within the hCA II active site (Figure 4(B)).

Compound 42 reported a strong observed omit map electron density, which may indicate stronger binding and/or a high binding occupancy (PDB: 7SUY, Figure 3(B)). Again, the swap from a furyl (41) to a 4-F-benzyl (42) in T1 induced the halo aromatic ring to lie over the lipophilic cleft made by Val131, Val135, Pro202, and Ala204. In contrast, the phenethyl portion in T2 interacts with the opposite side of the hCA IX-mimic hydrophobic half, namely with...
Trp5, Phe20, and Pro201 (Figure 4(C)). The protonated amino-propyl tail was exposed to bulk solvent.

Compound 46 had a weak observed omit map electron density around the benzene ring and carboxylic acid tail (PDB: 7SV8, Figure 3(C)). The furylmethyl tail exhibited lipophilic interactions with Gly132 and Val135. The COO⁻ in T3 was involved in a water-mediated H-bond network with Gln92 that included the amide carbonyl group, while the T3 alkyl tail showed interactions with Leu91 (Figure 4(D)).

Compound 48 showed a weak observed omit map electron density around the benzene ring in T2 (PDB: 7SV1, Figure 3(D)). Expectedly, not being able to occupy the lipophilic pocket nearby Leu198 for steric hindrance reasons, the naphthyl ring in T1 lied over the region lined by Trp5, Phe20, Pro201, and Pro202. The benzene ring in T3 was interestingly found to interact with the outer portion of the α-helix including residues 130–136 and is partially exposed to bulk solvent, whereas the carboxyethyl tail folded back towards the cavity, making an intramolecular water bridge with the protonated amine group branching T2 and T3. However, it held water-bridged H-bonds to Gln92 together with the amide carbonyl group (Figure 4(E)).

Again, the binding mode exhibited by 46 was related to the best hCA IX inhibition measured in vitro (Kᵢ of 1.2 nM), though a minor difference was detected among the co-crystallised ligands with respect to hCA II. Likewise, the binding mode of 42, that mostly deviated from that of the other ligands and also within hCA IX-mimic, led to an efficient hCA IX inhibition of 4.8 nM. The 20-fold drop of efficacy passing from 46 to 48 (Kᵢ from 1.2 to 22.1 nM) might be related again to the furyl/naphthyl switch that provoked a significant loss of favourable contacts within the active site.

Figure 5 depicts the superimposed binding orientations of compounds 41, 42, 46, and 48 within the active site of hCA II and hCA IX-mimic. Although partially missing electron density was observed for compound 41 in the hCA IX-mimic active site, a similar orientation to the ligand binding mode in hCA II was observed for T1 and for the linker up to the T2/T3 branching junction. Hence, very similar ligand orientations exist for inhibitors 41 (Figure 5(A)) and 42 (Figure 5(B)) bound to hCA II and hCA IX-mimic. In contrary, greater differences were detected when ligands 46 and 48 bound to the active site of the two isoforms. Nonetheless, it should be stressed that all four compounds showed a significantly greater efficacy as inhibitors of hCA IX than hCA II, with selectivity that spans between 2- and 6-fold. In fact, hCA II had an average Kᵢ of 41 nM (for compounds 41, 42, 46, and 48), whereas hCA IX had an average Kᵢ of 10 nM for these same inhibitors. In this context, Val131 in hCA IX compared to Phe131 in hCA II is the major difference in active sites between the two isoforms. Inhibitors more easily enter the active site in hCA IX due to the smaller amino acid at residue 131. What is more, Phe131 of hCA II can produce less favourable positioning and conformational geometry of the inhibitors with respect to hCA IX-mimic.

Comparing compound 46 between hCA II and hCA IX-mimic shows that in the latter, the amide linker shifted towards the hydrophobic region most likely as a result of less steric hindrance from Val131. As a result, the two cyclic tails rotate in towards the active site, preventing clashes with the enzymes surface residues (Figure 5(C)). As for compound 48, there was also less steric hindrance from Val131 which allowed the phenyl tails to rotate about the linker (Figure 5(D)). The clearer case was that of compound 48.
although a very similar orientation was observed when bound within the two active sites, the inhibitor tails slightly moved towards the hydrophobic half of hCA IX-mimic accordingly to the less steric hindrance from Val131 (Figure 5(B)) just enough to improve the binding and thus inhibition efficacy.

3.3. Antiproliferative studies

Hypoxic tumours are a heterogeneous mass of cells with different degrees of oxygen supply[1,6–8,54]. The cells in the internal part of the mass grow under hypoxic conditions while the external ones have a more physiological supply of oxygen. In normoxic cells (where CA IX and XII are normally expressed on the membranes), CAIs could predominantly act on the cytosolic isoforms, blocking the ability of cells to maintain the intracellular and extracellular pH values compliant for their survival. Instead, in the hypoxic ones, CAIs also inhibit the tumour-membrane-associated isoforms of CA IX and XII that result overexpressed in these conditions. Obviously, achieving selective inhibition of CA IX and XII, expressed in both cell types, is preferable to avoid the side effects related to the inhibition of the off-target isoform also present in the healthy cells with CAIs.

To evaluate in vitro the effects on the viability of colon (HT29), prostate adenocarcinoma (PC3) and breast cancer (ZR75-1) cell

![Figure 5. Superimposition of the crystallographic binding orientations adopted in the active site of hCA II (grey) [36] and hCA IX-mimic (light blue) for A) 41, B) 42, C) 46, and D) 48. Colours are as in Figure 6 of Bonardi et al. [36] and Figure 4.](image)

![Figure 6. In vitro cell viability assay of colon adenocarcinoma (HT-29), prostate adenocarcinoma (PC3), and breast cancer (ZR75-1) cell lines after 48 h of treatment with three different concentrations (10, 30, and 100 μM) of three-tailed inhibitors 28 (orange), 34 (yellow), 36 (blue), and 50 (green) in normoxic (21% O2) and hypoxic (3% O2) conditions. Control cells are arbitrarily set at 100% and results are expressed as the mean ± SEM of three experiments. One-way ANOVA was performed followed by a Bonferroni’s significant difference procedure. *p < .05, **p < .01, and ***p < .001 vs. control; †p < .05 and ‡p < .01 vs. normoxia.](image)
lines, TTIs 28, 34, 36, and 50 were selected among all the synthesised derivatives for their selectivity against CA IX and XII and also considering the nature of the tails. Cells were incubated for 48h with three different concentration of inhibitors (10, 30, and 100 μM) in normoxic (21% O₂) and hypoxic (3% O₂) conditions.

All compounds act in a dose-dependent manner similarly in normoxia and hypoxia against HT29 and PC3 cancer cell lines, resulting in a possession of a slightly most potent effect in hypoxic conditions vs. the cell line ZR75-1. Moreover, inhibitors 28 and 36 were the most active against all the used cell lines while derivative 50 reduced the ZR75-1 cell viability.

In detail, inhibitor 28 was effective at the lowest concentration (10μM) only against HT-29 cells, decreasing viability by about 55% in normoxia and 40% in hypoxia. The other concentrations (30–100μM) further reduced viability by more than 90% in HT-29 and PC3 cell lines and were slightly less effective in ZR75-1 where the reduction was around 80%.

Inhibitors 34, 36, and 50, on the other hand, have no effect at a concentration of 10μM, except for compound 36 on PC3 cells in normoxia and on ZR75-1 cells in both conditions (15% of decrease). Interestingly, compound 50 at 10μM on ZR75-1 was effective in hypoxic condition compared to normoxia, inducing a 15% of reduction (Figure 6).

Inhibitor 34 at a concentration of 30μM affected the viability under normoxic condition up to 40% in HT-29 cells and 15–20% in PC3 and ZR75-1 cells. In hypoxic condition, it was less effective on HT-29 and PC3 cell lines, while ZR75-1 cell viability decreased by 30% compared to control and by 10–15% respect to normoxic condition. Even the concentration 100μM of inhibitor 34 was able to induce cell death in a similar percentage for the three cell lines (50–60%) under normoxic condition, and in hypoxia ZR75-1 cells were confirmed as the most sensitive compared to the other cell lines.

Derivative 36 was more effective against PC3 and ZR75-1 at 30μM and 100μM in both conditions, with a reduction of cell viability by around 80–90%, except for the 30μM in hypoxia on PC3 cells where the viability decreased only by 50%. On HT-29 cells inhibitor 36 induced a 40% and 90% reduction at 30 and 100μM, respectively, in both conditions.

Finally, inhibitor 50 had no effect at 30μM on HT-29 cells, and at 100μM a 40% reduction was observed in both conditions; on PC3 cells under hypoxic condition the concentration 30μM of derivative 50 induced a reduction by 30%, while at 100μM a more effective action under hypoxic condition was determined (almost 50% compared to normoxia); also ZR75-1 cells were sensitive to inhibitor 50, which was active at 30μM (respectively, 20% and 30% of reduction in normoxia and hypoxia) and at 100μM could be induced 65% of death in normoxia and a significant further decrease in hypoxia (5–10% less).55-58

4. Conclusion

In recent decades the human (h) CAs (EC 4.2.1.1) isoforms IX and XII were validated as anticancer targets against solid hypoxic tumours. The lack of selectivity of sulphonamide CAIs prevents their wider use as therapeutic agents owing to the side effects onset, mainly due to the inhibition of the ubiquitous human (h) CA I and II. To overcome this issue the “three-tails approach” is here proposed as an extension of the forerunner “tail” and “dual-tail approach” to fully exploit the amino acid differences at the medium/outer active sites rim among the different hCA active sites. The majority of the thirty-three synthesised TTIs resulted in a higher selectivity against the tumour-associated isoforms hCA IX and XII with respect to the off-targets hCA I and II than the mono-tailed compounds (CA I/CA IX = 1.8–225.5; CA II/CA IX = 1.3–91.8; CA I/CA XII = 2.3–752.3; CA II/CA XII = 1.3–90.0). X-ray crystallography studies were performed to investigate the binding mode of four TTIs (41, 42, 46, and 48) in complex with hCA IX mimic. Moreover, the ability of the most potent and selective TTIs (28, 34, 36, and 50) to reduce in vitro the viability of colon (HT-29), prostate adenocarcinoma (PC3), and breast cancer (ZR75-1) cell lines was evaluated in normoxic (21% O₂) and hypoxic (3% O₂) conditions. In particular, all tested compounds act in a concentration-dependent manner similarly in normoxia and hypoxia against HT-29 and PC3 cancer cell lines, and with a slightly most potent effect in hypoxic conditions against ZR75-1 cell line. Moreover, inhibitors 28 and 36 resulted in the most active derivatives against all the cell lines used while derivative 50 was able to strongly affect PC3 and ZR75-1 cell viability under hypoxic condition compared to normoxia.

Disclosure statement

CT Supuran is Editor-in-Chief of the Journal of Enzyme Inhibition and Medicinal Chemistry. He was not involved in the assessment, peer review, or decision-making process of this article. The authors have no relevant affiliations of financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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