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# Phthalazine Sulfonamide Derivatives as Carbonic Anhydrase Inhibitors. Synthesis, Biological and *in silico* Evaluation

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Carbonic Anhydrases (CAs) are a large family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide involved in several biological processes. They show a wide diversity in tissue distribution and their subcellular localization. Twenty-two novel phthalazine derivatives were designed, synthesized, and evaluated against four human isoforms: hCA I, hCA II, hCA IX, and hCA XII. Compounds appeared to be very active mostly against hCA IX (**7**) and hCA I (**6**) isoforms being more potent than reference drug acetazolamide (**AAZ**). Some compounds appeared to be very selective with a selectivity index up to 13.8. Furthermore, docking was performed for some of these compounds on all isoforms to understand the possible interactions with the active site. Additionally, the most active compounds against hCA IX were subjected to cell viability assay. The anticancer activity of the compounds (**3 a–d**, **5 d**, **5 i**, and **5 m**) was investigated using two human breast cancer cell lines, *i.e.* MCF-7 and MDA-MB-231 cells, and the normal counterpart, namely MCF10-A cells.

## Introduction

In recent years, there has been a growing recognition of the significance of carbonic anhydrases (CAs; EC 4.2.1.1), a family of zinc metalloproteins, in the field of cancer biology. Among the 15 isoforms identified in humans that catalyse the hydration of carbon dioxide to bicarbonate and proton, two cell surface CA isoforms, namely CA IX (predominantly associated with tumours) and CA XII (over expressed in certain tumour types), assume pivotal roles in tumorigenesis.[1–5] The connection between CAs and cancer arises from their ability to regulate the tumour microenvironment, influencing essential processes in cancer progression, such as angiogenesis, invasion, and metastasis. Moreover, CAs have been implicated in enabling cancer cells to adapt to the acidic and hypoxic conditions prevalent within tumours, providing them with a survival advantage.[5,6] This is particularly relevant given that poor oxygen supply and hypoxia-related acidosis are common phenotypic characteristics in many malignancies. Additionally, it is important to highlight that hypoxia-related acidosis not only triggers inactivation but also prompts metabolic adaptations in highly malignant cell phenotypes.[4]

Focusing on the tumour-associated hCA IX is now recognized as a promising strategy in the development of novel anticancer drugs designed for hypoxic tumours. Different approaches and novel families of pharmacological agents have been explored to create compounds that selectively target CA IX while minimizing interactions with the ubiquitous cytosolic off-target isoforms CA I and CA II. The majority of oncology drugs belong to the class of heterocyclic compounds, featuring one or more nitrogen atoms.[7] In this context, phthalazine scaffold has been effectively incorporated into therapeutic agents with diverse applications, including antimicrobial,[7,8] anti-inflammatory,[9,10] anticancer,[11,12] antidiabetic,[13,14] anticonvulsant,[15,16] and cardiotonic[17] activities. Furthermore, there are many drugs with phthalazine scaffold used, such as azelastine, H1 receptor-blocker to treat allergic rhinitis and eye drops for allergic conjunctivitis, zopolrestat, aldose reductase inhibitor for the treatment of diabetes type II, MY 5445 – specific inhibitor of phosphodiesterase against human platelet

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aggregation, hydralazine, medication used to treat high blood pressure and heart failure, tepotinil, kinase inhibitor for the treatment of non-small cell lung cancer (NSCLC), olapanil and talazopanil PARP (poly ADP ribose polymerase) inhibitors – medications to treat BRCA – mutated advanced ovarian cancer in adults (Figure 1).

Recently, phthalazine derivatives have also been under investigation as carbonic anhydrase inhibitors.[18–22] Turkes *et al.*[18] reported CA inhibitory activity of novel *N*-substituted phthalazine sulfonamide derivatives against human isoform hCA I and hCA II with $K_i$ in the range of 0.12–12.46 nM and 0.63–7.54 nM respectively. Berber *et al.*[19] synthesized new phthalazine urea and thiourea derivatives and demonstrated that all compounds inhibited hCA I and hCA II isoforms with inhibition values in the range of 6.00–24.00 $\mu$M. In another paper, Berber[20] reported the synthesis and evaluation of new phthalazine substituted β-lactam derivatives as carbonic anhydrase inhibitors against hCAI and hCA II with $IC_{50}$ ranging from 6.97–20.51 $\mu$M and 8.48–24.84 $\mu$M, respectively. Nevertheless, the potential use of this scaffold to develop inhibitors targeting the tumour-associated isoforms CA IX and CA XII has not yet been explored. Based on all mentioned above we designed and synthesized twenty-two phthalazine derivatives and evaluated for their CA inhibitory activity against the off-target isoforms (hCA I and hCA II) and the tumor-associate isoforms hCA IX and hCA XII.

## Results and Discussions

### Chemistry

Compounds **3a–d**, **5a–m**, and **6** were synthesized according to Scheme 1 and Scheme 2, while derivatives of the condensed tricyclic system of triazolophthalazine **8a–d** according to Scheme 3. Compounds **3a–d**, **5a–m**, and **6** were obtained by the reaction of nucleophilic substitution of the chlorine atom in 1-chloro-4-R-phthalazines by various amines at boiling during 2–3 hours (Scheme 2). The best solvent for this reaction was methyl cellosolve providing good yield and purity of the reaction products. It was noted that the addition of ammonia to convert the salts of the product into bases should be carried out at 35 °C (for compounds **3a–d**, **6**) and 40–45 °C (for compounds **5a–m**) since neutralization at room temperature required a long time.

Compounds **8a–d** were obtained by the interaction of phenyl-substituted triazolophthalazine **7a–d** with chlorosulfonic acid. The reaction started at 0–5 °C and proceeded at 150 °C. To convert the resulting hydrochlorides into bases, they were treated with 20% $NH_4OH$ solution during vigorous stirring for 2 hours at 0–5 °C.

The phthalazine structures were confirmed by $^1$H NMR spectroscopy. The nature of the aromatic proton signals in these compounds (**3a–d**, **5a–m**, **6**, **8a–d**), corresponds to the proposed structures and differs from the signals of 1-chloro-4-R-phthalazines.[23] In the $^1$H NMR spectra of compounds **3a**, **5a–m**, **6**, and **8a–d** in deuterochloroform, the signals of the methyl groups of 2-methylbenzenesulfonamide of phthalazine are

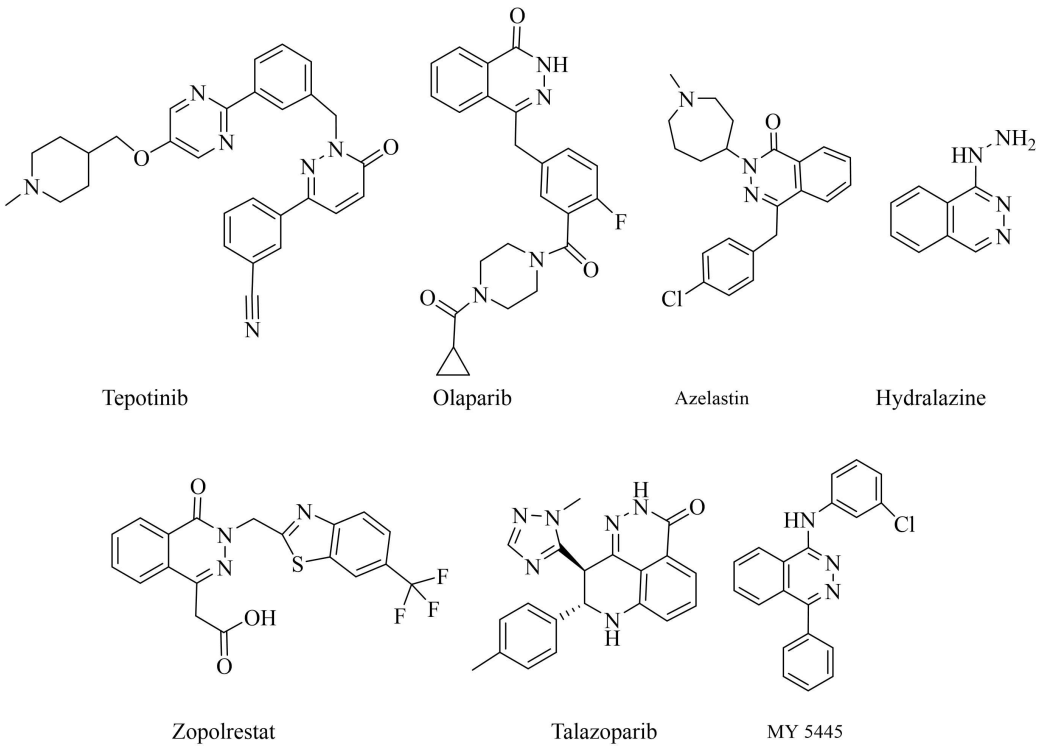


**Figure 1.** FDA approved drugs with phthalazine scaffold.


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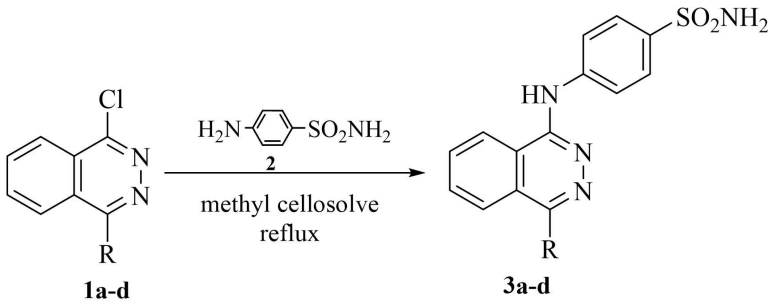
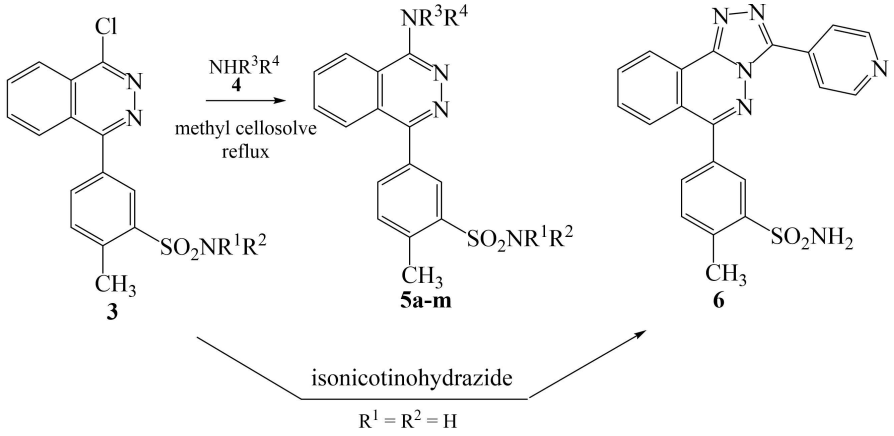


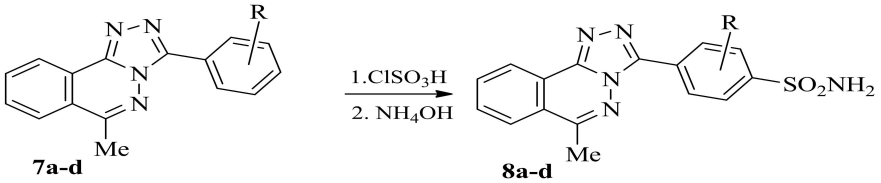

**1, 3** R = Me (**a**), 4-$HOC_6H_4$, (**b**), 4-$C(O)NH_2C_6H_4$ (**c**), 4-$OCH_2CHC_6H_4$ (**d**)

**Scheme 1.** Synthesis of compounds **3 a–d**. *Reagents and conditions:* 4-substituted of 1-chlorophthalazine, 4-aminobenzenesulfonamide, methyl cellosolve, refluxed, 3 h, 20% $NH_4OH$, 20 °C, 1 h.




**5** $R^1 = R^2 = H$, $R^3 = H$: $R^4$ = 3-$HOC_6H_4$ (**a**), 4-$HOC_6H_4$ (**b**), 4-$CH_3OC_6H_4$ (**c**), 4-$C(O)NH_2C_6H_4$ (**d**), 3-$C(O)NH_2$-4-$CH_3OC_6H_3$ (**e**), 4-$NH_2SO_2C_6H_4$ (**f**), 4-$OCH_2C(O)NHCH_3C_6H_4$ (**g**), $R^3R^4$ = morfoline-4-yl (**h**); $R^1 = R^3 = H$, $R^2 = CH_3$, $R^4$ = 4-$NH_2SO_2C_6H_4$ (**i**); $R^1 = R^2 = CH_3$, $R^3 = H$, $R^4$ = 4-$NH_2SO_2C_6H_4$ (**j**); $R^1 = R^3 = H$, $R^2 = (CH_2)_3OH$, $R^4$ = 4-$NH_2SO_2C_6H_4$ (**k**); $R^1R^2$ = morfoline-4-yl, $R^3 = H$, $R^4$ = 4-$NH_2SO_2C_6H_4$ (**l**); $R^1R^2$ = 4-methylpiperazine-1-yl, $R^3 = H$, $R^4$ = 4-$NH_2SO_2C_6H_4$ (**m**).

**Scheme 2.** Synthesis of compounds **5 a–m** and **6**. *Reagents and conditions:* 4-substituted of 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide, substituted amine, methyl cellosolve, refluxed, 2 h, 5% $NH_4OH$, 20 °C, 0.5 h.




**7, 8** R = 2-Me (**a**), R = 4-Me(**b**), R = 2-OMe (**c**), R = 4-OMe

**Scheme 3.** Synthesis of compounds **8**. *Reagents and conditions:* chlorosulfonic acid, 0 °C, 10 min, phenylsubstituted triazolophthalazine **7 a–d**, 0–5 °C, 1 h, 200 g of crushed ice 15 min, 20% $NH_4OH$, 20 °C, 2 h.

located in the upfield part of the spectra (2.73–2.74 ppm). The NH proton in 4-substituted phthalazine (**3**, **5**) appears as a singlet in the region 8.69–9.50 and is absent in compounds **8**. The spectra of the studied compounds are also characterized by the presence of phthalazine multiplets and a substituted phenyl ring in the region of 7.10–8.70 ppm in their aromatic region.

### Carbonic Anhydrase Inhibition

All compounds, here reported, were evaluated for their inhibitory activity against four human CA isoforms, namely: hCA I, hCA II, hCA IX, and hCA XII. The results are shown in Table 1.


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**Table 1.** Inhibition data of human CA isoforms I, II, IX, and XII with compounds **3a–d**, **5a–m**, **6**, **8a–d**, and **AAZ** by a stopped-flow $CO_2$ hydrase assay.[24]

| | $K_i$ (nM)* | | | |
|---|---|---|---|---|
| Cmp | hCA I | hCAII | hCA IX | hCA XII |
| **3a** | 24.9 | 23.7 | 25.5 | 45.6 |
| **3b** | 55.5 | 5.5 | 4.6 | 15.1 |
| **3c** | 72.7 | 1.6 | 3.7 | 471.6 |
| **3d** | 759.8 | 52.8 | 8.9 | 405.3 |
| **5a** | 802.7 | 25.7 | 1080 | 497.8 |
| **5b** | 899.4 | 55.4 | 700.0 | 29.7 |
| **5c** | 1531 | 33.6 | 33.3 | 126.2 |
| **5d** | 78.4 | 40.9 | 4.8 | 361.1 |
| **5e** | 6607 | 656.5 | 238.9 | 885.4 |
| **5f** | 4210 | 19.5 | 1410 | 65.0 |
| **5g** | 6206 | 67.1 | 2831 | 414.3 |
| **5h** | 4185 | 176.2 | 979.0 | 375.1 |
| **5i** | 83.5 | 4.3 | 23.6 | 592.6 |
| **5j** | 5719 | 346.8 | 129.5 | 6761 |
| **5k** | 83.8 | 23.1 | 27.0 | 435.4 |
| **5l** | 1410 | 52.7 | 35.3 | 64.5 |
| **5m** | 589.1 | 15.0 | 19.7 | 597.7 |
| **6** | 4810 | 39.8 | 43.2 | 463.6 |
| **8a** | 6517 | 2694 | 2398 | 8390 |
| **8b** | 7523 | 2451 | 3040 | 5698 |
| **8c** | >10000 | 6124 | >10000 | >10000 |
| **8d** | 7661 | 4147 | 1341 | 5612 |
| **AAZ** | 250.0 | 12.1 | 25.8 | 5.7 |

*Mean from 3 different assays, by a stopped-flow technique (errors were in the range of ±5–10% of the reported values).

From the data in Table 1, it is obvious that all compounds demonstrated inhibition against all hCA isoforms but with different inhibition constants. Among all tested isoforms the compounds tested displayed the best activity against the hCA IX isoform followed by hCA I, hCA II, and finally, hCA XII. The $K_i$ values of compounds against the hCA I isoform were in the range of 24.9->10000 nM. Nevertheless, six out of twenty-two tested compounds appeared to be more potent ($K_i$ = 24.9–83.8 nM) compared to the reference drug acetazolamide **AAZ** ($K_i$ = 250 nM). The most active compound appeared to be compound **3a** with a $K_i$ value of 24.9 nM almost ten times better that the reference compound, followed by compound **3b** ($K_i$ = 55.5 nM), while the lowest activity was shown by compound **6**. The order of activity of compounds towards hCA I can be presented as follows: **3a** > **3b** > **3c** > **5d** > **5i** > **5k** > **5m** > **3d** > **5a** > **5b** > **5e** > **5c** > **5h** > **5f** > **6** > **5j** > **5g** > **5l** > **8a** > **8b** > **8d** > **8c**.

The structure-activity relationship studies revealed that the presence of methyl group at position 4′ of phthalazine ring as well as 4-aminobenzensulfonamide substitution (**3a**) is very beneficial for inhibition of hCA I isoform. The introduction of the 4-hydroxybenzene group at position 4′ of phthalazine ring (**3b**) decreased activity 2 folds, while the introduction of benzamide at position 4′ of the phthalazine ring, led to less active compound **3c**, neverthless remaining among three the most active compounds. It is interesting to notice that the presence of 4-methyl-3-sulfamoylphenyl group at position 1′ of the phthalazine ring as well as 4-sulfamoylphenylamino substitution resulted in compound **5d**, slightly less active than compound **3c**. On the other hand, the presence of 5-(3-(pyridin-4-yl)-[1,2,4]-triazolo[3,4-a]phthalazin-6-yl) on position 5 of 2-methylbenzensulfonamide (**6**) ring was detrimental for hCA I inhibitory activity.

As far as hCA II is concerned three compounds among tested exhibited activity with $K_i$ from 1.6–5.5 nM higher than that of **AAZ** ($K_i$ = 12.1 nM). The order of activity can be presented as: **3c** > **5i** > **3b** > **5m** > **5f** > **5k** > **3a** > **5a** > **5c** > **6** > **5d** > **5e** > **3d** > **5b** > **5g** > **5h** > **5j** > **5l** > **8b** > **8a** > **8d** > **8c**. Thus, the most active against hCA II was found to be compound **3c** ($K_i$ = 1.8 nM) and selectivity index (SI) towards hCA I and hCA XII of 45.44 nM and 294.75 nM, respectively. Compound **5i** was the next more active ($K_i$ = 4.3 nM) compared to **AAZ** ($K_i$ = 12.1 nM) and SI 19.42. 5.49 and 137.81 against hCA I, hCA IX, and hCA XII, respectively. It should be mentioned that compound **3c** also was among the most active against the hCA I isoform.

According to structure-activity relationship studies the presence of benzamide at position 4′ of the phthalazine ring has a positive influence on hCA II inhibitory activity. Introduction of 4-methyl-*N*-2-methylsulfamoylbenzene at position 4′ of the phtalazine ring led to compound **5i** with decreased approximately 3 times activity but with SI 19.42,5.49 and 137.81 towards hCA I, hCA IX and CA XII, respectively. The presence of 4-hydroxybenzene substitution resulted in slightly less active compound **3b** compared to the previous one, while compound **8c**, as in the case of inhibition of hCA I had a very negative influence on hCA II inhibitory activity.

In the case of hCA IX seven compounds ($K_i$ = 3.7–25.5 nM) appeared to be more potent than **AAZ** ($K_i$ = 25.8 nM). The order of activity of tested compounds towards hCA IX isoform can be presented as follows: **3c** > **3b** > **5d** > **3d** > **5m** > **5i** > **3a** > **5k** > **5c** > **5e** > **6** > **5j** > **5l** > **5b** > **5h** > **8d** > **5a** > **5f** > **8a** > **5g** > **8b** > **8c**. Thus, compound **3c** as in the case of hCA II isoform achieved the best activity with $K_i$ = 3.7 nM and SI 19.65 and 127.46 against hCA I and hCA XII isoforms, respectively. The next more active compound was **3b** ($K_i$ = 4.6 nM), which was also the second active against hCA I isoform followed by compound **5d** ($K_i$ = 4.8 nM) and SI 16.33, 8.52 and 75.23 towards hCA I, hCA II and hCA XII isoforms, respectively. The fourth active compound appeared to be compound **3d** with $K_i$ = 8.9 nM compared to **AAZ** ($K_i$ = 25.8 nM) and SI 85.37, 5.93 and 45.54 against hCA I, hCA II and hCA XII isoforms, respectively.

It was observed that the presence of benzamide at position 4′ of the phthalazine ring, as in the case of hCA II inhibition was beneficial for hCA IX inhibitory activity. Replacement of benzamide substitution by 4-hydroxybenzene led to compound **3b** slightly less active than **3c**. The presence of 2-methylbenzensulfonamide group at position 1 of the phthalazine ring,


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as well as 4-sulfamoylphenylamino substitution (**9**) decreases slightly the activity being almost equipotent with compound **3b**. It was observed that the introduction of 3-methoxy-4-benzensoulfonamide group on triazole ring fused with phthalazine one in all cases was detrimental to the inhibition of all studied isoforms.

It should be mentioned that no one compound was found to be more potent than **AAZ** against hCA XII isoform. Nevertheless, the most active compound was **3b** ($K_i = 15.1$ nM) followed by compound **5b** with $K_i = 20.7$ nM and SI 30.28,1.87 and 23.57 towards hCA I, hCA II and hCA IX isoforms, respectively according to activity order: **3b** > **5b** > **3a** > **5e** > **5f** > **5c** > **5d** > **5h** > **3c** > **5g** > **5k** > **6** > **3c** > **5a** > **8d** > **5i** > **5m** > **5l** > **8b** > **5j** > **8a** > **8c**. In this case, compound **6** was the least active ($K_i$ > 10000 nM).

In conclusion, we can say that compounds **3a**, **3b**, **3c** and **5i** were the most active against hCA I and hCA II isoforms, whereas, **3b**, **3c**, **3d** and **5d** against hCA IX isoform. Thus, compounds **3c** and **3b** were the most active ones against all isoforms tested. Furthermore, compounds **3c**, **5d** and **3d** were the most selective towards hCA XII isoform.

## Molecular Docking Studies

To predict the possible mechanism of inhibition of the tested compounds, molecular docking studies were performed on compounds **8c**, **3c**, **3d** and **5i** as representative of the whole set of compounds. The isoforms of human CAs have a similar active site that is 15 Å in depth and 15 Å broad, with conserved residues His94, His96, and His119 acting as zinc ligands, while conserved residues Thr199 and Glu105 acting as "gatekeepers".[25–28] However, these isoforms differ mostly in the residues around the entrance and exit of the active site cavity. The results of the molecular docking studies of the tested compounds on the hCA I, II, IX, and XII isoforms are shown in Table 2. All tested compounds bind enzymes, chelating the Zn (II) ion in a deprotonated form as anions (negative nitrogen of the sulfonamide group), according to docking results.[29]

According to docking studies, the compounds' selectivity and inhibitory profiles for each isoform are influenced by variations in the active sites of the enzymes. More specifically, the kind of amino acids present in the enzyme active site considerably affects the final conformation and interactions that compounds will adopt and form within the enzyme active site, influencing the inhibitory profile of the compounds.

For instance, compound **3d**, which binds both hCAs and has a $K_i$ value of 8.9 nM for hCA IX and 52.8 nM for hCA II, adopts a different conformation. This is possible because the

**Table 2.** Molecular docking free binding energies (kcal/mol) and interactions of selected compounds on hCA I, II, IV and IX isoforms.

| No | *h*CA isoform | Estimated free binding energy (Kcal/mol) | Chelating the Zn (II) ion | Residues involved in H-Bond interactions | Residues involved in Hydrophobic interactions |
|---|---|---|---|---|---|
| **3d** | hCA I | −5.22 | No | – | Val121, Leu198, Thr200 |
| | hCA II | −5.02 | No | – | Val143, Val121, Leu198, Thr200, Val207, Trp209 |
| | hCA IX | −11.06 | Yes | Gln67 (N··H, 2.42 Å), Thr199 (O··H, 1.79 Å) | Leu91, Val121, Val131, Leu141, Leu198, Thr200 |
| | hCA XII | −4.76 | No | – | Val121, Leu198 |
| **3c** | hCA I | −9.52 | Yes | Thr199 (O··H, 2.63 Å) | Val121, Ala135, Leu198 |
| | hCA II | −10.74 | Yes | Trp5 (O··H, 1.97 Å), Thr199 (N··H, 1.85 Å) | Trp5, Val121, Phe131, Leu198, Thr200 |
| | hCA IX | −11.84 | Yes | Gln67 (N··H, 2.54 Å), Gln92 (N··H, 2.41 Å), Thr199 (O··H, 2.05 Å), Thr200 (O··H, 2.17 Å) | Leu91, Val131, Leu198, Thr200 |
| | hCA XII | −6.25 | No | – | Leu198, Thr200 |
| **5i** | hCA I | −9.11 | Yes | Thr199 (O··H, 2.75 Å) | Ala132, Ala135, Leu198 |
| | hCA II | −9.14 | Yes | His94 (O··H, 1.94 Å) | Val121, Phe131, Leu198 |
| | hCA IX | −8.86 | Yes | Thr199 (N··H, 2.21 Å) | Val143, Leu198, Thr200 |
| | hCA XII | −5.27 | No | – | Val121, Leu198 |
| **8c** | hCA I | −2.11 | No | – | – |
| | hCA II | −6.86 | No | Thr200 (N··H, 3.11 Å) | Trp5, Val121, Phe131, Leu198 |
| | hCA IX | −3.71 | No | – | – |
| | hCA XII | -4.66 | No | – | Val121, Thr200 |
| **AAZ** | hCA I | −8.28 | Yes | Gln92 | Leu198, Thr199, Pro201, Trp209 |
| | hCA II | −8.87 | Yes | Thr199, Thr200 | Val121, Phe131, Leu198, Trp209 |
| | hCA IX | −9.02 | Yes | Thr199, Thr200 | Val121, Val143, Val131, Leu198, Trp209 |
| | hCA XII | −9.15 | Yes | Thr199, Thr200 | Val121, Val143, Leu198, Trp209 |


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hCA II enzyme has the hydrophobic residue Phe131, whereas the hCA IX enzyme in exchange has the minor residue Val131. The presence of this smaller residue in the hCA IX enzyme permits ligands, such as compound **3 d**, to freely reach the active site of the enzyme. On the other hand, residue Phe131 prevents this compound from interacting with the enzyme's active site, orientating the compound with the sulphonamide group outside of the active center, thus explaining its weak inhibitory effect (Figure 2).

Conversely, compound **3 c** docked into isoform hCA II, forms two hydrogen bonds between the sulfonamide group and the backbone of Thr199 and another H-bond between the O atom of the amide group of the compound and residue Trp5, which further stabilizes the complex and explains the high inhibition potency. The superposition of compound **3 c** in both isoforms II and IX shows that this compound is able to enter the active site of the hCA IX enzyme (Figure 3). This is because this compound is a more flexible molecule and capable of avoiding the steric hindrance of the bulky residue Phe131 of the hCA II isoform, increasing the inhibition potency. Moreover, in both complexes, the negative nitrogen of the sulfonamide group chelates the Zn (II) ion and forms hydrogen bonds. Furthermore, in both enzymes, hydrophobic interactions were detected contributing to the stabilization of the complexes and decreasing their free energy of binding (Figure 3).

On the other hand, compound **8 c** differs from compounds **3 c** and **3 d** because it is a less bulky molecule and unable to avoid the steric hindrance of the bulky residue Phe131 of hCA II isoform, decreasing dramatically its inhibition potency. As illustrated in Figure 4 superposition of both compounds **8 c** and **3 c** in the hCA II isoform clearly shows this phenomenon where compound **8 c** can't reach the active site of the enzyme.

## ADME Properties

The ADME properties, of all compounds are presented in Table 3. How well a drug will be absorbed from an oral solution is one of the main concerns. In this case, all compounds showed high gastro-internal absorption. Moreover, most of the com-

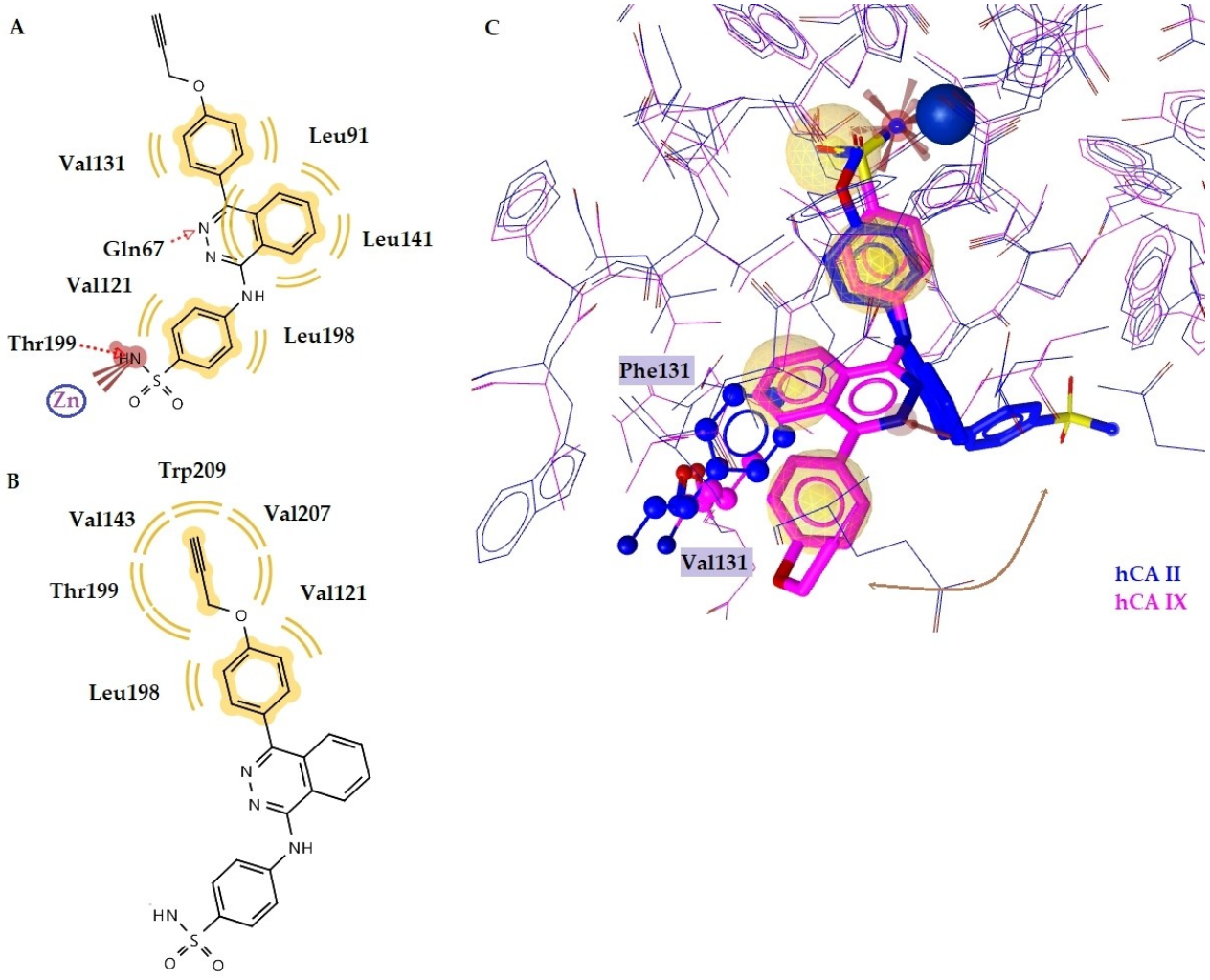


**Figure 2. A**) 2D interaction diagram of compound **3 d** docking pose interactions with the key amino acids in hCA IX; **B**) in hCA II; **C**) Superposition of compound **3 d** bound to hCA II (blue) in comparison to hCA IX (magenta), with specific residues labeled. Active site zinc is shown as a blue sphere, red dotted arrows indicate H-bond, and yellow spheres hydrophobic interactions.


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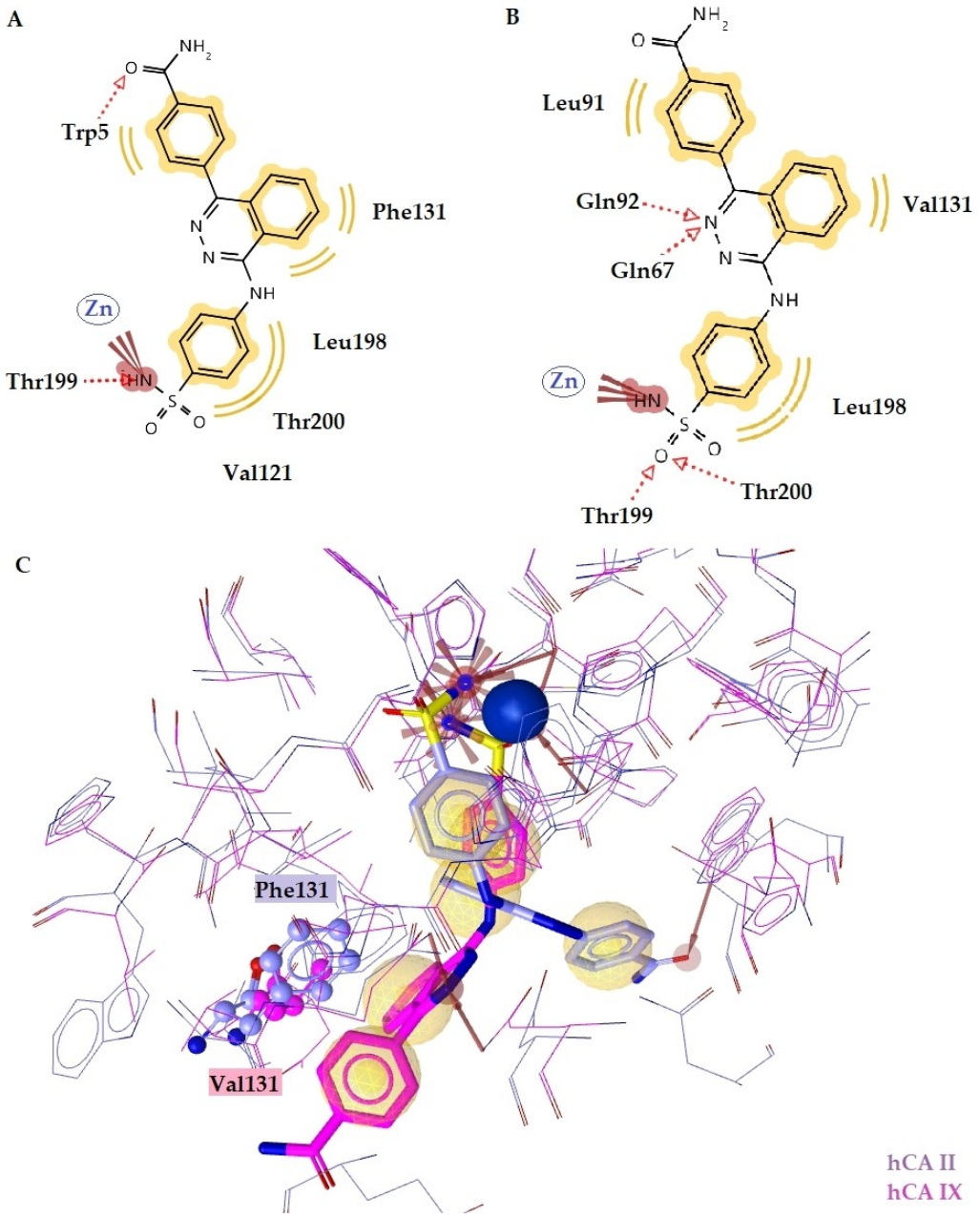


**Figure 3. A**) 2D interaction diagram of compound **3c** docking pose interactions with the key amino acids in hCA II, **B**) in hCA IX. **C**) Superposition of compound **3c** bound to hCA II (purple) in comparison to hCA IX (magenta), with specific residues labeled. Active site zinc is shown as blue sphere, red dotted and green arrows indicate H-bond and yellow spheres hydrophobic interactions.

pounds can be transported across the cell membrane by the ATP-binding cassette (ABC) transporter, which is a component of P-glycoprotein. However, the compounds were also predicted to be P-glycoprotein I and II inhibitors which can inhibit their transport. For skin permeability, a drug cannot be permeable if the logKp is more than −2.5. Within this theoretical framework, every compound showed improved skin permeability. The Volume of Distribution (VDss) suggests the total amount of drug needed to be homogeneously distributed in blood. Low VDss values are represented by below 0.15 log VDss, and high VDss values by $>0.45$ log VDss. In this case, only compounds **6** and **5c** can be characterised as high VDss compounds. The permeability of the blood brain barrier (BBB) indicates whether a substance can enter the brain or not. Since it is assumed that compounds with logBB $>0.3$ can pass BBB, all compounds' logBB values, indicate their low ability to pass BBB. Similarly, the majority of the compounds have low Central Nervous System (CNS) permeability. Metabolism prediction suggested that most of the compounds are both substrates and inhibitors of CYP3A4. Maximum Tolerated Dose (MRTD) is a crucial criterion for toxicity evaluation; a value of less than 0.44 log (mg/kg/day) is considered low, while a value of more than 0.477 log (mg/kg/day) is considered high. Thus, compounds, **3b**, **3d**, **5a**, **5b**, **5g**, **5l** and reference drug showed an


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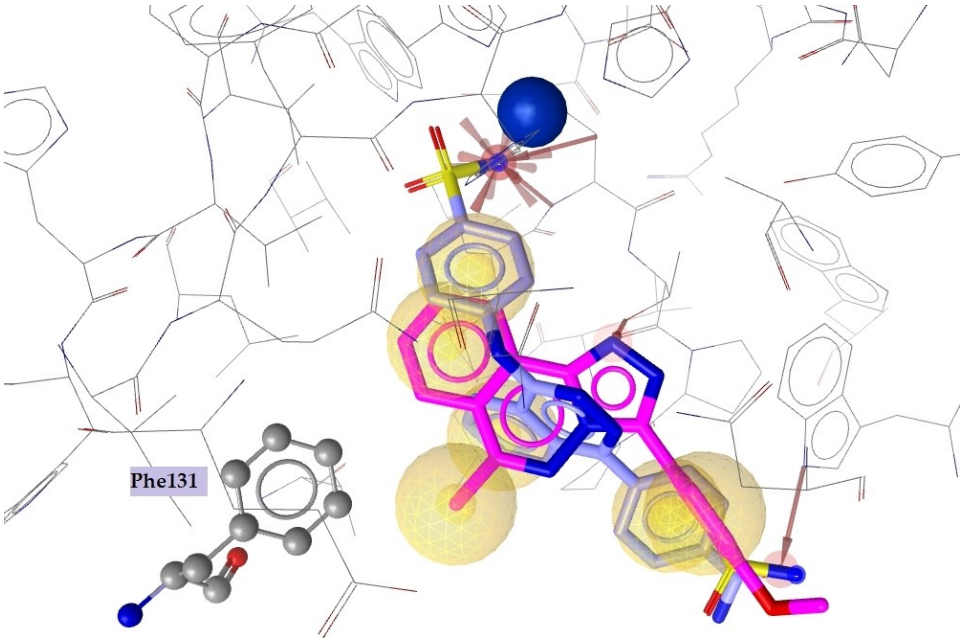


**Figure 4.** Superposition of compound **8c** (magenta) in comparison to compound **3c** (blue) bound to hCA II, with specific residues labeled. Active site zinc is shown as blue sphere, red dotted and green arrows indicate H-bond and yellow spheres hydrophobic interactions.

optimal MRTD. Moreover, most of the compounds showed no AMES toxicity, considered non-toxic, except for compounds, **5c**, **5g**, **5l**, **5m** and **8a**. Unfortunately, most of the compounds predicted to have hepatotoxicity except compound **6** with the most promising ADMET profile.

### Cell Viability Assay

The anticancer activity of the compounds (**3a–d**, **5d**, **5i** and **5m**) was investigated using two human breast cancer cell lines, *i.e.* MCF-7 and MDA-MB-231 cells, and the normal counterpart, namely MCF10-A cells. Cells were treated with the compounds for 72 hours, then their viability was assessed through the MTT assay, as reported in the experimental section, and the calculated $IC_{50}$ values are resumed in Table 4.

Compound **3d** exhibited the best activity against the MDA-MB-231 cells, with an $IC_{50}$ value of $14.2 \pm 0.2$ μM, without affecting the viability of the MCF-10 cells, at least until the concentration of 100 μM and under the adopted experimental conditions. However, a slight activity was evidenced against the MCF-7 cells ($IC_{50} = 85.5 \pm 0.8$ μM). Compound **3m** showed mild activity against both the MDA-MB-231 and MCF-7 cells, with $IC_{50}$ values of $64.3 \pm 1.1$ and $68.7 \pm 1.5$ μM, respectively, and as well in this case with no toxicity against the MCF-10 cells ($IC_{50} > 100$ μM). The other compounds were found inactive or non-selective (see Table 4). It should be noticed that the most active compound (**3d**) is the only one that possesses a prop-2-yn-1-yloxy group at position 4″ of the phenyl substituent. Compound **5m**, instead, presents two substituents on the phenyl moiety, namely a (4-methylpiperazin-1-yl)sulfonyl at position 1 and a methyl at position 2, which reduce the anticancer activity. Notably, the presence of a 4″-*p*-hydroxyphenyl (**3b**) and a 4″-benzamide scaffold (**3c**) leads to a loss of selectivity amongst cancer and normal breast cells (Table 4). Finally, compounds **3a**, **5d** and **5i** resulted inactive toward all the tested cell lines. As reference molecule, Ellipticine, a well-known heterocyclic compound with anti-breast cancer activity, has been adopted.

## Conclusions

In conclusion, the exploration of twenty-two novel phthalazine derivatives has revealed promising inhibitory activity against four human carbonic anhydrase isoforms: hCA I, hCA II, hCA IX, and hCA XII. Notably, these compounds exhibited heightened efficacy against hCA IX and hCA I, surpassing the potency of the reference drug **AAZ**. Furthermore, selectivity indices reached up to 13.8, indicating some compounds′ ability to selectively target specific isoforms. Docking studies provided insights into potential interactions with the active sites of the isoforms, enhancing our understanding of the molecular mechanisms underlying their inhibitory activity. Additionally, a subset of the most active compounds against hCA IX underwent cell viability assays, demonstrating their anticancer potential. Specifically, compounds **3a–d**, **5d**, **5i**, and **5m** exhibited noteworthy anticancer activity against human breast cancer cell lines (MCF-7 and MDA-MB-231) while sparing the normal counterpart, MCF10-A cells.

The major goal of this manuscript was the evaluation of the inhibitory activity toward one of the most studied targets implied in cancer onset and progression, namely the human carbonic anhydrases (hCA). Our data indicated an extraordinary inhibition toward some hCA isoforms, particularly hCA IX, even


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**Table 3.** ADMET values of Indomethacin and tested compounds.

| No | Model name – Predicted value | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Water solubility | Caco2 permeability | Intestinal absorption (human) | Skin permeability | P-Glycoprotein substrate | P-Glycoprotein I inhibitor | P-Glycoprotein II inhibitor | VDss (human) | Fraction unbound (human) | BBB permeability | CNS permeability |
| **3a** | −3.787 | 0.788 | 85.488 | −2.738 | Yes | No | Yes | −0.211 | 0.057 | −0.153 | −2.462 |
| **3b** | −3.734 | −0.239 | 78.733 | −2.737 | Yes | No | Yes | −0.344 | 0.06 | −1.069 | −2.727 |
| **3c** | −3.809 | −0.195 | 84.309 | −2.735 | Yes | Yes | Yes | −1.283 | 0.123 | −1.432 | −2.708 |
| **3d** | −3.885 | 0.649 | 92.458 | −2.735 | Yes | Yes | Yes | −0.683 | 0.149 | −0.361 | −2.488 |
| **5a** | −3.324 | 0.377 | 98.507 | −2.735 | Yes | No | Yes | −1.038 | 0.101 | −1.094 | −2.288 |
| **5b** | −3.318 | 0.203 | 95.406 | −2.735 | Yes | Yes | Yes | −1.05 | 0.095 | −1.102 | −2.290 |
| **5c** | −3.084 | 0.310 | 99.935 | −2.735 | Yes | Yes | Yes | 0.269 | 0.078 | −0.175 | −2.304 |
| **5e** | −3.620 | 0.265 | 87.287 | −2.735 | Yes | Yes | Yes | −0.843 | 0.076 | −1.293 | −3.356 |
| **5g** | −3.613 | 0.054 | 91.561 | −2.735 | Yes | Yes | Yes | −1.007 | 0.059 | −1.231 | −3.367 |
| **5h** | −3.072 | 0.386 | 100 | −2.749 | Yes | No | Yes | −0.014 | 0.074 | −0.784 | −2.571 |
| **5i** | −3.932 | −0.108 | 83.179 | −2.735 | Yes | Yes | Yes | −1.297 | 0.124 | −1.642 | −3.09 |
| **5j** | −3.919 | −0.321 | 87.694 | −2.735 | Yes | Yes | Yes | −1.179 | 0.147 | −1.174 | −3.436 |
| **5k** | −3.905 | 0.218 | 70.295 | −2.735 | Yes | Yes | Yes | −1.301 | 0.118 | −1.755 | −3.786 |
| **5l** | −3.896 | −0.070 | 85.288 | −2.735 | Yes | Yes | Yes | −1.211 | 0.165 | −1.300 | −3.581 |
| **5m** | −3.688 | 0.314 | 83.597 | −2.735 | Yes | Yes | Yes | −0.316 | 0.172 | −1.340 | −3.363 |
| **6** | −3.141 | 1.381 | 100 | −2.735 | Yes | Yes | Yes | 0.306 | 0.277 | −1.073 | −1.819 |
| **8a** | −3.332 | 1.406 | 93.483 | −2.741 | Yes | No | Yes | −0.057 | 0.216 | −1.000 | −2.425 |
| **8b** | −3.378 | 1.307 | 93.410 | −2.744 | Yes | No | No | −0.091 | 0.233 | −1.006 | −2.425 |
| **8c** | −3.360 | 1.323 | 93.098 | −2.710 | Yes | No | Yes | −0.120 | 0.226 | −1.235 | −3.338 |
| **8d** | −3.235 | 0.235 | 93.702 | −2.735 | No | Yes | Yes | −0.332 | 0.146 | −1.178 | −3.416 |
| **AAZ** | −2.428 | −0.062 | 59.043 | −2.879 | No | No | No | −0.548 | 0.639 | −0.622 | −3.249 |

| No | Model name – Predicted value | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CYP2D6 substrate | CYP3A4 substrate | CYP2D6 inhibitor | CYP3A4 inhibitor | Total Clearance | Renal OCT2 substrate | AMES toxicity | Max. Tolerated dose (human) | Oral Rat Acute Toxicity (LD50) | Hepatotoxicity | Skin Sensitization |
| **3a** | No | Yes | No | No | 0.456 | No | No | 0.336 | 2.201 | Yes | No |
| **3b** | No | No | No | No | 0.33 | No | No | 0.652 | 2.130 | Yes | No |
| **3c** | No | Yes | Yes | Yes | 0.366 | No | No | 0.361 | 2.801 | Yes | No |
| **3d** | No | Yes | No | Yes | 0.516 | No | No | 0.550 | 2.958 | Yes | No |
| **5a** | No | Yes | No | Yes | 0.433 | No | No | 0.452 | 2.514 | Yes | No |
| **5b** | No | Yes | No | Yes | 0.353 | No | No | 0.446 | 2.566 | Yes | No |


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**Table 3.** continued

| No | Model name – Predicted value | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Water solubility | Caco2 permeability | Intestinal absorption (human) | Skin permeability | P-Glycoprotein substrate | P-Glycoprotein I inhibitor | P-Glycoprotein II inhibitor | VDss (human) | Fraction unbound (human) | BBB permeability | CNS permeability |
| **5c** | No | Yes | Yes | Yes | 0.423 | No | Yes | 0.397 | 2.729 | Yes | No |
| **5e** | No | Yes | No | Yes | 0.281 | No | Yes | 0.484 | 2.582 | Yes | No |
| **5g** | No | Yes | No | Yes | 0.342 | No | Yes | 0.485 | 2.850 | Yes | No |
| **5h** | No | Yes | No | No | 0.742 | Yes | No | −0.142 | 2.358 | Yes | No |
| **5i** | No | Yes | No | Yes | 0.668 | No | No | 0.343 | 2.689 | Yes | No |
| **5j** | No | Yes | No | Yes | 0.825 | No | No | 0.286 | 2.101 | Yes | No |
| **5k** | No | Yes | No | Yes | 0.671 | No | No | 0.374 | 2.807 | Yes | No |
| **5l** | No | Yes | No | Yes | 0.621 | No | No | 0.232 | 2.624 | Yes | No |
| **5m** | No | Yes | No | Yes | −0.108 | No | Yes | 0.425 | 2.701 | Yes | No |
| **6** | No | Yes | No | Yes | 0.853 | No | No | 0.435 | 2.846 | No | No |
| **8a** | No | Yes | No | Yes | 0.741 | No | Yes | 0.435 | 2.521 | Yes | No |
| **8b** | No | Yes | No | No | 0.724 | No | No | 0.337 | 2.519 | Yes | No |
| **8c** | No | Yes | No | Yes | 0.694 | No | No | 0.426 | 2.500 | Yes | No |
| **8d** | No | Yes | No | No | 0.687 | No | No | 0.398 | 2.475 | Yes | No |
| **AAZ** | No | No | No | No | −0.01 | No | No | 1.263 | 2.292 | No | No |



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**Table 4.** $IC_{50}$ values of compounds **3a–d**, **5d**, **5i**, **5m** and Ellipticine (reference molecule) expressed in μM.[a]

| Compounds | MDA-MB-231 | MCF-7 | MCF10-A |
|---|---|---|---|
| **3a** | >100 | >100 | >100 |
| **3b** | 36.5 ± 1.1 | 50.4 ± 2.1 | 41.6 ± 2.2 |
| **3c** | 96.5 ± 1.2 | 49.7 ± 2.2 | 89.3 ± 1.5 |
| **3d** | 14.2 ± 0.2 | 85.5 ± 0.8 | >100 |
| **5d** | >100 | >100 | >100 |
| **5i** | >100 | >100 | >100 |
| **5m** | 64.3 ± 1.1 | 68.7 ± 1.9 | >100 |
| **Ellipticine** | 1.8 ± 0.8 | 1.5 ± 0.5 | 1.1 ± 0.5 |

[a] Values are the mean ± SD of three independent experiments, each performed in triplicate.

better than the adopted reference molecule. We also evaluated the potential anticancer activity of these compounds adopting two different models of breast cancer, obtaining good results as well. Furthermore, results by QSAR (Quantitative structure-activity relationship) will allow to modify the individuated lead structure, to prepare more active compounds and optimize the activity. The *in silico* evaluation outcomes support the above mentioned strategy and, taken together, the presented data will open the road for a multitarget approach, aiming at individuating other cancer targets, related or not to hCA, enlarging the panel of cancer cells against which the compounds will be tested. The most promising compounds will be tested *in vivo*, as themselves or properly vehicles, to improve their pharmacological properties. It should be also recalled that some hCA inhibitors have already entered clinical trials, showing promising results that could be further improved. Moreover, the presented data may provide alternative tools, useful to overcome the classic issues of anticancer therapies, such as the resistance phenomena onset, and the lack of selectivity causing acute/chronic toxicity. These findings underscore the potential of the designed phthalazine derivatives as promising candidates for further development in the pursuit of selective carbonic anhydrase inhibitors with potential applications in anticancer therapy. The observed selectivity, potency, and anticancer activity warrant continued investigation into the therapeutic potential of these compounds for targeted cancer treatment.

## Experimental Section

### General

NMR $^1$H spectra of newly synthesized compounds were recorded on a spectrometer Bruker AC-300 (300 MHz) in DMSO-$d_6$. Chemical shifts of nuclei $^1$H were measured relatively the residual signals of deuterosolvent (δ = 2.50 ppm). Coupling constants (*J*) are reported in Hz. Melting points were determined by using Fisher-Johns Melting Point Apparatus (Fisher Scientific) and are uncorrected. Elemental analysis was performed using a EuroEA 3000 analyzer by burning a portion of the sample in an oxygen flow followed by chromatography of the combustion gases. The reaction and purity of the obtained compounds were monitored by TLC (plates with $Al_2O_3$ III activity grade, eluent $CHCl_3$, development of TLC plates by exposition to iodine vapors in "iodine chamber"). The solvents were purified according to standard procedures. The starting compounds and compounds **1**, **3**, **7** were provided by InterBioscreen Ltd. (Russia).

### General Procedure for the Synthesis of Compounds 3a–d

A mixture of a corresponding 4-substituted of 1-chlorophthalazine (0.002 mol) **1a–c**, 4-aminobenzenesulfonamide **2** (0.34 g, 0.002 mol), 6 mL methyl cellosolve was refluxed for 3 h. Then the mixture was cooled, 20% $NH_4OH$ solution (15 mL) was added and stirred for 1 h at 20 °C, the precipitate formed was filtered off and washed with water (30 mL).

#### *4-((4-Methylphthalazin-1-yl)amino)benzenesulfonamide (3a)*

Compound **3a** was obtained from 1-chloro-4-methylphthalazine **1a** and 4-aminobenzenesulfonamide **2**, yield 0.54 g (86%), colorless crystals, m.p. 295–296 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.83 (s, 3H, Me), 3.04 (s, 2H, $SO_2NH_2$), 6.98 (s, 1H, H-5′), 7.78 (d, *J* 8.8, 2H, H-2, H-6), 7.92-7.97 (m, 2H, H-3, H-5,), 8.06-8.11 (m, 2H, H-6′, H-7′), 8.56-8.59 (m, 1H, H-8′), 9.26 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 167.7 (C–$CH_3$), 152.0 (C–NH), 151.9, 144.7, 136.7, 132.7, 132.2, 127.1, 126.9, 125.5 (2 C), 123.0, 119.4, 118.9, 19.7 ($CH_3$). Anal. Calcd. For $C_{15}H_{14}N_4O_2S$., %: C 57.31; H 4.49; N 17.82; S 10.20. Found, %: C %: C 57.20; H 4.28; N 17.68; S 10.26.

#### *4-((4-(4-Hydroxyphenyl)phthalazin-1-yl)amino)benzenesulfonamide (3b)*

Compound **3b** was obtained from 1-chloro-4-hydroxyphenylphthalazine **1b** and 4-aminobenzenesulfonamide **2**, yield 0.57 g (73%), colorless crystals, m.p. 310–312 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.51 (s, 2H, $SO_2NH_2$, residual signal DMSO-$d_6$),7.03-7.11 (m, 3H, H-3′′, H-5′′, H-5′), 7.56 (d, *J* 8.5, 2H, H-3, H-5), 7.89 (dd, *J* 8.6, 1.4, 2H, H-2′′, H-6′′), 8.02 (d, *J* 8.6, 2H, H-2, H-6), 8.11-8.29 (m, 3H, H-5′-H-7′), 9.49 (d, *J* 8.2, 1H, H-8′), 11.49 (s, 1H, OH).Anal. Calc.for $C_{20}H_{16}N_4O_3S$, %: C 61.21; H 4.11; N 14.28; S 8.17. Found, %: C 61.16; H 4.02; N 14.18; S 8.23.

#### *4-(4-((4-Sulfamoylphenyl)amino)phthalazin-1-yl)benzamide (3c)*

Compound **3c** was obtained from 4-(4-chlorophthalazin-1-yl)benzamide **1c** and 4-aminobenzenesulfonamide **2**, yield 0.59 g (70%), colorless crystals, m.p. 321–323 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 3.02 (s, 2H, SO $_2NH_2$), 7.00 (s, 2H, $CONH_2$), 7.73 (d, *J* 8.0, 2H, H-2′′, H-6′′), 7.82 (d, *J* 8.6, 2H, H-3′′, H-5′′), 7.91-8.01 (m, 3H, H-5′–H-7′), 8.09 (d, *J* 8.1, 2H, H-3, H-5), 8.17 (d, *J* 8.5, 2H, H-2, H-6), 8.69 (d, *J* 8.2, 1H, H-8′), 9.49 (s, 1H, NH). Anal. Calc.for $C_{21}H_{17}N_5O_3S$, %: C 60.13; H 4.09; N 16.70; S 7.64. Found, %: C 60.07; H 4.03; N 16.64; S 7.69.

#### *4-((4-(4-(Prop-2-yn-1-yloxy)phenyl)phthalazin-1-yl)amino)benzenesulfonamide (3d)*

Compound **3d** was obtained from 1-chloro-4-(4-(prop-2-yn-1-yloxy)phenyl)phthalazine **1d** and 4-aminobenzenesulfonamide **2**, yield 0.53 g (62%), colorless crystals, m.p. 238–240 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.87 (s, 2H, $SO_2NH_2$), 3.12 (s, 1H, CH), 4.83 (s, 2H, $CH_2$), 6.89-6.92 (m, 2H, H-3′′, H-5′′), 7.10–7.21 (m, 2H, H-3, H-5), 7.61 (d, *J* 8.2, 2H, H-2′′, H-6′′), 7.82 (d, *J* 8.9, 2H, H-2, H-


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6), 7.93-7.99 (m, 1H, H-6′), 8.15 (d, *J* 8.2, 2H, H-5′, H-7′), 8.63-8.65 (m, 1H, H-8′), 9.36 (s, 1H, NH). Found, %: C 64.12; H 4.16; N 12.90; S 7.54. $C_{23}H_{18}N_4O_3S$, %: C 64.17; H 4.21; N 13.01; S 7.45.

### General Procedure for the Synthesis of Compounds 5 a–m

A mixture of a corresponding 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide **3 a**–**m** (0.002 mol), substituted amine **4** (0.002 mol), 5 mL methyl cellosolve was refluxed for 2 h. The hot reaction mixture was poured into 5% $NH_4OH$ solution (20 mL). Then the mixture was stirred for 0.5 h at 20 °C, the precipitate was filtered off and washed with water (15 mL).

#### *5-(4-((3-Hydroxyphenyl)amino)phthalazin-1-yl)-2-methylbenzenesulfonamide (5 a)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methyl benzenesulfonamide and 3-aminophenol. Yield 0.56 g (69%), colorless crystals, m.p. 275–276 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.75 (s, 3H, $CH_3$), 3.00 (s, 2H, $SO_2NH_2$,), 6.44–6.47 (m, 1H, H-2′′), 7.10 (t, *J* 8.0, 1H, H-4′′), 7.26-7.28 (m, 2H, H-5′′, H-6′′), 7.52 (d, *J* 7.8, 1H, H-3), 7.61-6.64 (m, 1H, H-4), 7.73-7.76 (m, 1H, H-6′), 7.86-7.98 (m, 3H, H-5′, H-7′, NH), 8.21 (s, 1H, OH), 8.66 (d, *J* 8.0, 1H, H-8′), 9.01 (d, *J* 5.8, H-6). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 155.6 (2 C), 146.8, 143.8, 143.1, 138.2, 134.4, 133.2 (2 C), 131.6, 130.4, 127.7 (2 C), 126.3 (2 C), 124.7, 123.2, 122.9, 122.8, 20.3, 20.2 ($CH_3$).Anal. Calc.for $C_{21}H_{18}N_4O_3S$, %: C 62.05; H 4.46; N 13.78; S 7.89. Found, %: C 62.00; H 4.39; N 13.72; S 7.94.

#### *5-(4-((4-Hydroxyphenyl)amino)phthalazin-1-yl)-2-methylbenzenesulfonamide (5 b)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 4-aminophenol. Yield 0.61 g (75%), colorless crystals, m.p. 306–310 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.73 (s, 3H, $CH_3$), 3.02 (s, 2H, $SO_2NH_2$), 6.75–6.78 (d, *J* 7.8, 2H, H-3′′, H-5′′), 7.26 (s, 1H, OH), 7.50 (d, *J* 7.8, 1H, H-3), 7.62 (d, *J* 8.4, 2H, H-2′′, H-6′′), 7.69-7.73 (m, 1H, H-4), 7.84-7.94 (m, 3H, H-5′ - H-7′), 8.17 (s, 1H, NH), 8.60 (d, *J* 8.0, 1H, H-8′), 8.87 (s, 1H, H-6). $^{13}$C NMR (126 MHz, dmso) δ 132.8, 125.9, 124.2, 115.3. Anal. Calc. for $C_{21}H_{18}N_4O_3S$. %: C 62.05; H 4.46; N 13.78; S 7.89. Found, %: C 62.01; H 4.42; N 13.74; S 7.96.

#### *5-(4-((4-Methoxyphenyl)amino)phthalazin-1-yl)-2-methylbenzenesulfonamide (5 c)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 4-methoxyaniline. Yield 0.77 g (91%), colorless crystals, m.p. 228–230 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.73 (s, 3H, $CH_3$), 3.03 (s, 2H, $SO_2NH_2$), 3.80 (s, 3H, $OCH_3$), 6.89–6.94 (m, 2H, H-3′′, H-5′′), 7.27–7.32 (m, 2H, H-2′′, H-6′′), 7.51 (d, *J* 7.8, 1H, H-3), 7.70–7.74 (m, 1H, H-4), 7.81 (d, *J* 7.8.6, 1H, H-5′), 7.87–7.92 (m, 2H, H-6′, H-7′), 8.18 (s, 1H, NH), 8.62 (d, *J* 8.1, 1H, H-8′), 9.03 (s, 1H, H-6). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 153.4 (C–$OCH_3$), 152.2, 144.2, 139.6, 137.6, 137.3, 134.9, 133.7, 133.2, 132.5, 129.8, 126.9, 126.1, 123.3, 120.1, 119.2, 60.4 (O–$CH_3$), 20.2 ($CH_3$). Anal. Calc. for $C_{22}H_{20}N_4O_3S$, %: C 62.84; H 4.79; N 13.32; S 7.63. Found, %: C 62.93; H 4.66; N 13.26; S 7.72.

#### *4-((4-(4-Methyl-3-sulfamoylphenyl)phthalazin-1-yl)amino)benzamide (5 d)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 4-aminobenzamide. Yield 0.68 g (79%), colorless crystals, m.p. 252–258 °C (methyl cellosolve). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.74 (s, 3H, $CH_3$), 3.06 (s, 2H, $SO_2NH_2$), 7.31 (s, 2H, $CONH_2$), 7.53 (d, *J* 7.8, 1H, H-3′′), 7.75 (dd, *J* 7.7, 1.9, 1H, H-5′′), 7.86-8.01 (m, 5H, H-5′–H-7′, H-2′′, H-6′′), 8.05 (d, *J* 8.3, 2H, H-3, H-5), 8.20 (s, 1H, H-2′′), 8.69 (d, *J* 8.1, 1H, H-8′), 9.39 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 168.0, 152.2, 142.8, 136.8, 134.8, 133.2, 133.1, 132.9, 132.4, 128.6 (3 C), 128.5 (2 C), 126.0 (2 C), 119.8 (4 C), 119.1, 20.2 ($CH_3$). Anal. Calc.for $C_{22}H_{19}N_5O_3S$. %: C 60.96; H 4.42; N 16.16; S 7.40. Found, %: C 60.84; H 4.36; N 16.05; S 7.51.

#### *2-Methoxy-5-((4-(4-methyl-3-sulfamoylphenyl)phthalazin-1-yl)amino)benzamide (5 e)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 5-amino-2-methoxybenzamide. Yield 0.63 g (68%), colorless crystals, m.p. 221–222 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.74 (s, 3H, $CH_3$), 3.03 (s, 2H, $SO_2NH_2$), 3.98 (s, 3H, $OCH_3$), 7.13 (d, *J* 9.0, 1H, H-3), 7.29 (s, 2H, $CONH_2$), 7.51 (d, *J* 7.8, 1H, H-6), 7.57–7.61 (m, 1H, H-6′′), 7.73 (dd, *J* 7.7, 1.8, 1H, H-5′′), 7.90–7.95 (m, 2H, H-6′, H-7′), 8.19–8.25 (m, 2H, H-5′, H-8′), 8.39 (d, *J* 2.8, 1H, H-2′′), 8.69 (d, *J* 8.0, 1H, H-4), 9.22 (s, 1H, NH). S 7.51. $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 168.9 (C=O), 144.2, 137.4, 133.2, 126.9 (2 C), 126.3 (2 C), 126.2 (2 C), 123.5, 122.6, 120.1 (3 C), 119.9 (2 C), 119.5 (3 C), 110.0, 20.7. Anal. Calc. For $C_{23}H_{21}N_5O_4S$., %: C 59.60; H 4.57; N 15.11; S 6.92. Found, %: C 60.84; H 4.36; N 16.05.

#### *2-Methyl-5-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl)benzenesulfonamide (5 f)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 4-aminobenzenesulfonamide. Yield 0.61 g (65%), colorless crystals, m.p. 280–282 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.75 (s, 3H, $NCH_3$), 2.3 (s, 4H, $2SO_2NH_2$), 7.22 (s, 1H, H-3) 7.54 (d, *J* 7.8, 1H H-4), 7.76 (d, *J* 8.0, 1H, H-5′), 7.83 (d, *J* 8.5, 2H, H-2′′, H-6′′), 7.96-8.01 (m, 2H, H-3′′, H-5′′), 8.16 (d, *J* 8.6, 2H, H-6′, H-7′), 8.23 (s, 1H, H-8′), 8.69 (d, *J* 8.2, 1H, H-6), 9.45 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 168.0 (C–NH), 152.2, 143.8, 142.8, 136.8, 134.8, 132.9, 132.4, 128.6 (3 C), 128.6 (3 C), 127.9, 126.0 (3 C), 119.8 (3 C), 119.1, 20.2 ($CH_3$). Anal. Calc. for, %: C 53.72; H 4.08; N 14.92; S 13.66. Found, %: C 53.64; H 4.38; N 14.83; S 13.76.

#### *N-Methyl-2-(4-((4-(4-methyl-3-sulfamoylphenyl)phthalazin-1-yl)amino)phenoxy)acetamide (5 g)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and 2-(4-aminophenoxy)-N-methylacetamide. Yield 0.64 g (67%), colorless crystals, m.p. 238–240 °C (methyl cellosolve). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.72, 2.74 (2 s, 6H, $2CH_3$), 3.04 (s, 2H, $SO_2NH_2$), 4.43 (s, 2H, $CH_2$), 6.95 (d, *J* 9.2, 2H, H-3′′, 5′′), 7.30 (s, 2H, H-2′′, H-6′′), 7.51 (d, *J* 7.9, 1H, H-5′), 7.72 (dd, *J* 7.8, 1.9, 1H, H-6′), 7.82-7.88 (m, 2H, H-6, H-7), 7.90-7.95 (m, 2H, H-5), H-8), 8.17 (d, *J* 2.0, 1H, $NHCH_3$), 8.63 (d, *J* 8.1, 1H, H-2′), 9.08 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 168.6 (2 C, C=O, C–O), 152.5, 142.7 (2 C), 136.6, 134.5, 133.1 (2 C), 132.8, 128.5, 123.4 (4 C), 115.1 (4 C), 67.9 (O–$CH_2$-CO), 25.8 ($CH_3$), 20.2 ($CH_3$). Anal. Calc. for $C_{24}H_{23}N_5O_4S$, %: C 60.36; H 4.85; N 14.67; S 6.71. Found, %: C 60.29; H 4.80; N 14.62; S 6.78.

#### *2-Methyl-5-(4-morpholinophthalazin-1-yl)benzenesulfonamide (5 h)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide and morfoline. Yield 0.73 g (95%), colorless crystals, m.p. 240–241 °C (EtOH). $^1$H NMR (300 MHz), DMSO-$d_6$, δ,


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ppm: 2.74 (s, 3H, $CH_3$), 3.02 (s, 2H, $SO_2NH_2$), 3.13–3.16 (m, 4H, morfoline-4-yl), 3.67-3.70 (m, 4H, morfoline-4yl), 7.64 (d, J 7.9, 1H, H-3), 7.81–7.84 (m, 2H, H-6′, H-7′), 7.87–7.90 (m, 1H, H-4), 8.12-8.17 (m, 2H, H-5′, H-8′), 8.69 (s, 1H, H-6). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 159.4, 155.1, 142.8 (C–S), 137.1, 134.6, 133.2, 132.9, 132.5 (2 C), 128.5, 126.8, 126.4, 125.2, 121.1, 66.6 (2 C), 51.7 (2 C), 20.2 ($CH_3$). Anal. Calc. For, %: C 59.36; H 5.24; N 14.57; S 8.34. Found, %: C 59.32; H 5.22; N 14.54; S 8.37.

### *N,2-dimethyl-5-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl)benzenesulfonamide (5 i)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-N,2-dimethylbenzenesulfonamide and 4-aminobenzenesulfonamide. Yield 0.65 g (67 %), colorless crystals, m.p. 238–240 °C (methyl cellosolve). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.55-2.57 (m, 2H, $SO_2NH_2$), 2.73 (s, 3H, NHCH$_3$), 2.98 (s, 3H, $CH_3$), 6.97 (s, 2H, H-2′′, H-6′′), 7.40 (s, 1H, H-3), 7.57 (d, J 7.8, 1H, H-4), 7.80–7.84 (m, 2H, H-3′′, H-5′′), 7.93-7.96 (m, 2H, H-6′, H-7′), 8.12-8.21 (m, 3H, NHCH$_3$, H-5′, H-8′), 8.69 (s, 1H, H-6), 9.49 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 153.4 (C-NH), 152.2, 144.2, 138.3, 137.6, 137.3, 134.9, 133.8, 133.3, 133.2, 132.5, 130.1, 126.9, 126.1 (2 C), 126.0, 120.1, 119.1 (2 C), 31.1, 28.9 (CH$_3$), 20.2 ($CH_3$). Anal. Calc. for %: C 54.64; H 4.38; N 14.48; S 13.26. Found, %: C 54.61; H 4.35; N 14.46; S 13.29.

### *N,N,2-Trimethyl-5-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl)benzenesulfonamide (5 j)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-N,N,2-trimethylbenzenesulfonamide and 4-aminobenzenesulfonamide. Yield 0.72 g (72 %), colorless crystals, m.p. 286–288 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.73 (s, 3H, $CH_3$), 2.83–2.86 (m, 8H, N(CH$_3$)$_2$, $SO_2NH_2$), 6.88 (s, 2H, H-2′′, H-6′′), 7.58 (d, J 7.9, 1H, H-3), 7.83 (d, J 8.5, 2H, H-3′′, H-5′′), 7.92-7.97 (m, 2H, H-6′, H-7′), 8.11 (s, 1H, H-4), 8.16-8.19 (m, 2H, H-5′, H-8′), 8.67–8.69 (m, 1H, H-6), 9.44 (s, 1H, NH). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 155.7, 148.4, 143.3, 142.9, 142.4, 134.5, 131.6, 128.5, 127.9, 127.7, 127.0, 123.7, 123.0, 20.4 (N–(CH$_3$)$_2$), 20.0 (C–$CH_3$). Anal. Calc. for $C_{23}H_{23}N_5O_4S_2$, %: C 55.52; H 4.66; N 14.07; S 12.89. Found, %: C 55.50; H 4.63; N 14.05; S 12.93.

### *N-(2-Hydroxyethyl)-2-methyl-5-(4-((4-sulfamoylphenyl)amino)phthalazin-1-yl)benzenesulfonamide (5 k)*

The starting compounds were 5-(4-chlorophthalazin-1-yl)-N-(2-hydroxyethyl)-2-methylbenzenesulfonamide and 4-aminobenzenesulfonamide. Yield 0.80 g (78 %), colorless crystals, m.p. 248–249 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.74 (s, 3H, $CH_3$), 2.93-3.01 (m, 4H, $CH_2$, $NH_2$), 3.41-3.45 (m, 2H, $CH_2$), 4.41 (s, 1H, OH), 6.99 (s, 2H, H-2′′, H-6′′), 7.50 (s, 1H, $SO_2NH$), 7.57 (d, J 7.8, 1H, H-3), 7.78-7.83 (m, 3H, H-4, H-3′′, H-5′′), 7.95 (s, 2H, H-6′, H-7′), 8.15–8.18 (m, 2H, H-5′, H-8′), 8.69 (d, J 8.1, 1H, H-6), 9.50 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 153.4, 152.2, 144.2, 139.6, 137.6, 137.3, 134.8, 133.7, 133.2, 132.5, 129.8, 126.9, 126.1, 123.3, 120.1, 119.2, 110.0, 60.4, 45.3, 39.9, 20.2. Anal. Calc. for $C_{23}H_{23}N_5O_5S_2$: %: C 53.79; H 4.51; N 13.64; S 12.49. Found, %: C 53.76; H 4.48; N 13.60; S 12.55.

### *4-((4-(4-Methyl-3-(morpholinosulfonyl)phenyl)phthalazin-1-yl)amino)benzenesulfonamide (5 l)*

The starting compounds were 4-((5-(4-chlorophthalazin-1-yl)-2-methylphenyl)-sulfonyl)morpholine and 4-aminobenzenesulfonamide. Yield 0.82 g (76 %), colorless crystals, m.p. 308–310 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.74 (s, 3H, $CH_3$), 3.02 (s, 2H, $SO_2NH_2$), 3.13–3.16 (m, 4H, morfoline-4-yl), 3.67-3.70 (m, 4H, morfoline-4-yl), 7.00 (s, 2H, H-2, H-6), 7.64 (d, J 7.9, 1H, H-5′′), 7.82 (d, J 9.0, 2H, H-3, H-5), 7.84–7.90 (m, 1H, H-6′′), 7.94 (d, J 2.4, 1H, H-2′′), 8.11-8.20 (m, 3H, H-5′ - H-7′), 8.70 (d, J 8.1, 1H, H-8′), 9.52 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 142.8 (3 C), 136.6 (6 C), 132.8 (2 C), 126.1 (5 C), 124.5 (2 C), 110.0 (2 C), 56.6, 20.2. Anal. Calc. for $C_{25}H_{25}N_5O_5S_2$, %: C 55.64; H 4.67; N 12.98; S 11.88. Found, %: C 55.61; H 4.65; N 12.95; S 11.94.

### *4-((4-(4-Methyl-3-((4-methylpiperazin-1-yl)sulfonyl)phenyl)phthalazin-1-yl)amino)benzenesulfonamide (5 m)*

The starting compounds were 1-chloro-4-(4-methyl-3-((4-methylpiperazin-1-yl)sulfonyl)phenyl)phthalazine and 4-aminobenzenesulfonamide. Yield 0.73 g (66 %), colorless crystals, m.p. 281–283 °C (methyl cellosolve). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.24 (s, 3H, $NCH_3$), 2.43-2.46 (m, 4H, piperazine-1-yl), 2.73 (s, 5H, $SO_2NH_2$, $CH_3$), 3.18-3.22 (m, 4H, piperazine-1-yl), 6.92 (s, 2H, H-2′′, H-6′′), 7.58 (d, J 7.9, 1H, H-5), 7.83 (d, J 8.7, 2H, H-3′′, H-5′′), 7.92 (s, 2H, H-6′, H-7′), 8.11–8.19 (m, 3H, H-6, H-5′, H-8′), 8.67–870 (m, 1H, H-2), 9.46 (s, 1H, NH). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 153.4, 152.2, 144.2, 138.3, 137.6, 137.3, 134.9, 133.8, 133.3, 133.2, 132.5, 130.1, 126.9 (3 C), 126.1, 126.0, 120.1 (2 C), 119.1, 28.9 (2 C), 20.2 (2 C). Anal. Calc. for $C_{26}H_{28}N_6O_4S_2$, %: C 56.50; H 5.11; N 15.21; S 11.60. Found, C 56.46; H 5.08; N 15.17; S 11.66.

### *2-Methyl-5-(3-(pyridin-4-yl)-[1,2,4]triazolo[3,4-a]phthalazin-6-yl)benzenesulfonamide (6)*

A mixture of 5-(4-chlorophthalazin-1-yl)-2-methylbenzenesulfonamide (0.67 g, 0.002 mol), isonicotinohydrazide (0.27 g, 0.002 mol), 6 mL methyl cellosolve was refluxed for 3 h and hot reaction mixture was poured into 20 % $NH_4OH$ solution (15 mL). Then the mixture was stirred for 1 h at 20 °C, the precipitate was filtered off and washed with water (3×10 mL). Yield 0.66 g (79 %), colorless crystals, m.p. 336–339°C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.81 (s, 5H, $CH_3$, $SO_2NH_2$), 7.30 (s, 1H, H-3), 7.62 (d, J 7.8, 1H, H-4), 7.83 (d, J 7.8, 1H, H-7′), 7.88-7.96 (m, 1H, H-9′), 7.99 (d, J 8.1, 1H, H-10′), 8.11 (t, J 7.4, 1H, H-8′), 8.29 (d, J 1.9, 1H, H-6), 8.35-8.41 (m, 2H, H-3′′, H-5′′), 8.73-8.77 (m, 2H, H-2′′, H-6′′). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 156.6, 150.9 (2 C), 146.6, 144.4, 143.0, 138.7, 134.9, 133.9, 133.4, 132.4, 132.1, 129.0, 128.6, 123.8, 123.5, 123.1, 121.4 (3 C), 20.3 ($CH_3$). Anal. Calc. for $C_{21}H_{16}N_6O_2S$., %: C 60.56; H 3.87; N 20.18; S 7.70. Found, %: C 60.54; H 3.84; N 20.15; S 7.76.

## General Procedure for the Synthesis of Compounds 8 a–d

Corresponding phenylsubstituted triazolophthalazine **7 a–d** (0.002 mol) was added in portions over 10 min at 0–5 °C to chlorosulfonic acid (6 mL) cooled to 0 °C. Then, with intensive stirring for 1 h, the bath temperature was raised to 150 °C, stirred 0.5 h, and poured into 200 g of crushed ice. After 15 min the mixture was filtered off, carefully squeezed out and added in portions to 20 mL of 20 % $NH_4OH$. After 2 h the precipitate was filtered off, washed with water (40 mL).

### *4-Methyl-3-(6-methyl-[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (8a)*

The starting compound was 6-methyl-3-(o-tolyl)-[1,2,4]triazolo[3,4-a]phthalazine. Yield 0.36 g (52 %), colorless crystals, m.p. 257–259 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.44 (s, 3H, 6′-$CH_3$), 2.82 (s, 3H, 4-$CH_3$), 2.92 (s, 2H, $SO_2NH_2$), 7.18 (s, 1H, H-5), 7.58 (d, J


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8.1, 1H, H-6), 7.94 (s, 1H, H-7′), 8.02-8.07 (m, 1H, 10′), 8.15–8.22 (m, 2H, H-8′, H-9′), 8.65 (d, *J* 7.9, H-2). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 153.3 (C–$CH_3$), 152.3, 144.2, 138.3, 137.5, 135.2, 134.3, 133.7, 133.2, 132.5, 130.7, 126.9, 126.2, 125.9, 123.4, 120.2, 119.2, 40.7, 40.5, 40.3, 40.1, 39.9, 39.6, 39.4, 37.4, 20.6 (2 C, $CH_3$). Anal. Calc. for $C_{17}H_{15}N_5O_2S$, %: C 57.78; H 4.28; N 19.82; S 9.07. Found, %: C 57.71; H 4.23; N 19.80; S 9.11.

### *2-Methyl-5-(6-methyl-[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (8 b)*

The starting compound was 6-methyl-3-(p-tolyl)-[1,2,4]triazolo[3,4-a]phthalazine. Yield 0.49 g (69 %), colorless crystals, m.p. 303–305 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.75 (s, 3H, 6′-$CH_3$), 2.90 (s, 2H, $SO_2NH_2$), 2.93 (s, 3H, 2-$CH_3$), 7.25 (s, 1H, H-3), 7.51 (d, *J* 8.01, 1H, 7′), 7.90 (t, *J* 7.7, 1H, H-8′), 8.02 (t, *J* 7.7, 1H, 9′), 8.20 (d, *J* 8.00, 1H, H-10′), 8.52 (d, *J* 7.7, H-6), 8.62–8.64 (s, 1H, H-4). $^{13}$C NMR (126 MHz, DMSO-$d_6$) δ 155.5 (N-C=N), 146.9, 143.8, 143.1, 138.2, 134.4, 133.2, 131.5, 130.4, 127.7, 126.3, 124.7, 123.2, 122.9, 122.7, 20.3 ($CH_3$), 20.2 ($CH_3$).Anal.Calc.for $C_{17}H_{15}N_5O_2S$. Calculated, %: C 57.78; H 4.28; N 19.82; S 9.07. Found, %: C 57.73; H 4.26; N 19.78; S 9.12.

### *2-Methoxy-5-(6-methyl-[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (8 c)*

The starting compound was 6-methyl-3-(4-methoxyaniline)[1,2,4]triazolo[3,4-a]phthalazine. Yield 0.45 g (61 %), colorless crystals, m.p. 290–292 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.93 (s, 5H, $CH_3$, $SO_2NH_2$), 4.02 (s, 3H, $OCH_3$), 6.93 (s, 1H, H-3), 7.35-7.38 (m, 1H, H-7′), 7.88-7.93 (m, 1H, H-8′), 8.03 (t, 1H, *J* 7.5, H-6), 8.21 (d, *J* 8.2, H-7′), 8.63 (d, *J* 8.2, H-10′). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 157.6, 155.5, 147.1, 143.7, 134.5, 133.0, 132.2, 131.5, 127.9, 127.3, 123.4, 123.1, 122.9, 118.8, 113.7, 56.9, 20.3. Anal. Calc. for $C_{17}H_{15}N_5O_3S$, %: C 55.27; H 4.09; N 18.96; S 8.68. Found, %: C 55.23; H 4.07; N 18.93; S 8.71.

### *4-Methoxy-3-(6-methyl-[1,2,4]triazolo[3,4-a]phthalazin-3-yl)benzenesulfonamide (8 d)*

The starting compound was 6-methyl-3-(4-methoxyaniline)-[1,2,4] triazolo[3,4-a]phthalazine. Yield 0.50 g (68 %), colorless crystals, m.p. 293–297 °C (DMF). $^1$H NMR (300 MHz), DMSO-$d_6$, δ, ppm: 2.80 (s, 1H, $CH_3$), 2.94 (s, 2H, $SO_2NH_2$), 3.91 (s, 3H, $OCH_3$), 7.32–7.35 (m, 1H, H-3), 7.87–7.92 (m, 1H, H-2), 7.99-8.08 (m, 3H, H-7–H-9′), 8.18 (d, *J* 8.0, 1H, H-6), 8.64 (d, *J* 7.8, 1H, H-10′). $^{13}$C NMR (101 MHz, DMSO-$d_6$) δ 160.9, 155.1, 147.0, 143.3, 136.8, 134.4, 131.6, 130.5, 130.0, 127.9, 123.6, 122.9, 116.3, 113.0, 57.0, 19.9. Anal. Calc. $C_{17}H_{15}N_5O_3S$. Calculated, %: C 55.27; H 4.09; N 18.96; S 8.68. forFound, %: C 55.25; H 4.06; N 18.94; S 8.74.

## Carbonic Anhydrase Inhibition

An Applied Photophysics stopped-flow instrument was used to assay the CA catalyzed $CO_2$ hydration activity[24] Phenol red (at a concentration of 0.2 mM) was used as an indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) as a buffer, and 20 mM $Na_2SO_4$ (to maintain constant ionic strength), following the initial rates of the CA-catalyzed $CO_2$ hydration reaction for a period of 10–100 s. The $CO_2$ concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants.[30] Enzyme concentrations ranged between 5–12 nM. For each inhibitor, at least six traces of the initial 5–10 % of the reaction were used to determine the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of the inhibitor (0.1 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with the assay buffer. Inhibitor and enzyme solutions were preincubated together for 15 min at r.t. prior to the assay, to allow the formation of the E–I complex. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3 and the Cheng-Prusoff equation as reported earlier and represent the mean from at least three different determinations. All CA isoforms were recombinant proteins obtained in house, as reported earlier.[31–35]

## Docking Studies

Molecular modelling studies were performed using software AutoDock 4.2.[36] Protein Data Bank was also used in order to obtain the crystal structures of *hCA I* (PDB code 3 W6H) and *hCA II* (PDB code 3HS4) cytosolic isoforms, *hCA IX* (PDB code 3IAI) and *hCA XII* (PDB code 1JD0) isoforms.[37] All the procedure was carried out as in our previous work.[38] In particularly, for ligand preparation, all structures were sketched in chemdraw12.0.[39] The geometry was optimized using the molecular mechanical force fields 94 (MMFF94) energy via program LigandScout, partial charges were also calculated, conformers of each ligand were generated and the one with the best conformation was maintained and saved as mol2 in order to pass to ADT for pdbqt file preparation. For protein preparation the PDB files were added to SPDBV program[40] in order to fix any missing residues. Then the co-crystallized ligands and water molecules were removed, polar hydrogens and charges were added using AutoDock tools.

For docking, the Lamarckian genetic algorithm was applied for minimization using default parameters. The number of docking runs was 100 that were set to terminate after a maximum of 2,500,000 energy evaluation. All rotatable torsions were released and the population size was set to 150. A translational step of 0.2 Å, and quaternion and torsion steps of 5 were applied during the search. After docking, the 100 solutions were clustered into groups with RMS lower than 1.0 E. The clusters were ranked by the lowest energy representative of each cluster. To describe the ligand-binding pocket interactions, the top ranked binding mode was found by Autodock in complex with the binding pocket of the enzyme. The resulting poses and potential interactions were visualized in LigandScout program.[41] As a first step of docking studies, for the validation of the docking method, the initial inhibitor of each enzyme was removed, docked back and compared with its initial position. The RMSD values 0.885, 0.966, 1.034, and 1.176 Å for hCA I, II, IX, and XII, respectively validated the protocol.

## ADME Properties

For the prediction of the ADMET properties of all compounds, the free web pkCSM server was used.[42] All the molecules were first converted into SMILES (Simplified Molecule Input Line Entry Specification) for the prediction.

## Cell Culture

The cell lines employed in the experiments were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Human breast cancer cells MCF-7 and MDA-MB-231, and human mammary epithelial cell line MCF-10A were cultured as described,[43] and maintained at the temperature of 37 °C, in a humidified atmosphere containing 5 % $CO_2$.


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### MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Sigma Aldrich (St. Louis, MO, USA)) was used to determine the *in vitro* anticancer activity of the tested compounds, as previously reported.[43] Briefly, cells were exposed for 72 h to the indicated concentrations of the complexes (1, 5, 10, 20, 40, 80 and 100 μM) and the $IC_{50}$ values were extrapolated, versus the percent (%) of control, using the software GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA). Each value is the mean ± SD of three separated experiments, each performed in triplicate.

## Associated Content

$^{1}H$, $^{13}C$ NMR spectra of compounds.

## Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## Conflict of Interests

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** carbonic anhydrase · triazolophthalazines · sulfonamides · docking · drug-likeness

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