Dependence on linkers’ flexibility designed for benzenesulfonamides targeting discovery of novel hCA IX inhibitors as potent anticancer agents

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ABSTRACT
Herein we reported the design and synthesis of two series comprising twenty-two benzenesulfonamides that integrate the s-triazine moiety. Target compounds successfully suppressed the hCA IX, with IC50 ranging from 28.6 to 871 nM. Compounds 5d, 11b, 5b, and 7b were the most active analogues, which inhibited hCA IX isoform in the low nanomolar range (Ki = 28.6, 31.9, 33.4, and 36.6 nM, respectively). Furthermore, they were assessed for their cytotoxic activity against a panel of 60 cancer cell lines following US-NCI protocol. According to five-dose assay, 13c showed significant anticancer activity than 5c with GI50-MID values of 25.08 and 189.01 μM, respectively. Additionally, 13c’s effects on wound healing, cell cycle disruption, and apoptosis induction in NCI-H460 cancer cells were examined. Further, docking studies combined with molecular dynamic simulation showed a stable complex with high binding affinity of 5d to hCA IX, exploiting a favourable H-bond and lipophilic interactions.

HIGHLIGHTS
- Carbonic anhydrase (CA) inhibitors comprising rigid and flexible linkers were developed.
- Compound 5d is the most potent CA IX inhibitor in the study (IC50: 28.6 nM).
- Compounds 5c and 13c displayed the greatest antiproliferative activity towards 60 cell lines.
- Compound 13c exposed constructive outcomes on normal cell lines, metastasis, and wound healing.
- Molecular docking and molecular dynamics (MDS) simulation was utilised to study binding mode.

GRAPHICAL ABSTRACT

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Introduction

The carbonic anhydrase (CA) enzymes are zinc-metalloenzymes family that catalyse the conversion of CO2 and H2O to the dissociated products of H2CO3 (HCO3− and H+ ions) reversibly in all organisms.1,2 In humans, there are 15 distinct CA isoforms, each with its own molecular features, subcellular localisation, and tissue distribution.3,4 These enzymes are required for a variety of physiological and cellular activities, including electrolyte secretion, acid-base balance, carbon dioxide transport, and biosynthetic pathways.5,6 Compared to normal tissues, where CA IX expression is modest, the variety of solid tumours have some of the most over-expressed transmembrane proteins.7,8 CA IX is used by tumour cells to keep the tumour microenvironment acidic, preventing tumour hypoxia-related responses and assisting tumour cell survival and proliferation.9 CA IX’s overexpression in tumour cells made it an ideal candidate as a viable target for developing novel small compounds for both tumour diagnostics and treatments.10 Many selective CA IX inhibitors have recently been described in the literature, which are in various stages of clinical trials and have shown good activity against different types of solid tumours.11 The sulfonamide derivative SLC-0111, an efficient inhibitor of CA IX and CA XII, which was advanced in subsequent clinical investigations, is a well-known selective anticancer drug candidate.12 The well-known tail approach was used to develop SLC-0111, which includes a ureido linker between the zinc-binding group (ZBG) benzenesulfonamide and the tail of the inhibitor. The tail portion of the inhibitor, which offers isoenzyme specificity over off-target isoenzymes, is made more flexible by the linker moiety to engage with the individual amino acid residues on the active site.13 There are several bioisosteric groups reported exchanging urea as a tailing linker in various benzenesulfonamide derivatives, which is the most effective class of CA inhibitors.

Hydrazones are an important family of compounds because of their flexibility and structural similarities to a variety of biologically important natural chemicals.14,15 The imine (N=C) group in hydrazone derivatives plays an important role in the mechanism of transformation and racemisation in biological systems16,17 in addition to its chemical stability towards liver microsomal enzymes.18,19 Reported CAs inhibitors having aforesaid chemical features are illustrated in Figure 1. In previous studies, isatin, phenoxy, or pyrazole-moieties (I–III) that carry aromatic sulfonamides via hydrazone linker were declared as potent inhibitors of cancer-related hCA IX isoenzyme with the hCA IX Ks values of 8.3 nM,20 14.6 nM,21 and 19.7 nM,22 respectively (Figure 1). Numerous studies and experiences pointed out that heterocyclic rings, such as pyrazoline IV, pyrazole V, and triazole VI bearing benzenesulfonamides are an attractive group of compounds with significant CAs inhibitory activity profiles towards CA IX isoform with Ks values of 5.5 nM,23 302 nM,24 and 180 nM,25 respectively (Figure 1). In previous studies reported by our group,26 compound 1 was considered a cornerstone to which any part of the above can be added to design more potent and selective inhibitors. Therefore, we have designed series I (3a–c, 5a–d, and 7a–e), having the hydrazone linker while in Series II (9, 11a,b, 13a–e, and 15a,b), we have fixed the configuration of the hydrazone linker via incorporation in five-membered heterocyclic rings seeking to solve dilemma of hCA IX selectivity. Moreover, benzenesulfonamide was retained as a zinc-binding group in target compounds for Series I and II. Different lipophilic tails were constructed in Series I such as substituted isatins (3a–c), substituted benzenes (5a–d), and substituted phenylpyrazoles (7a–e). Regarding Series II, the lipophilic tails were designed to be substituted pyrazolindines (9 and 11a,b), substituted pyrazoles (13a–e), and substituted triazoles (15a,b).

Results and discussion

Chemistry

New s-triazine-based benzenesulfonamide derivatives 3a–c, 5a–d, 7a–e, 9, 11a,b, 13a–e, and 15a,b were synthesised by the chronological reactions sequence depicted in Schemes 1 and 2. The hydrazine derivative 1 was reacted with selected reagents to install a variety of phenyl or heterocyclic moieties connected to the s-triazine scaffold. Reaction of 1 with isatin derivatives 2a–c in hot, dry methanol and glacial acetic acid as catalyst yielded 3a–c (Scheme 1). The analogues 3a–c may exist as the Z- or E-isomer relying on several factors which estimate the preferred configuration.27 The development of a single stereoisomer was established by the 1H NMR spectra of the compounds 3a and 3b. 1H NMR of 3a displayed a singlet at δ 10.68 ppm for the introduced NH of hydrazine moiety as an E-configured structure.28 The downfield shift of the NH proton peak of isatin as in 3b, which appears at 12.74 ppm, suggests that the NH proton of the hydrazine moiety is intramolecularly hydrogen-bonded with the carbonyl group of the indolinone ring, which resulted in the construction of the pseudo-six-membered ring as Z-configured structure29 as shown in Figure 2.

Hydrazones 5a–d and 7a–e were easily synthesised in high yields (>70%) by condensing equimolar amounts of 1 with different carbonyl compounds in boiling absolute MeOH. The geometry of target hydrazones 5a–c and 7a–e was considered as E isomers rather than Z isomers depending on 1H NMR spectra that were assigned for the methine proton (¼CH) between 8.07 and 8.35 ppm,30 in addition to our reported results of NOESY study.26

Reagents and conditions: (i) Dry MeOH, gl. AcOH, reflux 25 h; (ii) Dry MeOH, gl. AcOH, reflux 5 h.

In Scheme 2, the hydrazinyl derivative 1 reacted, under neutral conditions, with the different active methylene compounds, namely ethyl cyanoacetate and dicarbonyl ketones, to afford products 9 and 13a–e, respectively, in good yields. The target compounds 11a,b were obtained through a cyclocondensation reaction of the corresponding hydrazino-triazine derivative 1 and the appropriate propenones, 10a,b in absolute methanol and potassium hydroxide. Furthermore, the triazolotriazine derivatives 15a,b were successfully synthesised by heating the hydrazine 1 in pyridine with either ethyl chloroformate to give 15a or carbon disulphide to give 15b. In the case of compound 15b, duplication of the signals in its 1H NMR spectrum was detected, even though only one spot in different TLC eluents was observed, which proves the presence of two isomers (depending on the position of nitrogen of the triazine ring that can be cyclised with carbon disulphide in basic medium). The proportion of the two isomers in this mixture, as indicated by 1H NMR, was 1: 1 approximately. In addition, the absence of symmetrical exchangeable singlets protons after the addition D2O at (10.02 and 10.23) and (12.60 and 12.82) ppm were assigned for two protons of NH of each isomer.

Furthermore, the lack of exchangeable singlets of D2O at 7.29 and 14.17 ppm were assigned for SO3NH2 and SH protons, respectively, of each isomer. All of that indicates the presence of another isomer (Figure S1, see Supporting information). The presence of the two isomers was also confirmed using HPLC due to the presence of a twin peak at 6.734 and 7.666 min (Figure S2, see Supporting information). The two proposed isomers of compound 15b, when refluxed with CS2 in pyridine, are shown in Scheme 3, and the plausible mechanism of the formation of one of two isomers of compound 15b is shown in Scheme 4.31,32

Reagents and conditions: (i) gl. AcOH, reflux 36 h; (ii) KOH, abs. MeOH, reflux 72 h; (iii) abs. MeOH, reflux 5 h; (iv) Ethyl
Twenty-two new compounds were designed and synthesised in this study. Their chemical structures were confirmed using $^1$H, $^{13}$C NMR, and EI-MS. Spectra are in the Supplementary file. In addition to elemental analysis results, the molecular ion peaks were in good harmony with the target compounds’ molecular formula within the permitted range (±0.4). Some representative compounds were also measured their purity by HPLC (Agilent Technologies, Santa Clara, CA).

**Biological evaluation**

**Carbonic anhydrase isoforms inhibition assay**

Potency parameter. The four pharmacologically and physiologically significant CA isoforms, including the hCA I and II (cytosolic...
isoforms) and the hCA IX and XII (transmembrane tumour-associated isoforms), were investigated using a stopped-flow CO₂ hydrase assay.33–38 Table 1 illustrates the enzyme inhibition constants (Ki) and the dose-response curves for determining the four CAs activity induced by the representative compounds 3c, 5b–d, and 11a,b presented in the Supplementary file, while Table 2 shows the estimated selectivity ratios (SRs). Acetazolamide (AAZ), a clinically used sulphonamide CAI, and SLC-0111 (Phase Ib/II clinical trials) were also used as control compounds in the tests. Based on the inhibitory results (as Ki values) listed in Table 1 for the synthesised analogues, the following structure–activity relationship (SAR) was estimated:

i. Sulphonamide analogues with Ki values ranging from 26.6 to more than 50 000 nM minimally inhibited the cytosolic isoform hCA I, showing that all synthetic compounds are weaker inhibitors than AAZ (Ki = 250 nM) except compounds 7b, 9, 13d, and 11a,b with Ki values of 156.3, 195.9, 92.6, and 26.6 nM, respectively. The most active analogue in this group, 15b, Ki = 26.6 nM, has mercaptotriazole ring fused to the main scaffold triazine. The weakest inhibitors were 11a and 11b; Ki > 50 000 nM, which comprise substituted diphenyl pyrazoline ring.

ii. The target compounds showed Ki values ranging from 30.8 to 4585 nM, which showed lower activity than AAZ (Ki = 12.1 nM), according to analysis of the potency results for inhibiting hCA II. The investigated compounds exhibited better activity towards the hCA II than the hCA I isoform (5d, 9, 13a, and 15b were the exemption). The presence of diphenyl pyrazole moiety was described the most active inhibitor towards hCA II 13d, Ki = 30.8 nM, while analogue 9, Ki = 4585 nM, was the weakest inhibitor.

Regarding SAR for Series I, the lipophilic moiety attached to hydrazone linker-controlled potency of inhibitors, where substituted pyrazole group enhanced the potency and reported the best inhibitory effect for analogue 7a, Ki = 46.4 nM. Replacing pyrazole with isatin moiety reduced the potency as observed in analogue 3a, Ki = 98.2 nM, while the existence of substituted phenyl group diminished the activity as perceived for compound 5a, Ki = 175 nM. Concerning Series II, inclosing hydrazone linker within pyrazole ring (13a–e) exposed the strongest inhibitor 13d, Ki = 30.8 nM. Fusing the triazole ring with the triazine scaffold (15a,b) diminished the potency as reported in 15b, Ki = 46.2 nM. Meanwhile, partially saturated pyrazole moiety abolished activity of compounds 9 and 11a,b, Ki = 4585, 4192, and 2131 nM, respectively.

(iii) With Ki values, the investigated compounds significantly suppressed the transmembrane tumour-associated isoform hCA IX.

Scheme 1. Synthetic pathway of new sulphonamides analogues 3a–c, 5a–d, and 7a–e.
The diphenyl hydrazinyl methylidine analogue 5d showed a stronger inhibitory effect with a Kᵢ value (28.6 nM) comparable to AAZ (Kᵢ = 25.8 nM). Meanwhile, comparing with SLC-0111 (Kᵢ = 45 nM), compounds 3c, 5b, 5d, 7b, 11b, and 15b were more potent with Kᵢ values of 43.8, 33.4, 28.6, 36.6, 31.9, and 40.7 nM, respectively. SAR study for Series I revealed that potency relied on lipophilic moieties attached to the hydrazone linker and decreased in the following order: phenyl derivatives

Scheme 2. Synthetic pathway of target sulphonamides 9, 11a,b, 13a–e, and 15a,b.

Figure 2. The E-isomer of compound 3a and Z-isomer of compound 3b with the pseudo-six-membered ring (in blue color).

Scheme 3. The two proposed isomers of compound 15b when refluxed with CS₂ in pyridine.

(28.6-871 nM). The diphenyl hydrazinyl methylidine analogue 5d showed a stronger inhibitory effect with a Kᵢ value (28.6 nM) comparable to AAZ (Kᵢ = 25.8 nM). Meanwhile, comparing with SLC-0111 (Kᵢ = 45 nM), compounds 3c, 5b, 5d, 7b, 11b, and 15b were more potent with Kᵢ values of 43.8, 33.4, 28.6, 36.6, 31.9, and 40.7 nM, respectively. SAR study for Series I revealed that potency relied on lipophilic moieties attached to the hydrazone linker and decreased in the following order: phenyl derivatives
Scheme 4. A plausible mechanism for the formation of one of the two isomers regarding analogue 15b.\textsuperscript{31,32}

Table 1. Inhibition profile concerning human CA isoforms, off-target isoforms (hCA I and II), and the tumour-associated isoforms (hCA IX and XII) with triazine-based benzene-sulfonylamides 3a–c, 5a–d, 7a–e, 9, 11a,b, 13a–e, 15a,b, besides the standard inhibitors acetazolamide (AAZ) and SLC-0111 by a stopped-flow CO\textsubscript{2} hydrase assay.

| Compounds | hCA I  | hCA II | hCA IX | hCA XII |
|-----------|-------|--------|--------|---------|
| 3a        | 8164  | 98.2   | 818.4  | 59.2    |
| 3b        | 8223  | 399.3  | 95.9   | 82.2    |
| 3c        | 3924  | 1516   | 43.8   | 24.4    |
| 5a        | 5111  | 175.4  | 575.5  | 64.1    |
| 5b        | 3523  | 668    | 33.4   | 14.9    |
| 5c        | 5309  | 2352   | 75.4   | 36.7    |
| 5d        | 4083  | 4291   | 28.6   | 31.3    |
| 7a        | 5811  | 46.4   | 68.6   | 85.5    |
| 7b        | 156.3 | 74.4   | 36.6   | 78.1    |
| 7c        | 8896  | 3147   | 47.3   | 439.0   |
| 7d        | 980.4 | 68.4   | 56.9   | 2185    |
| 7e        | 3720  | 2224   | 52.8   | 888.3   |
| 9         | 195.9 | 4585   | 443.0  | 27.3    |
| 11a       | <50 000 | 4192 | 227   | 47.7    |
| 11b       | <50 000 | 2131 | 31.9  | 8.29    |
| 13a       | 583.1 | 645.8  | 91.9   | 82.4    |
| 13b       | 368.9 | 114.3  | 94.7   | 77.8    |
| 13c       | 4710  | 2253   | 92.9   | 82.4    |
| 13d       | 92.6  | 30.8   | 91.8   | 89.9    |
| 13e       | 476.8 | 96.0   | 70.0   | 751.3   |
| 15a       | 728.0 | 704.7  | 871.4  | 40.6    |
| 15b       | 26.6  | 46.2   | 40.7   | 194.8   |
| AAZ\textsuperscript{b} | 250.0 | 12.1   | 25.8   | 5.7     |
| SLC-0111\textsuperscript{b} | 5080 | 960   | 45.0   | 4.5     |

\textsuperscript{a}Mean from three different tests by a stopped-flow technique (approximately 5:10% of the reported numbers were erroneous).
\textsuperscript{b}AAZ, a standard sulphonamide carbonic anhydrase inhibitor. SLC-0111 is also provided for comparison.
(5d, $K_1 = 28.6\, \text{nM}$ and $5b, K_1 = 33.4\, \text{nM}$) > pyrazole derivatives $(7b, K_1 = 36.6\, \text{nM})$ > isatin analogues $(3c, K_1 = 43.8\, \text{nM})$.

Regarding the isatin analogues $3a$–c, substitution of isatin with an electron-withdrawing group (chlorine atom) in $3b$ and $3c (K_1 = 95.9$ and $43.8$ nM, respectively) potentiated the potency while an electron-donating group such as methyl group diminished the activity of analogue $3a (K_1 = 818.4$ nM). In addition, N-alkylation of 5-chloroisatin with benzyl group $3c (K_1 = 43.8$ nM) enhanced the activity more than the unsubstituted one, $3b (K_1 = 95.9$ nM). The diphenyl pyrazole analogues, $7b$–e $(K_1$ ranging from $36.6$ to $56.9$ nM), showed better inhibition of hCA IX than one phenyl analogue $7a (K_1 = 68.6$ nM). In the case of compounds $7c$–e, the substitution of phenyl ring at para position with either EWG or EDG reduced the inhibitory activity towards hCA IX than the unsubstituted analogue, $7b$.

Cyclic hydrazono linkers in five-membered rings reduced the potency of Series II compared to Series I, regarding SAR of Series II, potency declined in the following order: dihydropyrazoles $(11b, K_1 = 31.9$ nM) > fused triazole derivatives $(15b, K_1 = 40.7$ nM) > pyrazole derivatives $(13e, K_1 = 70.0$ nM). The fused triazol-3-ol analogue $15a (K_1 = 871.4$ nM) was noted the least inhibitor in the series. Replacing the hydroxyl group in $15a$ with the thiol group enhanced the inhibitory action against hCA IX by 21-fold, as noted in $15b (K_1 = 40.7$ nM).

(iv) Despite being less active than AAZ $(K_1 = 5.7$ nM), the target compounds significantly suppressed hCA XII. Their $K_1$ values ranged from $8.29$ to $2185$ nM. Analogue $11b$ with pyrazolone moiety directly attached to triazine scaffold exhibited the strongest inhibition of hCA XII with $K_1 = 8.29$ nM. Concerning SAR of Series I, phenyl hydrazono analogues $(5a$–d) exhibited the best activity as detected in $5b, K_1 = 14.9$ nM. Exchanging the phenyl group with isatin reduced the potency of analogues $3a$–c, $K_1$ ranging from $59.2$, $82.2$, and $24.4$ nM, respectively. Attaching phenylpyrazoles to the hydrazono linker $(7a$–e, $K_1$ from $78.1$ to $2185.0$ nM) diminished activity as observed in $7b, K_1 = 78.1$ nM. Moreover, alkylation of isatin enhances the activity towards hCA XII as shown in compound $3c (K_1 = 24.4$ nM) compared to compounds $3a$ and $3b$ with $K_1$ ranging from $59.2$ and $82.2$ nM, respectively. Target compounds in Series II ($K_1 = 8.29$–$751.3$ nM) were better inhibitors for hCA XII than series I $(K_1 = 14.9$–$2185.0$ nM). It was observed that SAR for Series II revealed that potency dropped in the following order: pyrazoline derivatives, $11b, K_1 = 8.29$ nM > fused triazoles, $15a, K_1 = 40.6$ nM > pyrazoles, $13b, K_1 = 77.8$ nM. In addition, replacing the hydroxyl group in $15a (K_1 = 40.6$ nM) with thiol group diminished the inhibitory activity against hCA XII by about five times, as informed in $15b (K_1 = 194.8$ nM). The dose-response curves for the determination of dissociation constants $(K_1)$ for inhibition of hCA I, II, IX, and XII isoforms induced by representative compounds $3c$, $5b$–d, and $11a,b$ are illustrated in Figures S2–S7 (see Supporting information).

As a result, the diphenyl hydrazinyl analogue $5d$ was the most effective anticancer substance. With $K_1$ of $28.6$ and $31.3$ nM for hCA IX and XII (the tumour-associated isoforms) and $4083$ and $4291$ nM for hCA I and II (the off-target isoforms), respectively, it showed the highest inhibitory impact relative to those isoforms.

Selectivity parameter. With high conservation in the all isoforms of CA active sites, the main sequence identity of the human CAs is at least 30.\(^{39}\) Designing isoform-selective CAs for CA IX with few off-target actions has been difficult due to the high conservation of amino acid standing between hCA isoforms.\(^{40}\) As demonstrated in Table 2, the compounds developed extraordinary selectivity towards hCA IX and XII (the tumour-associated isoforms) over the hCA I and II (off-target isoforms). The SRs, which are indicative parameters for enzyme selectivity and are pronounced in Table 2, were determined as the ratio between $K_1$ for hCA I and II related to hCA IX and XII.

Table 2. Selectivity ratios for the inhibition of hCA IX and XII over hCA I and II for target compounds $3a$–c, $5a$–d, $7a$–e, $9$, $11a,b$, $13a$–e, $15a,b$ and the standard inhibitors acetazolamide (AAZ) and SLC-0111.

| Compounds | I/IX | I/XII | I/IX | I/XII |
|-----------|------|------|------|------|
| AAZ       | 10   | 0.48 | 43.86| 2.10 |
| SLC-0111  | 112.9| 21.3 | 1128.9| 213.3|

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The $K_1$ ratios indicate isozyme selectivity: A low-value ratio indicates the presence of a weak selective inhibitor.

Selectivity as measured by the hCA I and II $K_1$ ratio in comparison to hCA IX and hCA XII.

- **i.** The calculated SR I/IX were ranged from $1567.40$ to $0.44$ in terms of the selectivity towards hCA IX over hCA I. Twelve compounds exhibited SR (I/IX) (from $1567.40$ to $17.23$) higher than AAZ value $(SR = 10)$ while four analogues displayed SR (I/IX) (from $1567.40$ to $142.76$) higher than SLC-0111 value $(SR = 112.9)$. Compound $11b$ with pyrazoline linker carrying benzodioxole and phenyl rings showed extraordinarily great hCA IX selectivity, with $SR$ (I/IX) = $1567.70$ (156-times that of AAZ). Replacement of benzodioxole ring with *para*-chlorophenyl ring reduced selectivity of analogue $11a$, SR (I/IX) = $220.26$. Compounds $5b$, $5d$, and $7c$ revealed remarkable selectivity over AAZ with SR (I/IX) = $105.48$, $142.76$, and $188.08$, respectively.

- **ii.** The compounds demonstrated higher hCA IX selectivity over hCA II, with SR (I/XII) ranged from $150.03$ to $0.12$ comparative to AAZ $(SR = 0.48)$. The analogue $5d$ with diphenyl hydrazono linker disclosed the highest hCA IX selectivity, with $SR$ (I/XII) = $150.03$ (312-times that of AAZ). Structural modifications to analogue $5d$ via substitution with either EWG or EDG upon the phenyl ring diminished selectivity while further target compounds presented lower selectivity with SR (I/XII) between $66.80$ and $0.12$.

- **iii.** The estimated SR (I/XII) for target molecules ranged from $6031.36$ to $0.14$ in terms of selectivity towards hCA XII over...
hCA I. Eleven compounds showed SR (I/XII) (from 6031.36 to 57.16) higher than AAZ value (43.86). Fortunately, compound 11b exhibited SR (I/XII) = 6031.36, about 6-times higher than SLC-0111 value with SR (I/XII) = 1128.9. Compounds 11a and 11b disclosed the best selectivity towards hCA XII, with SR (I/XII) = 1048.22 and 6031.36, which was 23 (1) and 137 (5) times that of AAZ (SLC-0111), respectively. The isatinylhydrazones, 3a-c and phenylhydrazones, 5a-d, reported the highest selectivity towards hCA XII, with SR (I/XII) ranging from 79.73 to 236.44. The selectivity was drastically reduced in 7a-e, 9, 13a-e, and 15a,b, having the pyrazole and triazole linkers.

iv. Thirteen analogues showed stronger selectivity for hCA XII than hCA II, with SR (II/XII) values ranging from 257.06 to 2.50 in comparison to AAZ, SR (II/XII) = 2.10. Compound 11b, SR (II/XII) = 257.06 showed higher selectivity than SLC-0111, SR (II/XII) = 213.3. Compound 11b with a pyrazoline linker exhibited the highest selectivity towards hCA XII, SR (II/XII) = 257.06, which was 122-times that of AAZ and higher than SLC-0111, SR (II/XII) = 213.3. Chemical modifications on the structure of compound 11b dropped selectivity. Analogues 9, SR (II/XII) = 167.95 and 5d, SR (II/XII) = 137.09 showed high selectivity while analogue 7d, SR (II/XII) = 0.03 reported the lowest selectivity.

Relying on the aforementioned findings, we have successfully developed new inhibitors with remarkable selectivity profiles towards hCAs IX and XII. Compound 11b displayed excellent selectivity concerning hCAs IX and XII (the tumour isoforms) over hCAs I and II (the off-target) with SR values = 1567.40, 66.80, 6031.36, and 257.06. Additionally, it demonstrated relatively high effectiveness against hCAsIX and XII, with K6 values of 31.9 and 8.29 nm, respectively. The most effective analogue, 5d, showed a respective selectivity profile with SR values of 142.76, 150.03, 130.44, and 137.09 against hCA IX and XII, respectively.

**In vitro evaluation of antiproliferative activity by NCI**

**In vitro preliminary screening anticancer activity at 10 µM towards 60 cancer cell panels.** Series I (3a-c, 5a-d, and 7a-e) and Series II (9, 11a,b, 13a-e, and 15a,b) of the newly synthesised triazine-based benzenesulphonanilides underwent initial anticancer screening activity at the National Cancer Institute (NCI) as part of a screening effort in the United States. The NCI’s preliminary *in vitro* 10 µM anticancer screening against the 60 cancer cell line panels representing nine types of cancer was carried out in accordance with the procedure using the novel analogues that were chosen and evaluated NCI. The treated cells’ mean graph percent growth (G%) in comparison to the control cells that were not treated was used to represent the results for the test compound. This graph includes values for cytotoxicity (less than 0) and inhibition (cytostatic) (between 0 and 100). The results of tested compounds against sixty cancer cell lines were evaluated using the COMPARE tool. When tested at 10 µM, the anticancer activity of the compounds ranged from poor to excellent, with a wide range of cytotoxic activity against several cancer cell lines. For target compounds, inhibition of percentage growth (G%) was estimated as (100 – G%) and given in Table 3. Compounds 3b, 3c, 5a, 7a, 7b, 7e, 9, 13a, and 15a,b that disclosed mean G% less than 10% did not declare in Table 3. All one-dose and five-dose charts are presented in Supplementary files.

Inspection of biological data in Table 3 revealed that analogues of Series II were more potent (mean G%, from 19 to 65) than target compounds of Series I (mean G%, from 12 to 58), while...
compounds in Series I displayed better selectivity than Series II. Regarding Series I, analogues 3a-c, the presence of methyl group at phenyl ring of isatin moiety enhanced the activity of 3a, mean GI% = 25 while exchanging methyl group with chlorine atom abolished the anticancer activity for analogues 3b and 3c. The anticancer activity of phenylhydrazones, 5a-d, was strongly potentiated upon the addition of two bromide atoms at the meta positions of the phenyl ring of analogue 5c. Moreover, adding another phenyl ring to the hydrazone linker in 5d, mean GI% = 41 enhanced the cytotoxic activity. Concerning phenyl pyrazole analogues 7a-e, analogues 7c (mean GI% = 17) and 7d (mean GI% = 12) with para chlorophenyl or para bromophenyl rings, respectively, showed better cytotoxic activity than unsubstituted phenyl analogue, 7b or para methoxy substituted one, 7e (both reported mean GI% less than 10).

The most active analogue 5c, GI% = 58, demonstrated very strong activity (lethal effect) and selectivity against two leukaemia cell lines (MOLT-4, GI% = 118 and 5R, GI% = 112), one of lung cancer cell lines (HOP-92, GI% = 104), one of CNS cancer cell lines (SF-539, GI% = 125), four of melanoma cell lines (GI% from 105 to 126), and four of breast cancer cell lines (GI% from 101 to 157). Analogue 5d, mean GI% = 41, disclosed selective and strong cytotoxic effect against five leukaemia cell lines (GI% from 66 to 81), one of lung cancer cell lines (GI% = 76), four of colon cancer cell lines (GI% from 64 to 73), one of renal cancer cell lines (GI% = 74), one of prostate cancer cell lines (GI% = 76), and one of breast cancer cell lines (GI% = 82). Analogue 3a, mean GI% = 25, reported strong and selective cytotoxicity towards two leukaemia cell lines only (GI% = 73 and 66). Compound 7c, mean GI% = 17, selectively exhibited strong anticancer effect against two leukaemia cell lines (GI% = 89 and 67) and breast cancer, MCF7 cell line (GI% = 66). Finally, 7d, mean GI% = 12, reported selective and strong cytotoxicity towards leukaemia, MOLT-4 cell line, GI% = 68.

Regarding Series II, the most active pyrazoline analogue 11a with two para chlorophenyl rings (mean GI% = 53) reported broad and strong cytotoxic activity towards all leukaemia cell lines, non-small cell lung cancer; NCI-H460, all colon cancer cell lines except SW-620, melanoma cell lines; LOX IMVI, M14, and MDA-MB-435, ovarian cancer; IGROV1 and OVCAR-3, renal cancer; ACHN, and breast cancer cell lines; MCF7 and MDA-MB-468 (Table 3) whereas it displayed lethal effect (GI% = 103) against renal cancer cell line; RXF 393. Replacement of one para chlorophenyl ring in compound 11a with a 1,3-benzodioxole ring of 11b (mean GI% = 19) diminished the antiproliferative activity while it showed selective and strong cytotoxic activity towards leukaemia (CCRF-CEM) cell line (GI% = 63). Considering pyrazole derivatives 13a-e, the most active analogue 13c (mean GI% = 67) with diphenyl pyrazole scaffold displayed broad and strong anticancer activity towards almost all tested cancer cell lines. Replacement of one phenyl ring of 13c with a pyridine ring reduced the cytotoxic activity of 13d (mean GI% = 27) and 13e (mean GI% = 22), while methyl phenyl analogue 13b (mean GI% = 23) showed lower activity as well. Analogue 13d revealed a selective and strong anticancer effect against three leukaemia cell lines (GI% from 64 to 61) and breast cancer, MCF7, cells (GI% = 60). Compound 13e disclosed strong selective anticancer activity towards renal cancer, CAKI-1, cells (GI% = 65).

In vitro anticancer screening at five doses towards 60 cancer cell panels. Because they met the NCI’s established threshold inhibition criteria, two compounds, 5c (NSC 834606) and 13c (NSC 832458), were screened and tested against the 60 cancer cell lines at 10-fold dilutions and five different concentrations (0.01, 0.1, 1,

Table 4. Five doses of in vitro anticancer activity results against all sixty cancer cell lines expressed as GI₅₀ (μM), TGI (μM), and LC₅₀ (μM) for compounds 5c and 13c.

| Subpanel/cell lines | 5c          | 13c         |
|---------------------|-------------|-------------|
| Leukaemia           |             |             |
| CCRF-CEM            | 11.1        |             |
| HL-60(TB)           | 22.0        |             |
| K-562               | 23.4        |             |
| MOLT-4              | 19.7        |             |
| RPMI-8226           | 22.8        |             |
| SB                 | 15.9        |             |
| NSC lung cancer     |             |             |
| A549/ATCC           | 21.8        | 3.18        |
| EKXV                | 21.4        | 2.96        |
| HOP-62              | 21.3        | 3.29        |
| HOP-92              | 26.7        | 2.16        |
| NCI-H226            | 26.5        | 2.52        |
| NCI-H23             | 20.9        | 2.84        |
| NCI-H322M           | 14.2        | 2.78        |
| NCI-H460            | 19.6        | 2.07        |
| NCI-H522            | 16.8        |             |
| Colon cancer        |             |             |
| COLO 205            | 16.0        | 1.78        |
| HCC-2998            | 14.8        | 2.98        |
| HCT-116             | 17.7        | 1.74        |
| HCT-15              | 32.3        | 3.08        |
| HT29                | 19.6        | 2.38        |
| SW-620              | 17.5        | 2.87        |
| SF-268              | 17.2        | 3.52        |
| CNS cancer          |             |             |
| SF-295              | 19.9        | 3.97        |
| SF-539              | 17.9        | 3.60        |
| SNB-19              | 14.2        | 3.44        |
| SNB-75              | 18.6        | 3.09        |
| U251                | 20.0        | 2.91        |
| Melanoma            |             |             |
| LOX IMVI            | 15.8        | 3.04        |
| MALME-3M            | 14.7        | 2.43        |
| M14                 | 18.8        | 3.22        |
| MDA-MB-435          | 17.8        | 3.32        |
| SK-MEL-2            | 15.7        | 3.29        |
| SK-MEL-5            | 18.1        | 3.87        |
| UACC-257            | 20.6        | 3.26        |
| UACC-62             | 14.5        | 3.76        |
| Ovarian cancer      |             |             |
| IGROV1              | 18.5        | 2.72        |
| OVCAR-3             | 21.4        | 2.44        |
| OVCAR-4             | 23.2        | 3.77        |
| OVCAR-5             | 22.7        | 3.34        |
| OVCAR-8             | 26.9        | 3.22        |
| NCI/ADR-RES         | >100        | 2.93        |
| Renal cancer        |             |             |
| 786-0               | 21.3        | 5.37        |
| A498                | 13.3        | 3.09        |
| ACNH                | 24.8        | 2.59        |
| CAKI-1              | 24.0        | 2.55        |
| RXF-393             | 14.7        | 4.95        |
| SN 12 C             | 15.6        | 1.47        |
| TK-10               | 27.7        | 3.87        |
| UO-31               | 26.7        | 3.20        |
| Prostate cancer     |             |             |
| PC-3                | 19.5        | 2.37        |
| DU-145              | 16.1        | 3.16        |
| Breast cancer       |             |             |
| MCF7                | 16.9        | 2.27        |
| MDA-MB-231          | 17.2        | 1.81        |
| HS 578T             | 77.6        | 4.31        |
| BT-549              | 16.8        | 2.24        |
| T-47D               | 7.39        | 2.96        |
| MDA-MB-468          | 2.94        | 3.21        |

*In vitro human cancer cell lines screen data from NCI. **Molar concentration necessary to inhibit 100% of growth of cancer cell line. ***Molar concentration necessary to kill 50% of cancer cell line. NT: not tested.
10, and 100 M). Following the described experimental techniques, the SRB (sulforhodamine-B) protein assay was used to compare the viability of treated versus untreated cells.

The results of this assay are stated in GI50 (molar concentration required to inhibit 50% of the growth of cancer cell line), TGI (molar concentration required to inhibit 100% of the growth of cancer cell line), and LC50 (molar concentration required to kill 50% of cancer cell line) after a 48-h incubation period for each cell line tested.

Compound 5c displayed strong cytotoxic activity with GI50 values ranging from 2.94 to 32.2 M (except against H575T (77.6 M) and NCI/ADR-RES (＞100 M)), TGI values ranging from 15.5 to ＞100 M, and LC50 values ranging from 52.4 to more than 100 M. Compound 5c demonstrated the greatest cytotoxic activity towards NCI-H322M NSC lung cancer cell line, LC50 = 52.4 M, while it exposed the most cytostatic activity towards MDA-MB-468 breast cancer cell line with GI50 = 2.94 M and TGI = 15.5 M followed by its effect on T-47D on same cancer with GI50 = 2.88 M, and TGI = 18.8 M as displayed in Table 4.

Compounds 5c and 13c showed outstanding action against cancer cells, according to the findings of anticancer screening of the five-dose. Compound 5c displayed strong cytotoxic activity with GI50 values ranging from 2.94 to 32.2 M (except against H575T (77.6 M) and NCI/ADR-RES (＞100 M)), TGI values ranging from 15.5 to ＞100 M, and LC50 values ranging from 52.4 to more than 100 M. Compound 5c demonstrated the greatest cytotoxic activity towards NCI-H322M NSC lung cancer cell line, LC50 = 52.4 M, while it exposed the best cytostatic activity towards MDA-MB-468 breast cancer cell line with GI50 = 2.94 M and TGI = 15.5 M followed by its effect on T-47D on same cancer with GI50 = 7.39 M and TGI = 27.3 M as displayed in Table 4.

Compound 13c reported stronger cytotoxic activity than 5c, with GI50 values ranging from 1.47 to 5.37 M (all in the single-digit micromolar range), TGI values ranging from 2.88 to ＞100 M, and LC50 values ranging from 5.63 to more than 100 M. It revealed the greatest cytostatic activity towards the majority of the cancerous cell lines, including; MOLT-4 "most affected one in leukemia" with GI50 = 1.51 M, NCI-H460 "most affected one in lung cancer" with GI50 = 2.07 M, HCT-116 "most affected one in colon cancer" with GI50 = 1.74 M, SF-539 "most affected one in CNS cancer" with GI50 = 2.66 M, UACC-62 "most affected one in melanoma" with GI50 = 1.81 M, OVCAR-3 "most affected one in ovarian cancer" with GI50 = 2.24 M, SN 12 C "most affected one in renal cancer" with GI50 = 1.47 M, PC-3 "most affected one in prostate cancer" with GI50 = 2.37 M, and MDA-MB-231 "most affected one in breast cancer" with GI50 = 1.81 M. It exhibited the best cytotoxic action towards the SN 12 C renal cancer cell lines with LC50 = 5.63 M (Table 4).

A mean graph midpoints (MG-MID) were computed, resulting in averaged activity parameters across all cell lines. The GI50-MID values for the compounds 5c, 13c, and 5-FU were 189.01, 25.08, and 65.16 M, respectively (Table 5 and Figure 3). The ratios were calculated by dividing the full panel MID by their individual sub-panel MID and were used to determine the selectivity of these compounds (the sensitivity average of the whole cell lines of a particular subpanel). SRs between 3 and 6 indicate moderate selectivity, whereas ratios of more than 6 reveal the best selectivity towards the associated cell line. The compounds that match none of these requirements are classed as non-selective. Accordingly, the studied compounds, 5c and 13c are non-selective.

### Table 5. Selectivity ratios of the analogues 5c and 13c in comparison to 5-FU towards nine tumours.

| Compound | 5c | 13c |
|----------|----|-----|
| Leukaemia | 19.15 | 21.06 |
| NSC lung cancer | 21.02 | 1.09 |
| Colon cancer | 19.30 | 1.09 |
| CNS cancer | 18.80 | 1.12 |
| Melanoma | 16.76 | 1.25 |
| Ovarian cancer | 33.42 | 0.63 |
| Renal cancer | 20.23 | 1.04 |
| Prostate cancer | 17.80 | 1.18 |
| Breast cancer | 23.13 | 0.91 |
| GI50-MID | 189.01 | 25.08 |

**Table 6. Cytotoxic activities of compounds 5d and 13c against MCF7 (breast cancer), and NCI-H460 (lung cancer) under hypoxia compared to 5-FU.**

| Compound No. | MCF7 (Breast cancer) | NCI-H460 (Lung cancer) |
|--------------|----------------------|------------------------|
| 5d           | 15.02 ± 0.02         | 10.12 ± 0.03           |
| 13c          | 3.03 ± 0.01          | 4.62 ± 0.02            |
| 5-FU         | 4.10 ± 0.02          | 6.77 ± 0.02            |

*IC50 values are the mean ± SD of three experiments.*

Figure 3. GI50-MID and average sensitivity of all cell lines (μM) of 5c (blue), and 13c (orange), in comparison to 5-FU (grey).
and have a broad-spectrum antitumor effect against the examined nine tumor subpanels, with SRs ranging from 0.63 to 1.25.

Compound 13c showed the best potency with average MID = 2.78 \( \mu \)M, which was better than 5-FU, average MID = 7.24 \( \mu \)M, and 5c, average MID = 21.06 \( \mu \)M. It disclosed the greatest potency and selectivity towards leukaemia, MID = 2.29 \( \mu \)M, with selectivity = 1.21, while it demonstrated better potency and selectivity towards NSCLC lung cancer, MID = 2.67 \( \mu \)M, selectivity = 1.04 compared to 5c, MID = 21.02 \( \mu \)M, selectivity = 1.00 and 5-FU, MID = 20.11 \( \mu \)M, selectivity = 0.36. Compound 5c with average MID = 21.06 \( \mu \)M, showed the greatest potency and selectivity against melanoma, MID = 16.76 \( \mu \)M, selectivity = 1.25 which were more selective than 13c, selectivity = 0.98 and 5-FU, selectivity = 0.91 (Table 5 and Figure 3).

**Activities of compounds 5d and 13c against MCF7 and NCI-H460 cancer cell lines under hypoxia**

Using the SRB assay in hypoxic circumstances (1% O2, 5% CO2) at 37°C, sulphonamide derivatives 5d and 13c were tested for their in vitro cytotoxic effects against the MCF7 (breast cancer) and NCI-H460 (lung cancer) cell lines. S-FU was applied as a positive control, and the concentration needed to inhibit cell viability by 50%, or IC\(_{50}\), was determined (Table 6). Compound 13c displayed the most potent activity towards MCF7, and NCI-H460, with IC\(_{50}\) values of 3.03 \( \pm \) 0.01 \( \mu \)M (by five-folds) and 4.62 \( \pm \) 0.02 \( \mu \)M (by 2-fold), respectively, compared to compound 5d, which showed IC\(_{50}\) values of 15.02 \( \pm \) 0.02 and 10.12 \( \pm \) 0.03 \( \mu \)M, respectively. Additionally, compound 13c has superior activity against MCF7 and NCI-H460 compared with positive reference drug (5-FU) with IC\(_{50}\) values of 4.10 \( \pm \) 0.02 \( \mu \)M and 6.77 \( \pm \) 0.02, respectively.

**Toxicity of 13c and 5-FU towards normal human cells**

13c demonstrated a strong tumour proliferation suppression effect in vitro as a possible anticancer cancer agent. We investigated 13c’s possible toxicity towards healthy human cells to learn more about its therapeutic properties. For this, LO2 (human normal liver cells) and HK2 (human kidney proximal convoluted tubule epithelial cells), two different types of nontumorigenic cell lines, were used. The results reported in Table 7 indicated that 13c exhibited a far safer impact on normal human cells (LO2 and HK2) with IC\(_{50}\) values of 30.88 \( \pm \) 0.98 and 53.39 \( \pm \) 1.58 \( \mu \)M, respectively, using 5-FU as a positive control, which presented IC\(_{50}\) values of 18.71 \( \pm \) 0.48 and 34.01 \( \pm \) 0.98 \( \mu \)M, respectively.

**Compound 13c suppresses the migratory of NCI-H460 cells**

The ability of 13c to prevent the metastasis of NSCLC cells in vitro was examined because tumour cell migration is one of the key factors contributing to the death of cancer patients. The effect of 13c on NSCLC cell migration was examined using transwell invasion assays and wound healing experiments. In contrast to cells treated with a vehicle, compound 13c greatly reduced the migration of NCI-H460 cells, as seen in Figure 4. This suggests that 13c may be a potential choice for preventing metastasis.
Colony formation assay in NSC lung cancer, NCI-H460 cells

The colony-forming assay, an in vitro test for cell survival, assesses a cell's capacity to multiply into a colony. Each cell in the population is tested to see if it divides widely and forms foci. Additionally, it keeps track of the cells that have kept their ability to form colonies after being exposed to agents that cause cell death (chemotherapeutic agents or radiations). Compound 13c's effective and broad-spectrum proliferative inhibition in this work motivated us to investigate how it affected NCI-H460 cells' ability to form cell colonies (one of the most sensitive cell lines as determined in previous NCI assays). Ten days following the compound 13c treatment, colony development was assessed. Compound 13c
was able to significantly reduce colony formation in the tested cells when compared to the untreated control, as shown in Figure 5.

**Annexin V–FITC apoptosis assay**

The primary method by which drugs kill cancer cells is by the activation of apoptosis.\(^50,51\) Cellular alterations brought on by apoptosis include translocating phosphatidylserine (PS) from the inside to the outside via the plasma membrane. PS can bind to Annexin-V, making it sensitive to PS on the plasma membrane’s outer side.\(^52,53\) We used cytometric assay to separate the apoptosis from the necrosis mechanism of NCI-H460 (melanoma) cells death caused by the most potent analog, 13c. NCI-H460 cells were stained with AV/PI for 24 h using compound 13c (10 mM). Results from treating NCI-H460 cells with compound 13c for 24 h are displayed in Figures 6 and 7. We find that the early apoptosis ratio increased from 0.59% in the negative control (DMSO) to 15.34% (Figure 6, lower-right quarter of the cytogram) and that the late apoptosis ratio increased significantly from 0.18 to 26.56%. These data demonstrate that the necrotic pathway is not the mechanism driving compound 13c-induced programmed cell death but rather the apoptotic pathway.

**In vitro cell cycle analysis**

Antitumor drugs can cause S-phase cell cycle arrest and apoptosis via activating signalling pathways.\(^54-58\) The proliferation of cells in various cell cycle phases (pre-G1, G1, S, and G2/M) is measured by flow cytometry.\(^59\) The NCI-H460 cell line was used to further examine the effects of the most active compound, 13c, on cell cycle progression (Figure 8). As a negative control, we employed the solvent DMSO. In a nutshell, we gave NCI-H460 cells 24 h of exposure to 10 \(\mu\)M of compound 13c. Compound 13c disrupted the NCI-H460 cells’ typical cell cycle. An increase in cells in the S phase (42.86%) in comparison to the control suggested that there was a considerable impact on the proportion of apoptotic cells (28.73%). Cell cycle arrest resulted from a considerable drop in the proportion of cells in the G0/G1 and G2/M phases (55.29% and 1.85%, respectively) as compared to the control (62.42% and 8.84%, respectively). The alteration of the S-phase arrest is a crucial observation for compound 13c to induce apoptosis in NCI-H460 cells (Figure 8).

**In silico analysis**

**Molecular docking analysis**

In hCA IX, having the active site at the bottom side of the conical cavity, the three residues of histidine (His 94, 96, and 119) make the coordination interaction with the zinc ion at the bottom of the active site.\(^60,61\) The target compounds demonstrated potential for being potent and selective hCA IX inhibitors. Consequently, the mechanism of action of target compounds were explored via evaluating the docking profiles and amino acid interactions for
analouges, 5d, and 13c within the active region of hCA IX (PDB, ID: 3IAI). The docking process was achieved by MOE program.33,62 The docking of compounds 5d and 13c on the hCA IX active site illustrated proper fitting and good energy scores (S), suggesting the inhibitory activity of these sulphonamides as displayed in Figure 9 and Table 8. The docking scores (S) and interactions of inhibitors 5d and 13c with various amino acids on the active site of hCA IX were reported in Table 8. The deprotonated sulphonamide group’s nitrogen and the triple histidines were coordinated to the Zn$^{2+}$ atom conferring to molecular docking of compound 5d (Figure 9). The gatekeeper amino acids of this enzyme, Thr199, and Thr200, were joined by two H-bonds and one H-bond, respectively, to the sulphonamide groups of docked inhibitors.63 Furthermore, the hydrophobic region of the hCA active site (Leu91, Val121, Val131, and Leu141) was attracted to the two phenyl rings that were linked to the methylidene hydrazone moiety. The hydrophobic interaction between the N-phenyl segment and Arg60 was observed (Figure 9(A,B)). In 13c, the nitrogen atom of sulphonamide showed coordination interaction with the Zn$^{2+}$ atom and could form H-bonds with Thr199 and Thr200. Val131 and Pro202 of the CA active site’s hydrophobic region revealed an interaction with the diphenylpyrazole moiety of 13c hydrophobically (Figure 9(C,D)).

**Molecular dynamics (MD) simulation**

The MD simulation using the GROMACS program64–66 was performed to study the behaviour of the most potent compound 5d within the target hCA IX through the time of the simulation (100 ns) under comparable physiological conditions.

**Analysis of the root mean square deviation (RMSD).** Quantitatively to measure the degree of divergence of complex protein structure with ligand from its initial behaviour, the root mean square deviation (RMSD) was explored.67 The RMSD aids in assessing the system’s stability during the simulation. For this, a control system (a ligand-free structure) and complex were set up in two separate MD simulations. A 100-ns MD simulation was used to examine the stability and convergence of compound 5d in its complex with hCA IX where the backbone atoms’ RMSD value was calculated as illustrated in Figure S8. The results suggested that complex-maintained equilibrium throughout the simulation time. The apoprotein and the compound 5d-bound complex’s RMSD values ranged from 0.17 to 0.33 nm. Over the duration of the simulation, compound 5d displayed consistent behaviours inside the receptor pocket and moved further into the binding pocket. This could account for the strong inhibitory activity of 5d against hCA IX.

**Analysis of the root mean square fluctuation (RMSF).** The root mean square fluctuation (RMSF) was studied to represent the local changes that occur within the protein structure due to the presence of the recommended inhibitor.68 It revealed the flexibility degree of the protein throughout the simulation time. The most fluctuation was observed within the 0.03 – 0.23 nm range. In general, the native unbound hCA IX was more flexible than the comparable residues in the compound 5d-bound complex. The values of the key residues implicated in intermolecular interactions, such as Arg60, Leu91, His94, His96, His119, Val121, Val131, Leu141, Thr199, and Thr200, were also found to be at the bottom of the curve (0.03–0.09 nm) after the RMSF analysis. The docked molecules’ stability at the binding site was aided by these low-fluctuating residues (supplemental Figure S9).

**Analysis for the radius of gyration (R$_g$).** The size and compactness of protein molecules are indicated by the radius of gyration (R$_g$). When ligands are bound, the R$_g$ can be utilised to monitor the folding and unfolding of protein structures.69 Generally, the R$_g$ values for the drug-bound complexes were nearer to the native unbound hCA IX (Figure S10). The average R$_g$ values for compound 5d and hCA IX were measured to be 1.74–1.80 nm. A higher R$_g$ denotes a less compact or more unfolded protein–ligand interaction. However, a protein is said to be securely folded if its R$_g$ value stays constant during the MD simulation. If the value of R$_g$ changes with time, it is seen as unfolded. As seen in Figure S10, each complex revealed extremely comparable characteristics in terms of compactness and practically consistent values of R$_g$ when compared to the unbound protein.

**Analysis of solvent-accessible surface area (SASA).** The protein’s solvent-accessible surface area (SASA) was investigated both in the absence and presence of ligands. The amount of conformational changes that the aqueous solvent can access is predicted with the help of the protein-ligand complex’s SASA computation.70 Therefore, throughout the 100-ns MD simulation, the SASA was employed to assess interactions between the complex and the solvent. Figure S11 displays the SASA versus simulation time curve for the unbound protein and protein-ligand complexes. The SASA averages for compound 5d and CA ranged from 120 to 133 nm$^2$. The extended surface formed by a piece of the bound ligand surface sticking out from the protein surface upon compound 5d binding triggered the SASA to rise slightly.

**Analysis of hydrogen bond.** Hydrogen bonds that developed between the receptor and ligand help to stabilise the protein-ligand complex. Additionally, it affects the specificity, metabolism, and adsorption of drugs and their design.71 Therefore, each ligand–protein complex’s hydrogen bonds were examined. Following a 100-ns simulation, Figure S12 shows the total number of hydrogen bonds found in the complex. One to three hydrogen bonds were found in the hCA-5d complex, and one of them was constantly present throughout the simulation time. In addition, during the course of the simulation, compound 5d revealed a consistent hydrogen-bonding pattern, as seen in Figure S12. We could infer from the above-described H-bond study that compound 5d was tightly and successfully attached to the hCA IX. The CA-5d complex’s hydrogen bonds contact frequency is disclosed in Figure S13.

**Binding energy estimation by MM/PBSA method.** The molecular mechanics/Poisson Boltzmann surface area (MM/PBSA) approach was chosen for rescoring complexes because it computes the free energy of binding more quickly than other force field-based

| Complex 5d-hCA IX | $\Delta$G (kJ/mol) |
|------------------|-------------------|
| Van der Waal energy | $-154.67 \pm 17.16$ |
| Electrostatic energy | $-133.32 \pm 27.50$ |
| Polar solvation energy | $192.19 \pm 25.08$ |
| SASA energy | $-22.96 \pm 1.55$ |

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### Table 9. Calculated binding free energy of the compound 5d (kJ/mol).

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methods like the free energy perturbation (FEP) or thermodynamic integration (TI) methods.\textsuperscript{55} The MM/PBSA calculation was performed using g_mmpbsa software. The calculated binding free energies are illustrated in Table 9. The van der Waals attraction, electrostatic interactions, and non-polar solvation energy were the key contributors to the binding, while the polar solvation free energy weakened the complexation, according to this study. The average overall binding free energy of the complex is – The va ± 30.583 kJ/mol.

**Conclusion**

With the use of the dual-tail method, we were able to develop potent and selective hCA IX inhibitors that could potentially act as cytotoxic agents. Twenty-two novel anticancer compounds were designed, synthesised, characterised, and biologically tested. With \( K_I \) values ranging from 25.8 nM (hCA I); 30.8–4585 nM (hCA II); 28.6–871 nM (hCA XI); and 8.29–2185 nM (hCA XII), all assayed hCA isoforms were inhibited by analogues to varying degrees. The majority of the target compounds displayed a strikingly better selectivity towards CA IX than AAZ. Superior to AAZ (mean \( GI_50 \) = 25.8 nM, SR (I/IX)) and to AAZ, making it more potent and selective. However, in accordance with US-NCI policy, all target compounds were examined for their anti-cancer efficacy at 10\( ^{–5} \) M towards 60 cancer cell lines. The strongest antiproliferative actions were demonstrated by analogues 13c (mean \( GI_50 \) = 65) and 5c (mean \( GI_50 \) = 58). Comprising hydrazine linker in a rigid cyclic structure such as pyrazole ring enhanced anticancer activity of analogue 13c compared to flexible hydrazine linker in analogue 5c. Bulky and lipophilic tails attached to either pyrazole linker in 13c or hydrazone linker in 5c enhanced anticancer activity due to the lipophilic nature and large cavity size of the hCA IX active site. Moreover, compound 13c was screened for apoptosis and disturbance of cell cycle in NCI-H460 cells, where it was arrested at the S phase of the cell cycle, and the percent of annexin V-FITC positive apoptotic cells increased (mean \( GI_50 \) = 2.6-H2 of phenylamine, 4-H of phenylamine, 7.22 (s, 1H, 6-H of isatin), 7.28 (s, 2H, SO2NH2), 7.36 (s, 2H, 3,5-H2 of phenylamine), 7.76–7.96 (m, 5H, 2,6-H2 of phenylamine, 4-H of isatin and 2,6-H2 of benzene sulfonamide), 8.05 (s, 2H, 3,5-H2 of benzene sulfonamide), 9.99 (s, 1H, 6-NH), 10.25 (s, 1H, 2-NH), 10.68 (s, 2H, 4-NH and CONH). \( ^{13} \)C NMR (100 MHz, DMSO-d6 and DMF): \( \delta \) = 24.45; S, 6.22. Found: C, 55.60; H, 4.15; N, 24.38; S, 6.15.

**General procedure for preparation of compounds 3a–c**

A mixture of isatin derivatives 2a–c (0.3 mmol) in hot, dry methanol (10 mL) and a few drops of acetic acid (glacial) was added to an equimolar amount of compound 1 (112 mg, 0.3 mmol) in dry methanol (10 mL). The reaction mixture was heated under reflux for 24 h. To obtain the pure products, 3a–c, the separated products were collected, washed with pet. ether, and recrystallised from isopropanol.

**Experimental protocols**

**Chemistry**

Using a Stuart SMP30 apparatus, melting points were found in open-glass capillaries and were not adjusted. The Sigma-Aldrich, Alfa-Aesar, and Merck companies provided all of the organic chemicals and solvents, which were all employed without additional purification. Pre-coated aluminium sheets and silica gel (Silica 60 F254, Supelco Co., Poole, UK) are frequently used in analytical thin-layer chromatography (TLC) to check reaction completion and verify the purity of the compounds utilising the developing system: n-hexane, ethyl acetate (2:3) eluent by using a UV light with a wavelength of 254 nm. The Faculty of Pharmacy and Science, Mansoura University, Mansoura, Egypt, performed \(^1\)H NMR, \(^{13}\)C NMR, and APT spectra using a Bruker or JEOl instrument at 400–500 MHz for \(^1\)H NMR and at 100–125 MHz for \(^{13}\)C NMR. TMS was used as an internal standard, and chemical shifts were recorded in ppm on the scale using DMSO-d6 as the solvent. Compounds 3a and 3b were dissolved in a mixture of DMSO-d6 and DMF. Values for the coupling constant (\( J \)) were calculated in Hertz (Hz). The following splitting patterns are identified: singlet (s), wide singlet (br, s), doublet (d), triplet (t), and multiplet (m). The extremely low solubility of some compounds was the cause of the absence of some signals in \(^{13}\)C NMR spectra. Thermo Scientific’s ISQ Single Quadrupole MS was used to record the electron impact mass spectra. C, H, N, and S underwent microanalysis at the Microanalytical Laboratory of the Microanalysis Centre, Al-Azhar University in Nasr City, Cairo, Egypt. The purity of selected most active compounds 5d and 13c was 97.46 and 98.99%, respectively, as determined by HPLC (Agilent Technologies, Santa Clara, CA). Ten \( \mu \)L of the solution was injected on a column (100 mm × 3.0 mm; 3.5 \( \mu \)m; ZORBAX\textsuperscript{\tiny{X}} XDB-C18). The column was kept in a thermostat at 25 °C. Water and acetonitrile (60:40) were used as the mobile phase at flow rate of 1.50 mL/min operated at 254 nm. Retention time (min), area peak, and the purity percentage obtained from HPLC analysis are summarised in Tables S1 and S2 and Figures S58 and S59 (see Supporting information).
(E)-4-((4-(2-(1-Benzyl-5-chloro-2-oxoindolin-3-ylidene)hydrazinyl)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzenesulfonamide 5a. A pale green powder, yield: 77%. Mp: 278–280 °C. 1H NMR (400 MHz, DMSO-d6): δ 3.21 (s, 4H, 2CH2 of morpholine), 3.76 (s, 4H, 2CH2 of morpholine), 7.03 (s, 3H, 4-H of phenylamine and 3-H5 of phenyl), 7.29 (s, 4H, SO2NH3 and 3,5-H5 of phenylamine), 7.60 (s, 2H, 2,6-H2 of phenylamine), 7.77 (br, s, 4H, 2,6-H2 of benzene-sulfonamide), 8.07 (s, 2H, 3,5-H2 of benzene-sulfonamide), 8.15 (s, 1H, N = CH), 9.38–9.88 (m, 2H, 6-NH and 2-NH), 10.92 (s, 1H, NH of 4-NH). 13C NMR (100 MHz, DMSO-d6) showed signals for CH appeared at the negative side (below the base line of the spectrum): 114.97, 119.57, 120.84, 122.64, 126.73, 128.18, 128.88, and 144.28 whereas CH2 quaternary carbons and carbons of deuterated DMSO solvent were observed at positive side (above the base line of the spectrum): 48.11, 66.46, 125.77, 137.06, 140.38, 143.88, 152.21, and 164.36. MS (ESI) (m/z): 545.52 [M+]. Anal. calcld for C28H24N8O2S: C, 62.67, H, 4.51; N, 20.88; C, 58.40; H, 4.51; N, 23.23; S, 5.53.

(E)-4-((4-(2-(3,5-Dibromo-4-hydroxybenzylidyne)hydrazinyl)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzenesulfonamide 5c. An off-white powder, yield: 70%. Mp: 225–227 °C. 1H NMR (500 MHz, DMSO-d6): δ 7.03 (t, 1H, 4-H of phenylamine, J = 8.0 Hz), 7.24 (s, 2H, SO2NH3), 7.33 (s, 2H, 3,5-H2 of phenylamine), 7.73–8.02 (m, 8H, Ar-Hs), 8.07 (s, 1H, N = CH), 9.49–9.93 (m, 2H, 2-NH and 6-NH), 10.37 (s, 1H, 4-NH), 11.36 (s, 1H, OH). 13C NMR (125 MHz, DMSO-d6): δ 112.28, 118.94, 119.12, 119.38, 126.34, 128.49, 130.25, 132.25, 132.07, 137.00, 151.63, 163.79. MS (ESI) (m/z): 631.05 [M+]. Anal. calcld for C32H19BrN3O2S: C, 58.44; H, 3.96; N, 21.98; S, 5.03. Found: C, 58.25; H, 3.90; N, 22.22; S, 5.16.
light brown powder, yield: 75%. Mp: 290–292 °C. 1H NMR (400 MHz, DMSO-d6): δ: 3.18 (s, 1H, 4-H of pyrazole), J = 16.8 Hz), 3.92–3.99 (m, 1H, 4-H pyrazole), 5.88 (s, 1H, 5-H pyrazole), 7.00 (s, 1H, 4-H of phenylamine), 7.26–7.46 (m, 8H, SO2NH2, 3,5-H2 of phenylamine and Ar-Hs), 7.58–8.13 (m, 10H, Ar-Hs), 9.45–9.79 (m, 2H, 6-NH and 2-NH). 13C NMR (100 MHz, DMSO-d6): δ: 42.28, 61.24, 119.49, 120.46, 122.57, 126.72, 127.56, 128.67, 128.85, 129.22, 129.42, 130.91, 131.23, 135.04, 137.19, 142.25, 152.30, 162.59, 164.4. MS (ESI) (m/z): 630.83 [M+]. Anal. calcld for C30H24Cl4N2O2S: C, 57.06; H, 3.83; N, 17.74; S, 5.19.

- **General procedure for preparation of compounds 13a–e**
  The mixture of the compound 1 (372 mg, 1 mmol) and 1,3-diketones derivatives 12a–e (1 mmol) in abs. methanol (5 mL) was refluxed for 5 h. The mixture was evaporated under a vacuum and refrigerated in cold water (20 mL) overnight. Products 13a–e were filtered, dried, and recrystallised from petroleum ether.

4-(4-((3-S-Bis[4-Chlorophenyl]-4-dihydro-1H-pyrazol-1-yl)-6-(phenylamo)-1,3,5-triazin-2-yl)amino)benzenesulfonamide 11a. A white powder, yield: 68%. Mp: 280–282 °C. 1H NMR (400 MHz, DMSO-d6): δ: 2.29 (s, 3H, CH3 pyrazole), 6.52 (s, 1H, 4-H pyrazole), 6.99 (t, 3H, 3,4,5-Hs of phenylamine, J = 7.2 Hz), 7.20–7.55 (m, 9H, SO2NH2, CH3 pyrazole and 2,6-Hs of phenylamine), 7.60–8.50 (m, 4H, 2,3,5,6-Hs of benzenesulfonamide), 10.04 (d, 1H, 6-NH), 10.50 (d, 1H, 2-NH). 13C NMR (100 MHz, DMSO-d6): δ: 13.84, 110.86, 119.78, 120.52, 123.17, 126.72, 128.62, 128.85, 129.28, 131.51, 137.91, 145.49, 150.64, 163.84, 164.87. MS (ESI) (m/z): 498.33 [M+]. Anal. calcld for C29H22N2O5S: C, 60.23; H, 4.45; N, 22.48; S, 6.43. Found: C, 60.55; H, 4.60; N, 22.80; S, 6.30.
4-((4-(3,5-Diphenyl-1H-pyrazol-1-yl)-6-(phenylamino)-1,3,5-triazin-2-yl)amino)benzenesulfonamide 13c. A yellow powder, yield: 54%. Mp: 198–200°C. HPLC analysis: retention time, 8.128 min; peak area, 98.99%. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$: 7.02–7.99 (m, 2H, Ar-Hs), 9.47–9.92 (m, 2H, 6-NH and 2-NH). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$: 108.03, 119.99, 120.76, 126.14, 126.75, 128.69, 129.10, 129.37, 131.23, 132.55, 138.12, 146.41, 152.56, 165.07. MS (ESI) (m/z): 560.70 [M$^+$]. Anal. calcd for C$_{29}$H$_{23}$N$_9$O$_2$S: C, 46.69; H, 3.32; N, 21.33; S, 5.57.

4-((4-(3-Hydroxy-7-(phenylamino)-[1, 2, 4]triazolo[4,3-a][1, 3, 5]triazin-2-yl)amino)benzenesulfonamide 13e. A red powder, yield: 59%. Mp: 268–270°C. $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta$: 7.28–8.61 (m, 2H, Ar-Hs), 9.69–10.16 (m, 2H, 6-NH, and 2-NH). $^{13}$C NMR (100 MHz, DMSO-d$_6$): $\delta$: 119.53, 120.15, 120.54, 122.74, 123.20, 126.72, 127.03, 128.86, 129.30, 130.54, 131.92, 137.26, 137.83, 149.71, 154.54, 163.90. MS (ESI) (m/z): 561.33 [M$^+$]. Anal. calcd for C$_{29}$H$_{23}$N$_9$O$_2$S: C, 48.24; H, 3.72; N, 3.58. Found: C, 48.19; H, 3.57; N, 3.23; S, 5.49.

General procedure for preparation of compounds 15a,b

The mixture of 1 (1 mmol, 0.176 g) and ethyl chloroformate (1 mmol, 0.109 g) in pyridine (2 mL) was refluxed for 16 h. The mixture was poured into cold water and then acidified using dil. hydrochloric acid. The formed precipitate was filtered washed several times with cold water, dried, and recrystallised to give compounds 15a,b.

Biological evaluation

The comprehensive procedures of biological assays of the target sulphonamides series I (3a–c, 5a–d, and 7a–e) and series II (9, 11a,b, 13a–e, and 15a,b) are presented in the Supplementary materials, including; CA I, II, IX, and XII inhibition studies, 3NCl-USA screening, 20,72 antiproliferative activities under hypoxic conditions, 73 toxicity towards normal human cells, 77 cell migration study, 76 colony formation assay, 79 apoptosis assay, 74 and cell cycle analysis. 75,76

In silico studies

The comprehensive procedures of in silico studies of the representative target sulphonamides Series I and II were presented in the Supplementary materials, including; molecular docking analysis and MD simulations. 64

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References

1. Feng CY, Chen ZF, Pei LL, et al. Genome-wide identification, phylogeny, and expression analysis of the CA gene family in tomato. Biotechnol Biotechnol Equip. 2020;34(1):70–83.
2. Momayyezi M, McKown AD, Bell SC, Guy RD. Emerging roles for carbonic anhydrase in mesophyll conductance and photosynthesis. Plant J. 2020;101(4):831–844.
3. Güttler A, Eiselt Y, Funtan A, et al. Betulin sulphonamides as carbonic anhydrase inhibitors and anticancer agents in breast cancer cells. Int J Mol Sci. 2021;22(16):8808–8821.
4. Becker HM, Deitmer JW. Proton transport in cancer cells: the role of carbonic anhydrases. UMS. 2021;22(6):3171.
5. Supuran CT, Scozzafava A. Carbonic anhydrases as targets for medicinal chemistry. Bioorg Med Chem. 2007;15(13): 4336–4350.
6. Osmaniye D, Türkç T, Demir Y, et al. Design, synthesis, and biological activity of novel dithiocarbamate-methylsulfonyl hybrids as carbonic anhydrase inhibitors. Arch Pharm. 2022; 355(8):2200132–15.

7. Zhang C, Fang L, Wang X, et al. Oncolytic adenovirus-mediated expression of decorin facilitates CAIX-targeting CAR-T therapy against renal cell carcinoma. Mol Ther Oncolytics. 2022;24:14–25.

8. Mahon BP, Pinard MA, McKenna R. Targeting carbonic anhydrase IX activity and expression. Molecules. 2015;20(2):2323–2348.

9. Pettersen EO, Ebbesen P, Gieling RG, et al. Targeting the liver stage of malaria. J Med Chem. 2013;56(11):4811–4815.

10. Lee SH, Griffiths JR. How and why are cancers acidic? Carbonic anhydrase IX and the homeostatic control of tumour extracellular pH. Cancers. 2020;12(6):1616–1625.

11. Supuran CT. How many carbonic anhydrase inhibition mechanisms exist? J Enzyme Inhib Med Chem. 2016;31(3):345–360.

12. Carta F, Vullo D, Osman SM, et al. Synthesis and carbonic anhydrase inhibition of a series of SLC-0111 analogs. Bioorg Med Chem. 2017;25(9):2569–2576.

13. Said MA, Eldehna WM, Nocentini A, et al. Sulfonamide-based ring-fused analogues for CAN508 as novel carbonic anhydrase inhibitors endowed with antitumor activity: design, synthesis, and in vitro biological evaluation. Eur J Med Chem. 2020;189:112019.

14. Katariya KD, Shah SR, Reddy D. Anticancer, antimicrobial activities of quinoline based hydrazone analogues: synthesis, characterization and molecular docking. Bioorg Chem. 2020;94:1–14.

15. Goff GL, Ouazzani J. Natural hydrazine-containing compounds: biosynthesis, isolation, biological activities and synthesis. Bioorg Med Chem. 2014;22(23):6529–6544.

16. Alaa A-M, El-Azab AS, El-Enin MAA, et al. Synthesis of novel isoindoline-1, 3-dione-based oximes and benzenesulfonamide hydrazones as selective inhibitors of the tumor-associated carbonic anhydrase IX. Bioorg Chem. 2018;80:706–713.

17. Queen A, Khan P, Idrees D, et al. Biological evaluation of p-toluene sulphonylhydrazone as carbonic anhydrase IX inhibitors: an approach to fight hypoxia-induced tumors. Int J Biol Macromol. 2018;106:840–850.

18. Rodrigues T, da Cruz FP, Lafuente-Monasterio MJ, et al. Quinolin-4 (1 H)-imines are potent antiproliferative drugs targeting the liver stage of malaria. J Med Chem. 2013;56(11):4811–4815.

19. Ressureição AS, Gonçalves D, Sitoe AR, et al. Structural optimization of quinolon-4 (1 H)-imines as dual-stage antimalarials: toward increased potency and metabolic stability. J Med Chem. 2013;56(19):7679–7690.

20. Abo-Ashour MF, Eldehna WM, Nocentini A, et al. 3-Hydrazinoisatin-based benzenesulfonamides as novel carbonic anhydrase inhibitors endowed with anticancer activity: synthesis, in vitro biological evaluation and in silico insights. Eur J Med Chem. 2019;184:111768.

21. Eldeeb AH, Abo-Ashour MF, Angeli A, et al. Novel benzenesulfonamides aryl and arylsulfone conjugates adopting tail/dual tail approaches: synthesis, carbonic anhydrase inhibitory activity and molecular modeling studies. Eur J Med Chem. 2021;221:113486–12.

22. Chandak N, Ceruso M, Supuran CT, Sharma PK. Novel sulfonamide bearing coumarin scaffolds as selective inhibitors of tumor associated carbonic anhydrase isozymes IX and XII. Bioorg Med Chem. 2016;24(13):2882–2886.

23. Alaa A-M, El-Azab AS, Bua S, et al. Design, synthesis, and carbonic anhydrase inhibition activity of benzenesulfonamide-linked novel pyrazoline derivatives. Bioorg Chem. 2019;87:425–431.

24. Tawfik HO, Petreni A, Supuran CT, El-Hamamys MH. Discovery of new carbonic anhydrase IX inhibitors as anticancer agents by toning the hydrophobic and hydrophilic rims of the active site to encounter the dual-tail approach. Eur J Med Chem. 2022;232:114190–21.

25. Jakusová K, Gáplovský M, Donovalová J, et al. Effect of reagents’ concentration on the ratio and yield of E, Z isomers of isatin-3-(4-phenyl) semicarbazone and N-methylisatin-3-(4-phenyl) semicarbazone. Chem Paper. 2013;67(1):117–126.

26. Ghorbah MM, Alsaied MS, Soliman AM, Ragab FA. VEGFR-2 inhibitors and apoptosis inducers: synthesis and molecular design of new benzo [g] quinazolin bearing benzenesulfonamide moiety. J Enzyme Inhib Med Chem. 2017;32(1):893–907.

27. Sharma PK, Balwani S, Mathur D, et al. Synthesis and anti-inflammatory activity evaluation of novel triazolyl-isatin hybrids. J Enzyme Inhib Med Chem. 2016;31(6):1520–1526.

28. AboulWafa OM, Daabees HM, Badawi WA. 2-Anilinopyrimidine derivatives: design, synthesis, in vitro anti-proliferative activity, EGFR and ARO inhibitory activity, cell cycle analysis and molecular docking study. Bioorg Chem. 2020;99:103798–19.

29. Salem MS, Sakr SI, El-Senousy WM, Madkour HM. Synthesis, antibacterial, and antiviral evaluation of new heterocycles containing the pyridine moiety. Arch Pharm (Weinheim). 2013;346(10):766–773.

30. Othman IM, Gad-Elkareem MAM, Anouar EH, et al. Novel fused pyridine derivatives containing pyrimidine moiety as prospective tyrosyl-tRNA synthetase inhibitors: design, synthesis, pharmacokinetics and molecular docking studies. J Mol Struct. 2020;1219:128651.

31. George RF, Said MF, Bua S, Supuran CT. Synthesis and selective inhibitory effects of some 2-oxindole benzenesulfonamide conjugates on human carbonic anhydrase isozymes CA I, CA II, CA IX and CA XII. Bioorg Chem. 2020;95:103514.

32. Ghorbah MM, Alsaied MS, Ceruso M, et al. Carbonic anhydrase inhibitors: synthesis, molecular docking, cytotoxic and inhibition of the human carbonic anhydrase isozymes I, II, IX, XII with novel benzenesulfonamides incorporating pyrrole, pyrrolopyrimidine and fused pyrrolopyrimidine moieties. Bioorg Med Chem. 2014;22(14):3684–3695.

33. Alaa A-M, El-Azab AS, Bua S, et al. Enhancement of the tail hydrophobic interactions within the carbonic anhydrase IX active site via structural extension: Design and synthesis of novel N-substituted isatins-SLC-0111
hybrids as carbonic anhydrase inhibitors and antitumor agents. Eur J Med Chem. 2019;162:147–160.

36. Nocentini A, Trailori E, Singh S, et al. 4-Hydroxy-3-nitro-5-ureido-benzensulfonamides selectively target the tumor-associated carbonic anhydrase isoforms IX and XII showing hypoxia-enhanced antiproliferative profiles. J Med Chem. 2018;61(23):10860–10874.

37. Demir-Yazıcı K, Bua S, Akγınes NM, et al. Indole-based hydrazones containing A sulfonamide moiety as selective inhibitors of tumor-associated human carbonic anhydrase isoforms IX and XII. Int J Mol Sci. 2019;20:1–14.

38. Shaldam M, Nocentini A, Elsayed ZM, et al. Development of novel quinoline-based sulfonamides as selective cancer-associated carbonic anhydrase isoform IX inhibitors. Int J Mol Sci. 2021;22(20):11191–116.

39. Aggarwal M, Kondeti B, McKenna R. Insights towards sulfonamide drug specificity in α-carbonic anhydrases. Bioorg Med Chem. 2013;21(6):1526–1533.

40. Pinard MA, Boone CD, Rife BD, et al. Structural study of interaction between brinzolamide and dorzolamide inhibition of human carbonic anhydrases. Bioorg Med Chem. 2013;21(22):7210–7215.

41. Boyd MR, Paull KD. Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen. Drug Dev Res. 1995;34(2):91–109.

42. Rashid M, Husain A, Mishra R, et al. Design and synthesis of benzimidazolides containing substituted oxadiazole, thia Diazole and triazolo-thiadiazines as a source of new anticancer agents. Arab J Chem. 2019;12(8):3202–3224.

43. Singla P, Luxami V, Paul K. Synthesis and in vitro evaluation of novel triazole analogues as anticancer agents and their interaction studies with bovine serum albumin. Eur J Med Chem. 2016;117:59–69.

44. Acton EM, Narayanan VL, Risbood PA, et al. Anticancer specificity of some ellipticineum salts against human brain tumors in vitro. J Med Chem. 1994;37(14):2185–2189.

45. Al-Saadi MS, Rostom SA, HM. Faidallah 3-Methyl-2-(4-substituted phenyl)-4, 5-dihydropaporphine [1, 2-c]-pyrazoles: synthesis and in vitro biological evaluation as antitumour agents. Arch Pharm (Weinheim). 2008;341(3):181–190.

46. Krymov SK, Scherbakov AM, Salnikova DI, et al. Synthesis, biological evaluation, and in silico studies of potential activators of apoptosis and carbonic anhydrase inhibitors on isatin-5-sulfonamide scaffold. Eur J Med Chem. 2022;228:113997.

47. An B, Liu J, Fan Y, et al. Novel third-generation pyrimidines-based EGFR tyrosine kinase inhibitors targeting EGFR T790M mutation in advanced non-small cell lung cancer. Bioorg Chem. 2022;122:105743–14.

48. Hassan RA, Hamed MI, Abdou AM, El-Dash Y. Novel antiproliferative agents bearing substituted thieno [2, 3-d] pyrimidine scaffold as dual VEGFR-2 and BRAF kinases inhibitors and apoptosis inducers; design, synthesis and molecular docking. Bioorg Chem. 2022;125:105861–19.

49. Kadagathur M, Sujat Shalik A, Panda B, et al. Synthesis of indolo/pyrroloazepinone-oxindoles as potential cytotoxic, DNA-intercalating and topo I inhibitors. Bioorg Chem. 2022;122:105706–105716.

50. Abdel-Aziz AA-M, El-Azab AS, AlSaif NA, et al. Synthesis, potential antitumor activity, cell cycle analysis, and multitarget mechanisms of novel hydrazones incorporating a 4-methylsulfonylbenzene scaffold: a molecular docking study. J Enzyme Inhib Med Chem. 2021;36(1):1521–1539.

51. Pfeffer CM, Singh AT. Apoptosis: a target for anticancer therapy. Int J Mol Sci. 2018;19:1–10.

52. Vermeiren H, Haenen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylinerse expression on early apoptotic cells using fluorescein labelled annexin V. J Immunol Methods. 1995;184(1):39–51.

53. Abdelrahman MA, Eldehna WM, Nocentini A, et al. Novel diamide-based benzensulfonamides as selective carbonic anhydrase IX inhibitors endowed with antitumor activity: synthesis, biological evaluation and in silico insights. Int J Mol Sci. 2019;20:1–16.

54. Qin JL, Shen WY, Chen ZF, et al. Oxoaporphine metal complexes (Co II, Ni II, Zn II) with high antitumor activity by inducing mitochondria-mediated apoptosis and S-phase arrest in HepG2. Sci. Rep. 2017;7:1–8.

55. Zhong S, Li YG, Ji DF, et al. Protocatechualdehyde induces S-phase arrest and apoptosis by stimulating the p27KIP1–cyclin A/D1-CDK2 and mitochondrial apoptotic pathways in HT-29 Cells. Molecules. 2016;21(7):934–121.

56. Musa MA, Gbadebo AJ, Latinwo VL, 7, 8-Dihydroxy-3-(4-nitrophenyl) coumarin induces cell death via reactive oxygen species–independent S-phase cell arrest. J. Biochem. Mol. Toxicol. 2018;32:794–801.

57. Nemr MT, AboulMagd AM, Hassan HM, et al. Design, synthesis and mechanistic study of new benzenesulfonamide derivatives as anticancer and antimicrobial agents via carbonic anhydrase IX inhibition. RSC Adv. 2021;11(42):26241–26257.

58. Dawood DH, Srour AM, Saleh DO, et al. New pyrimide and chromene scaffolds as potent vasorelaxant and anticancer agents. RSC Adv. 2021;11(47):29441–29452.

59. Kim KH, Sederstrom JM. Assaying cell cycle status using flow cytometry. Curr Protoc Mol Biol. 2015;11:1–11.

60. Cvijetić IN, Tanç M, Juranić IO, et al. S-Aryl-1H-pyrazole-3-carboxylic acids as selective inhibitors of human carbonic anhydrase IX and XII. Bioorg Med Chem. 2015;23(15):4649–4659.

61. Supuran CT. Multitargeting approaches involving carbonic anhydrase inhibitors: hybrid drugs against a variety of disorders. J Enzyme Inhib Med Chem. 2021;36(1):1702–1714.

62. Malebari AM, Ibrahim TS, Salem IM, et al. The anticancer activity for the bumetanide-based analogs via targeting the tumor-associated membrane-bound human carbonic anhydrase-IX enzyme. Pharmaceuticals. 2020;13(9):252–220.

63. Supuran CT, Scocciafa A. Carbonic anhydrase inhibitors: aromatic sulfonamides and disulfonamides act as efficient tumor growth inhibitors. J Enzyme Inhib. 2000;15(6):597–610.

64. Yousef RG, Ibrahim A, Khalifa MM, et al. Discovery of new nicotinamides as apoptotic VEGFR-2 inhibitors: virtual screening, synthesis, anti-proliferative, immunomodulatory, ADMET, toxicity, and molecular dynamic simulation studies. J Enzyme Inhib Med Chem. 2022;37(1):1389–1403.

65. Elraaz EZ, Seria RA, Ismail NS, et al. Discovery of potent thieno [2, 3-d] pyrimidine VEGFR-2 inhibitors: design, synthesis and enzyme inhibitory evaluation supported by molecular dynamics simulations. Bioorg Chem. 2021;113:105019–16.

66. Belhassan A, Zaki H, Choita S, et al. Camphor, artemisinin and sumac phytochemicals as inhibitors against COVID-19:
67. Guterres H, Im W. Improving protein-ligand docking results with high-throughput molecular dynamics simulations. J Chem Inf Model. 2020;60(4):2189–2198.

68. Jena AB, Kanungo N, Nayak V, et al. Catechin and curcumin interact with S protein of SARS-CoV2 and ACE2 of human cell membrane: insights from computational studies. Sci Rep. 2021;11(1):14.

69. Ali A, Ali A, Warsi MH, et al. Toward the discovery of a novel class of leads for high altitude disorders by virtual screening and molecular dynamics approaches targeting carbonic anhydrase. Int J Mol Sci. 2022;23(9):5054.

70. Costa RA, Rocha JA, Pinheiro AS, et al. A computational approach applied to the study of potential allosteric inhibitors protease NS2B/NS3 from dengue virus. Molecules. 2022;27(13):4118–4120.

71. Lee S, Wong AR, Yang AWH, Hung A. Interaction of compounds derived from the Chinese medicinal formula Huangqi Guizhi Wuwu Tang with stroke-related numbness and weakness targets: an in-silico docking and molecular dynamics study. Comput Biol Med. 2022;146:105568–105569.

72. Abdel-Aziz AAM, El-Azab AS, Alanazi AM, et al. Synthesis and potential antitumor activity of 7-(4-substituted piperazin-1-yl)-4-oxoquinolines based on ciprofloxacin and norfloxacin scaffolds: in silico studies. J Enzyme Inhib Med Chem. 2016;31(5):796–809.

73. Mussi S, Rezzola S, Chiodelle P, et al. Antiproliferative effects of sulphonamide carbonic anhydrase inhibitors C18, SLC-0111 and acetazolamide on bladder, glioblastoma and pancreatic cancer cell lines. J Enzyme Inhib Med Chem. 2022;37(1):280–286.

74. Eldehna WM, Abo-Ashour MF, Ibrahim HS, et al. Novel [(3-indolylmethylene) hydrazono] indolin-2-ones as apoptotic anti-proliferative agents: design, synthesis and in vitro biological evaluation. J Enzyme Inhib Med Chem. 2018;33(1):686–700.

75. Said MA, Eldehna WM, Nocentini A, et al. Sulfonamide-based ring-fused analogues for CAN508 as novel carbonic anhydrase inhibitors endowed with antitumor activity: design, synthesis, and in vitro biological evaluation. Eur. J. Med. Chem. 2020;189:1–14.

76. Eldehna WM, El-Naggar DH, Hamed AR, et al. One-pot three-component synthesis of novel spirooxindoles with potential cytotoxic activity against triple-negative breast cancer MDA-MB-231 cells. J Enzyme Inhib Med Chem. 2018;33(1):309–318.

77. Ewies EF, Sabry E, Bekheit MS, et al. Click chemistry-based synthesis of new benzenesulfonamide derivatives bearing triazole ring as selective carbonic anhydrase II inhibitors. Drug Dev Res. 2022;83(6):1281–1291.