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ABSTRACT

Design and synthesis of three novel series of aryl enaminones (**3a–f** and **5a–c**) and pyrazole (**4a-c**) linked compounds with sulphonamides, sulfaguanidine, or carboxylic acid functionalities were reported as carbonic anhydrase inhibitors (CAIs) using the "tail approach" strategy in their design to achieve the most variable amino acids in the middle/outer rims of the hCAs active site. The synthesised compounds were assessed *in vitro* for their inhibitory activity against the following human (h) isoforms, hCA I, II, IX, and XII using stopped-flow CO_2 hydrase assay. Enaminone sulphonamide derivatives (**3a–c**) potently inhibited the target tumour-associated isoforms hCA IX and hCA XII (KIs 26.2–63.7 nM) and hence compounds **3a** and **3c** were further screened for their *in vitro* cytotoxic activity against MCF-7 and MDA-MB-231 cancer cell lines under normoxic and hypoxic conditions. Derivative **3c** showed comparable potency against both MCF-7 and MDA-MB-231 cancer cell lines under both normoxic ((IC_{50} = 4.918 and 12.27 μM, respectively) and hypoxic (IC_{50} = 1.689 and 5.898 μM, respectively) conditions compared to the reference drug doxorubicin under normoxic (IC_{50} = 3.386 and 4.269 μM, respectively) and hypoxic conditions (IC_{50} = 1.368 and 2.62 μM, respectively). Cell cycle analysis and Annexin V-FITC and propidium iodide double staining methods were performed to reinforce the assumption that **3c** may act as a cytotoxic agent through the induction of apoptosis in MCF-7 cancer cells.

GRAPHICAL ABSTRACT
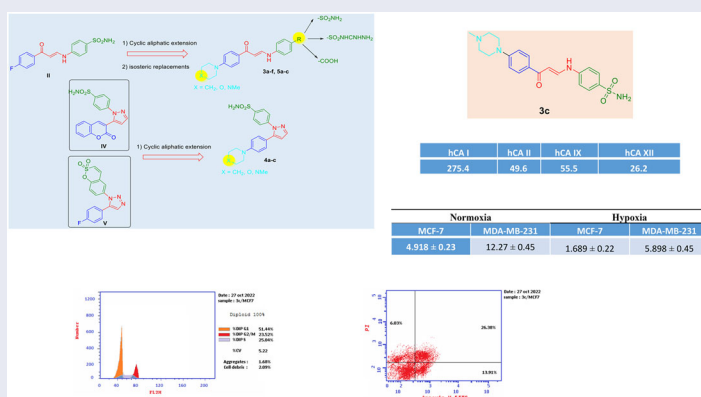
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Introduction

The reversible hydration of carbon dioxide into bicarbonate and a proton is an essential reaction for all living being and it is catalysed by the superfamily of metalloenzymes carbonic anhydrases (CAs, EC 4.2.1.1)[1]. This pivotal reaction is involved in several physiological processes such as respiration, pH, and homeostasis regulation, CO_2 and HCO_3^- transportation, electrolyte secretion in tissue and organs, biosynthetic reactions (e.g. gluconeogenesis, lipogenesis, and ureagenesis), photosynthesis (e.g. plants and cyanobacteria), bone resorption, calcification, and tumorigenesis. Nowadays, eight genetically unrelated CA families α, β, γ, δ, η, ζ, θ, and ι[1–8], were identified and only α-CAs are present in higher vertebrates and humans (h)[1]. In particular, among the 15 hCAs only 12 are catalytically active, differing for catalytic activity, subcellular/tissue distribution, and physiological role[9]. Thus, several studies highlighted that abnormal levels or activity of hCAs are linked to many human diseases such as retinopathies (e.g. glaucoma and retinitis pigmentosa), retinal/cerebral oedema, stroke, altitude sickness, cariogenesis, epilepsy, osteoporosis, neurodegeneration, sterility, obesity, and cancer[10–12]. Consequently, catalytically hCAs have been considered targets of great importance for the design of modulators, such as inhibitors (CAIs) and activators (CAAs), with biomedical applications[13]. Whereas initially CAIs were used as diuretics, antiglaucoma agents, antiepileptics and for the management of altitude sickness[1], new CAIs generations are being developed for the treatment of cancers, inflammation, obesity, neuropathic pain, infections, and neurodegenerative disorders[14–19].

Sulphonamides are the most potent CAI chemotype acting as a zinc-binding inhibitor. The "tail approach" is one of the most used strategies in the design of CAIs, which consists in appending a tail of various nature on a CA modulator scaffold (e.g. benzenesulfonamide) to achieve the most variable amino acids in the middle/outer rims of the hCAs active site[20–24]. This strategy improves the CAIs selectivity towards specific hCAs isoforms, preventing the onset of side effects due to the inhibition of the other CA isozymes that are not involved in the pathophysiology of the treated disease. An important result of the "tail approach" application is SLC-0111: a ureido-benzenesulfonamide derivative selective against the tumour-associated hCA IX and XII, that displayed promising antiproliferative effects in cancer cells *in vitro* and *in vivo* decreasing the tumoural growth, progression and metastases. Currently, SLC-0111 is in phase Ib/II clinical development for the treatment of advanced solid hypoxic tumours in patients and no significant dose-limiting toxicities were encountered[25–27]. These extraordinary results led our several research efforts to optimise the SLC-0111 structure based on using different linkers or aryl analogues with the aim to identify more potent and selective inhibitors against the tumour-associated isoform CA IX and XII[28,29]. Within the region of trials to use flexible linker compounds analogous to SLC-0111, compound II was synthesised before and showed sub-nanomolar activity against hCA IX but with a low selectivity ratio compared to hCA II[30]. Upon changing the phenyl group to coumarin to study the effect of the combination of both classical and non-classical inhibitors, compound (III) (Figure 1)

SLC-0111 (I)
K_I (IX) = 45 nM
SI (II/IX) = 21.3

II
K_I (IX) = 0.27 nM
SI (II/IX) = 1.8

III
K_I (IX, XII) = 15.1, 3.4 nM
SI (II/IX, II/XII) = 3.3, 14.9

IV
K_I (IX) = 36.4, 24.5 nM
SI (II/IX, II/XII) = 4.3, 6.4

V
K_I (IX, XII) = 8.3, 7.8 nM
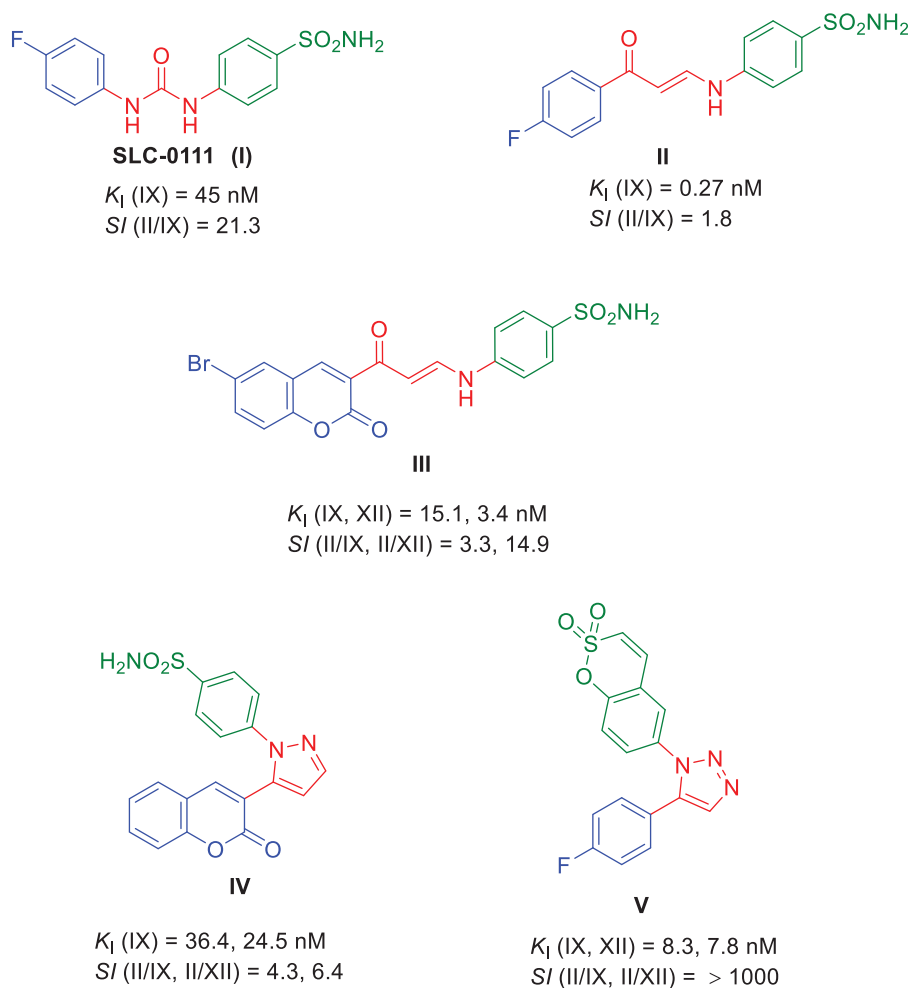SI (II/IX, II/XII) = > 1000

Figure 1. Different inhibitors of tumour-associated carbonic anhydrase XI including SLC-0111.

illustrated a significant nanomolar activity against hCA IX and XII which is more potent than SLC-0111 but unfortunately, the selectivity indices were not promising[31]. On the other hand, the part of this flexible linker is rigidified by the use of pyrazole, as in compound IV[31] or even triazole, as in compound V (Figure 1)[32,33]. Both of them with enhanced nanomolar activity against isoforms IX and XII but the selectivity ratio is in favour of compound V which is attributed to the presence of sulfocoumarin as ZBG.

Cyclic secondary amines proved their substantial effect on different molecules considering the effect on both activity and pharmacokinetic characteristics[34,35]. Cyclic secondary amines were employed to compound II to study the effect of these substitutions on the original inhibitory effect. These combinations were varied with different ZBGs as sulphonamide, sulfaguanidine, and carboxylic acid moieties as shown in compounds 3a–f, 5a–c in Figure 2. The concept of using cyclic secondary amines was also applied to compounds with rigid linkers as pyrazole as in compounds 4a–c as in Figure 2.

Materials and methods

Chemistry

General

The data of the devices used in the analytical experiments in the chemistry section were described in the supplementary data. Compounds 1a–c[36,37] and 2a–c[38–40] were synthesised according to the reported procedures.

General procedure for the preparation of compounds 3a–f

The appropriate enaminone intermediate 2a–c (1 mmol) was added to a hot-stirred solution of either sulphanilamide or sulfaguanidine (1 mmol) in glacial acetic acid (15 ml). The reaction mixture was refluxed for 5 h. The obtained precipitate upon cooling was filtered off, washed with water, dried, and recrystallised from isopropanol to furnish compounds 3a–f.

(E/Z)-4-((3-oxo-3-(4-(piperidin-1-yl) phenyl) prop-1-en-1-yl)amino) benzenesulfonamide (3a). Dark yellow crystals (yield 50%), m.p. 225–227 °C; IR (KBr, ν cm^{-1}): 3356, 3267 (NH, NH$_2$), 1635 (C=O), 1334 and 1153 (SO$_2$); ^{1}H NMR (DMSO-d_6) δ ppm: 1.59 (s, 6H, piperidine H), 3.35 (s, 4H, piperidine H), 6.16, 6.55 (2d, 1H, J = 8.1, 12.6, COCH=CHNH), 6.96 (d, 2H, J = 8.6, Ar-H), 7.21–7.25 (m, 3H, Ar-H and NH$_2$, D$_2$O exchangeable), 7.43 (d, 1H, J = 8.6, Ar-H), 7.73–7.82 (m, 4H, Ar-H), 7.85, 8.07 (d, t, 1H, J = 8.5, 12.6, COCH=CHNH), 10.17, 12.04 (2d, 1H, J = 12.6, 12.2, NH, D$_2$O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 24.4, 25.3, 48.4, 95.3, 100.0, 113.7, 115.0, 115.8, 127.4, 127.9, 129.7, 136.9, 137.9, 141.8, 143.3, 143.7, 144.7, 153.8, 186.1, and 189.3; Anal. Calcd. for C$_{20}$H$_{23}$N$_3$O$_3$S (385.48): C, 62.32; H, 6.01; N, 10.90; found C, 62.51; H, 6.28; N, 11.14.

(E/Z)-4-((3-(4-morpholinophenyl)-3-oxoprop-1-en-1-yl) amino) benzenesulfonamide (3b). Yellow crystals (yield 55%), m.p. 218–220 °C; IR (KBr, ν cm^{-1}): 3379, 3251 (NH, NH$_2$), 1635 (C=O), 1320 and 1149 (SO$_2$); ^{1}H NMR (DMSO-d_6) δ ppm: 3.28 (t, 4H, J = 4.7, morpholine H), 3.75 (t, 4H, J = 4.5, morpholine H), 6.19, 6.55 (2d, 1H, J = 8.1, 12.6, COCH=CHNH), 7.01 (d, 2H, J = 8.7, Ar-H), 7.21–7.27 (m, 3H, Ar-H and NH$_2$, D$_2$O exchangeable), 7.44 (d, 1H, J = 8.6, Ar-H), 7.73–7.85 (m, 4H, Ar-H), 7.89, 8.09 (d, t, 1H, J = 8.8, 12.7,
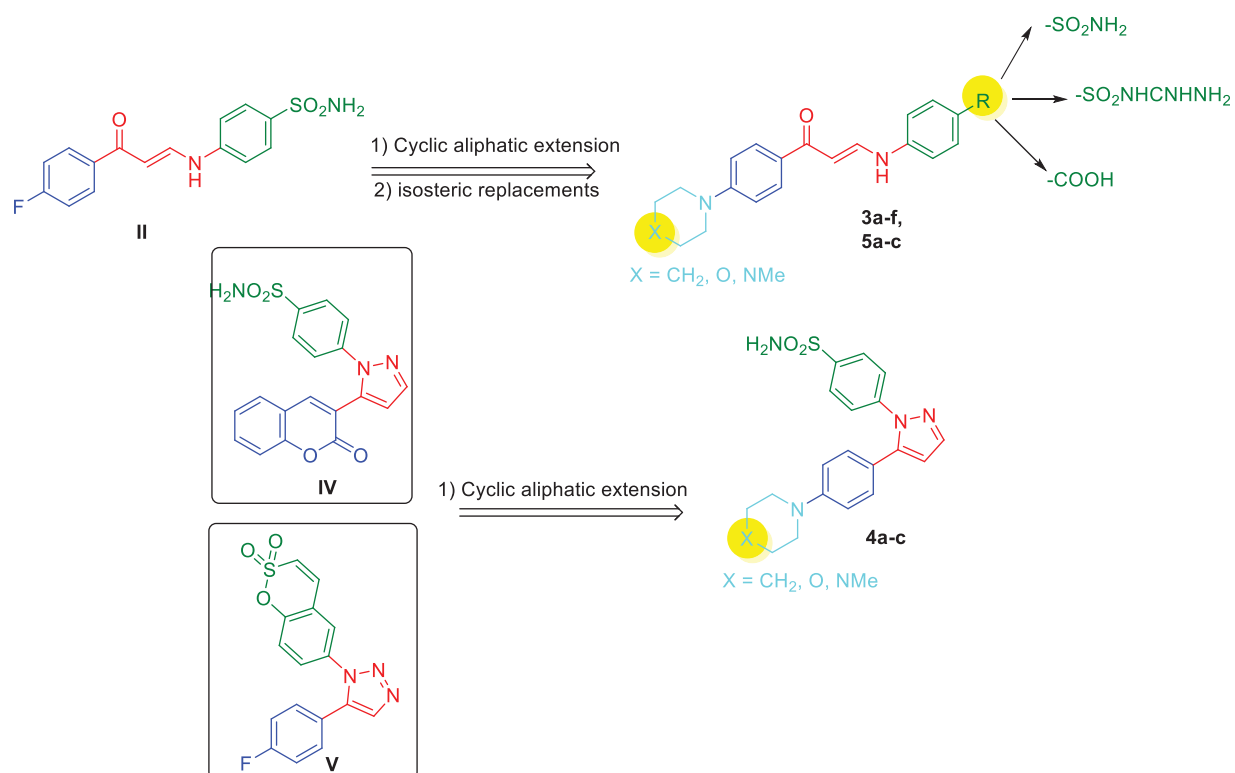

Figure 2. The rationale for the design of novel enaminones and pyrazoles as tumour-associated carbonic anhydrase inhibitors.

COCH=CHNH), 10.20, 12.04 (2d, 1H, J = 12.6, 11.9, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 47.4, 66.3, 95.2, 99.9, 113.7, 115.1, 115.9, 127.9, 128.7, 129.5, 137.0, 138.1, 142.1, 143.6, 144.68, 153.9, 189.4, and 189.4; Anal. Calcd. for $C_{19}H_{21}N_3O_4S$ (387.45): C, 58.90; H, 5.46; N, 10.85; found C, 59.21; H, 5.61; N, 11.08.

(E/Z)-4-((3-(4-(4-Methylpiperazin-1-yl)phenyl)-3-oxoprop-1-en-1-yl) amino)benzene sulphonamide (3c).
Yellow crystals (yield 46%), m.p. 248–250 °C; IR (KBr, ν cm^{-1}): 3302, 3155 (NH, NH_2), 1597 (C=O), 1319 and 1145 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 2.23 (s, 3H, CH$_3$), 2.45 (s, 4H, piperazine H), 3.32 (s, 4H, piperazine H), 6.17, 6.55 (2d, 1H, J = 7.7, 12.4, COCH=CHNH), 6.99 (d, 2H, J = 6.2, Ar-H), 7.21–7.25 (m, 3H, Ar-H and NH$_2$, D_2O exchangeable), 7.44 (d, 2H, J = 8.0, Ar-H), 7.74–7.88 (m, 3H, Ar-H), 7.87, 8.10 (d, t, 1H, J = 7.9, 12.0, COCH=CHNH), 10.19, 12.04 (2d, 1H, J = 12.0, 12.3, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 13.7, 21.5, 23.5, 55.5, 112.6, 121.4, 127.2, 127.6, 134.3, 142.3, 148.8, 172.4; Anal. Calcd. for $C_{20}H_{24}N_4O_3S$ (400.49): C, 59.98; H, 6.04; N, 13.99; found C, 60.24; H, 6.20; N, 14.17.

(E/Z)-N-Carbamimidoyl-4-((3-oxo-3-(4-(piperidin-1-yl)phenyl)prop-1-en-1-yl)amino) benzenesulfonamide (3d).
Yellow crystals (yield 52%), m.p. 256–258 °C; IR (KBr, ν cm^{-1}): 3444, 3344, 3217 (NH, NH_2), 1693 (C=O), 1369 and 1138 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 1.59 (s, 6H, piperidine H), 3.35 (s, 4H, piperidine H), 6.13 (d, $^1/_2$H, J = 8.2, COCH=CHNH), 6.51 (s, 1H, NH, D_2O exchangeable), 6.53 (t, $^1/_2$H, J = 12.6, COCH=CHNH), 6.68 (s, 2H, NH$_2$, D_2O exchangeable), 6.92–6.99 (m, 2H, Ar-H), 7.18 (d, 1H, J = 8.7, Ar-H), 7.35–7.40 (m, 2H, Ar-H and NH, D_2O exchangeable), 7.66–7.70 (m, 2H, Ar-H), 7.76–7.85 (m, 2$^1/_2$H, Ar-H and COCH=CHNH), 8.06 (t, $^1/_2$H, J = 12.6, COCH=CHNH), 10.12, 12.03 (2d, 1H, J = 12.7, 11.9, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 21.5, 24.4, 25.3, 48.7, 95.0, 99.7, 113.7, 114.8, 127.8, 128.2, 129.6, 137.6, 138.6, 143.1, 143.5, 144.2, 153.8, 158.5, 172.4, 186.1, and 189.2; Anal. Calcd. for $C_{21}H_{25}N_5O_3S$ (427.52): C, 59.00; H, 5.89; N, 16.38; found C, 58.79; H, 6.08; N, 16.62.

(E/Z)-N-Carbamimidoyl-4-((3-(4-morpholinophenyl)-3-oxoprop-1-en-1-yl)amino) benzenesulfonamide (3e).
Buff crystals (yield 48%), m.p. 168–170 °C; IR (KBr, ν cm^{-1}): 3444, 3348, 3217 (NH, NH_2), 1694 (C=O), 1369 and 1138 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 3.28 (t, 4H, J = 4.7, morpholine H), 3.75 (t, 4H, J = 4.5, morpholine H), 6.16 (d, $^1/_2$H, J = 8.1, COCH=CHNH), 6.51 (s, 1H, NH, D_2O exchangeable), 6.53 (t, $^1/_2$H, J = 12.6, COCH=CHNH), 6.67 (s, 2H, NH$_2$, D_2O exchangeable), 6.99–7.02 (m, 2H, Ar-H), 7.19 (d, 1H, J = 8.7, Ar-H), 7.36–7.38 (m, 2H, Ar-H and NH, D_2O exchangeable), 7.66–7.70 (m, 2H, Ar-H), 7.79–7.90 (m, 2$^1/_2$H, Ar-H and COCH=CHNH), 8.07 (t, $^1/_2$H, J = 12.5, COCH=CHNH), 10.15, 12.03 (2d, 1H, J = 12.8, 12, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 47.3, 66.3, 112.7, 113.4, 118.7, 127.0, 127.7, 130.4, 131.2, 139.0, 142.1, 151.8, 158.2, 158.4, and 169.2; Anal. Calcd. for $C_{20}H_{23}N_5O_4S$ (429.49): C, 55.93; H, 5.40; N, 16.31; found C, 56.15; H, 5.57; N, 16.58.

(E/Z)-N-Carbamimidoyl-4-((3-(4-(4-methylpiperazin-1-yl)phenyl)-3-oxoprop-1-en-1-yl)amino)benzenesulfonamide (3f).
Brown crystals (yield 50%), m.p. 265–267 °C; IR (KBr, ν cm^{-1}): 3564, 3433, 3336 (NH, NH_2), 1682 (C=O), 1315 and 1134 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 2.28 (s, 3H, CH$_3$), 2.51 (s, 4H, piperazine H), 3.34 (s, 4H, piperazine H), 6.15 (d, $^1/_2$H, J = 7.2, COCH=CHNH), 6.52–6.68 (m, 3$^1/_2$H, COCH=CHNH, NH and NH$_2$, D_2O exchangeable), 6.99–7.00

(m, 2H, Ar-H), 7.19 (d, 1H, J = 7.0, Ar-H), 7.37 (m, 2H, Ar-H and NH, D_2O exchangeable), 7.68–7.87 (m, 4$^1/_2$ H, Ar-H and COCH=CHNH), 8.07 (t, $^1/_2$ H, J = 12.5, COCH=CHNH), 10.15, 12.03 (2d, 1H, J = 11.7, 11.6, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 24.5, 46.1, 46.9, 54.7, 56.5, 113.5, 118.7, 118.9, 127.0, 139.0, 142.1, 142.9, 154.3, 154.4, 158.4, 169.2, and 169.4; Anal. Calcd. for $C_{21}H_{26}N_6O_3S$ (442.53): C, 57.00; H, 5.92; N, 18.99; found C, 57.24; H, 6.04; N, 19.21.

General procedure for the preparation of compounds 4a–c
Sulphanilamide hydrazine hydrochloride (0.22 g, 1 mmol) was added to a solution of the corresponding enaminoketone 2a–c (1 mmol) dissolved in glacial acetic acid and the mixture was heated under reflux for 12–24 h. After cooling, the separated solid was filtered and crystallised from chloroform/methanol to afford the pyrazole derivatives 4a–c in 50–60% yield.

4-(5-(4-(Piperidin-1-yl)phenyl)-1H-pyrazol-1-yl)benzenesulfonamide (4a).
Light brown crystals (yield 48%), m.p. 103–105 °C; IR (KBr, ν cm^{-1}): 3421 (NH$_2$), 1315 and 1161 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 1.58–1.67 (m, 6H, piperidine H), 3.26 (s, 4H, piperidine H), 6.61 (s, 1H, pyrazole H), 7.12–7.20 (m, 3H, Ar-H and NH$_2$, D_2O exchangeable), 7.45–7.47 (m, 3H, Ar-H), 7.79–7.85 (m, 3H, pyrazole H and Ar-H), 8.58–8.70 (m, 2H, Ar-H); ^{13}C NMR (DMSO-d_6) δ ppm: 24.2, 25.2, 48.5, 107.1, 112.4, 122.3, 123.4, 127.1, 127.6, 130.6, 134.0, 140.2, 142.4, and 148.9; Anal. Calcd. for $C_{20}H_{22}N_4O_2S$ (382.48): C, 62.80; H, 5.80; N, 14.65; found C, 63.06; H, 5.93; N, 14.86.

4-(5-(4-Morpholinophenyl)-1H-pyrazol-1-yl)benzenesulfonamide (4b).
Brown crystals (yield 45%), m.p. 281–283 °C; IR (KBr, ν cm^{-1}): 3394 (NH$_2$), 1327 and 1161 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 3.14 (t, 4H, J = 4.4, morpholine H), 3.72 (t, 4H, J = 4.4, morpholine H), 6.59 (d, 1H, J = 1.48, pyrazole H), 6.95 (d, 2H, J = 8.6, Ar-H), 7.10 (d, 2H, J = 8.6, Ar-H), 7.45 (d, 4H, J = 8.4, Ar-H and NH$_2$, D_2O exchangeable), 7.78 (d, 1H, J = 1.48, pyrazole H), 7.84 (d, 2H, J = 8.5, Ar-H); ^{13}C NMR (DMSO-d_6) δ ppm: 48.1, 66.4, 108.4, 115.0, 120.3, 125.4, 127.0, 129.7, 141.4, 142.8, 142.9, 143.5, and 151.1; Anal. Calcd. for $C_{19}H_{20}N_4O_3S$ (384.45): C, 59.36; H, 5.24; N, 14.57; found C, 59.58; H, 5.31; N, 14.68.

4-(5-(4-(4-Methylpiperazin-1-yl)phenyl)-1H-pyrazol-1-yl)benzenesulfonamide (4c).
Dark brown crystals (yield 51%), m.p. 285–287 °C; IR (KBr, ν cm^{-1}): 3379 (NH$_2$), 1319 and 1157 (SO$_2$); ^1H NMR (DMSO-d_6) δ ppm: 2.21 (s, 3H, CH$_3$), 2.42 (t, 4H, J = 6.8, piperazine H), 3.31 (t, 4H, J = 4.7, piperazine H), 5.83 (br s, 1H, pyrazole-H), 6.59 (d, 1H, J = 8.5, Ar H), 6.90–6.98 (m, 3H, pyrazole-H and Ar H), 7.25 (s, 2H, NH$_2$, D_2O exchangeable), 7.45 (d, 2H, J = 8.5, Ar-H), 7.73–7.80 (m, 3H, Ar-H), ^{13}C NMR (DMSO-d_6) δ ppm: 24.5, 26.5, 46.1, 46.9, 54.7, 112.8, 113.5, 118.8, 127.0, 127.8, 130.5, 138.4, 142.7, 152.4, 154.3, and 169.4; Anal. Calcd. for $C_{20}H_{23}N_5O_2S$ (397.49): C, 60.43; H, 5.83; N, 17.62; found C, 60.55; H, 5.97; N, 17.89.

General procedure for the preparation of compounds 5a–c
The para amino benzoic acid (1 mmol) was added to a solution of the enaminone derivatives 2a–c (1 mmol) dissolved in glacial acetic acid (10 ml) then the reaction mixture was heated under reflux for 6 h. After cooling, the precipitate was filtered off, washed with water, dried and recrystallised from isopropanol.

(E/Z)-4-((3-Oxo-3-(4-(piperidin-1-yl)phenyl)prop-1-en-1-yl)amino)-benzoic acid (5a). Yellow crystals (yield 44%), m.p. 270–272 °C; IR (KBr, ν cm^{-1}): 3465 (NH), 2665–2542 (OH), 1678, 1635 (2C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 1.59 (s, 6H, piperidine H), 3.33 (s, 4H, piperidine H), 6.16, 6.55 (2d, 1H, J = 8.2, 12.6, COCH=CHNH), 6.94–6.98 (m, 2H, Ar-H), 7.18 (d, 1H, J = 8.6, Ar-H), 7.35 (d, 1H, J = 8.7, Ar-H), 7.76 (d, 1H, J = 8, Ar-H), 7.81–7.90 (m, 3½ H, Ar-H and COCH=CHNH), 8.07 (t, ½H, J = 12.6, COCH=CHNH), 10.17, 12.06 (2d, 1H, J = 12.6, 11.9, NH, D$_2$O exchangeable), 12.61 (s, 1H, OH, D$_2$O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 24.4, 25.3, 48.3, 95.3, 100.0, 113.7, 114.8, 115.6, 123.7, 124.8, 127.4, 128.1, 129.7, 131.6, 141.7, 143.2, 144.7, 145.8, 153.8, 167.3, and 189.3; Anal. Calcd. for C$_{21}$H$_{22}$N$_2$O$_3$ (350.41): C, 71.98; H, 6.33; N, 7.99; found C, 71.79; H, 6.45; N, 8.21.

(E/Z)-4-((3-(4-Morpholinophenyl)-3-oxoprop-1-en-1-yl)amino)ben-zoic acid (5b). Yellow powder (yield 48%), m.p. >300 °C; IR (KBr, ν cm^{-1}): 3441 (NH), 2646–2549 (OH), 1697, 1643 (2C=O); 1369 and 1138 (SO$_2$); ^{1}H NMR (DMSO-d_6) δ ppm: 3.27–3.29 (m, 4H, morpho-line H), 3.75 (t, 4H, J = 4.6, morpholine H), 6.18, 6.57 (2d, 1H, J = 8.2, 12.6, COCH=CHNH), 6.99 (dd, 2H, J = 9.0, 3.2, Ar-H), 7.19 (d, 1H, J = 8.7, Ar-H), 7.37 (d, 1H, J = 8.7, Ar-H), 7.79 (d, J = 8.9, 1H, Ar-H), 7.85–7.91 (m, 3½H, Ar-H and COCH=CHNH), 8.09 (t, ½H, J = 12.6, COCH=CHNH), 10.12, 12.05 (2d, 1H, J = 12.7, 11.9, NH, D$_2$O exchangeable), 12.33 (s, 1H, OH, D$_2$O exchangeable); ^{13}C

NMR (DMSO-d_6) δ ppm: 53.5, 66.3, 113.7, 114.9, 115.7, 129.5, 131.6, 131.6, 136.4, 143.7, 144.7, 153.8, 172.6, and 189.6; Anal. Calcd. for C$_{20}$H$_{20}$N$_2$O$_4$ (352.38): C, 68.17; H, 5.72; N, 7.95; found C, 67.98; H, 5.85; N, 8.12.

(E/Z)-4-((3-(4-(4-Methylpiperazin-1-yl)phenyl)-3-oxoprop-1-en-1-yl)amino)benzoic acid (5c). Buff crystals (yield 50%), m.p. 260–262 °C; IR (KBr, ν cm^{-1}): 3425 (NH), 2669–2453 (OH), 1678, 1643 (2C=O); ^{1}H NMR (DMSO-d_6) δ ppm: 2.33 (s, 3H, CH$_3$), 2.34 (s, 4H, piperazine H), 3.33 (s, 4H, piperazine H), 6.18, 6.55 (2d, 1H, J = 8.1, 12.6, COCH=CHNH), 7.02 (dd, 1H, J = 8.8, 2.4, Ar-H), 7.18–7.25 (m, 2H, Ar-H), 7.37 (d, 1H, J = 8.6, Ar-H), 7.79–7.98 (m, 4½ H, Ar-H and COCH=CHNH), 8.19 (t, ½H, J = 12.6, COCH=CHNH), 10.21, 12.05 (2d, 1H, J = 12.68, 12.0, NH, D$_2$O exchangeable), 12.5 (s, 1H, OH, D$_2$O exchangeable); ^{13}C NMR (DMSO-d_6) δ ppm: 46.1, 46.9, 54.7, 96.4, 113.0, 118.6, 119.6, 128.4, 130.8, 131.6, 137.4, 143.9, 148.5, 170.8, and 188.4; Anal. Calcd. for C$_{21}$H$_{23}$N$_3$O$_3$ (365.43): C, 69.02; H, 6.34; N, 11.50; found C, 68.94; H, 6.51; N, 11.74.

Biological evaluation

CA inhibitory assay

For CA inhibitory assay, refer to Supplementary data.
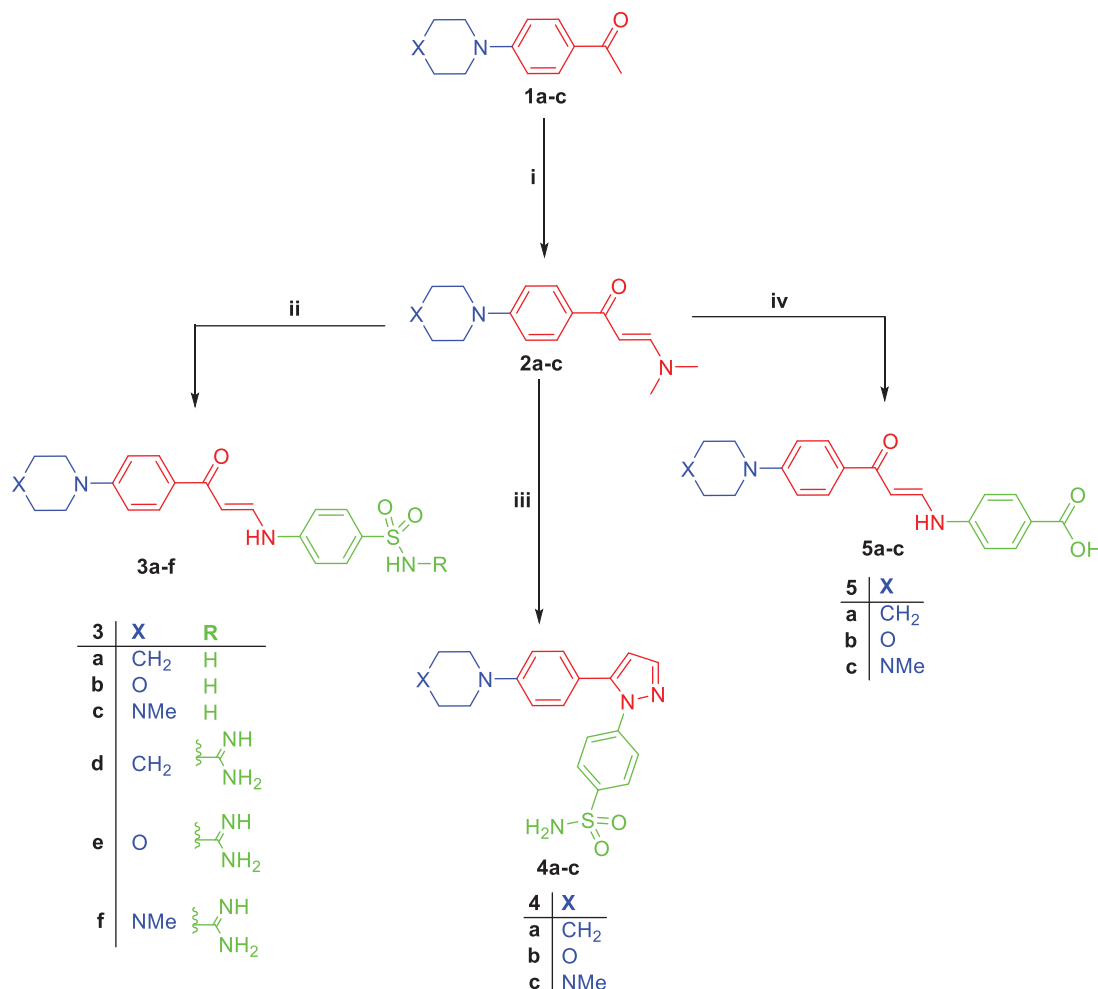
Scheme 1. Reagents and reaction conditions: (i) DMF-DMA, xylene reflux 8h; (ii) sulphanilamide or sulfaguanidine/gl. acetic acid, stirring overnight; (iii) sulphanilamide hydrazine HCl/gl. acetic, reflux; (iv) 4-aminobenzoic acid, gl. acetic acid, stirring overnight.
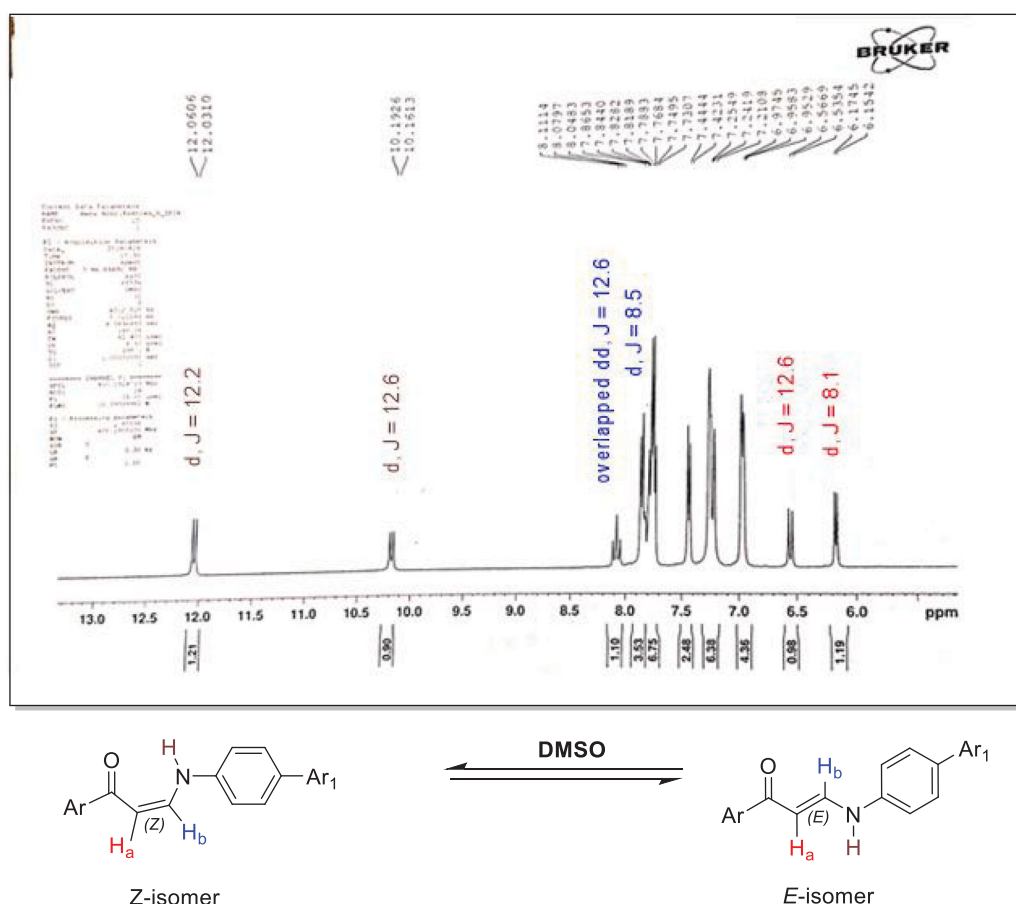
Figure 3. ^{1}H NMR of compound **3a** which showed the existing of Z/E geometric isomers in DMSO as represented example for **3a–f** and **5a–c**.

Anti-proliferative activity towards hormonal breast MCF-7 and non-hormonal breast MDA-MB-231 cancer cell lines

The MTT assay was used to determine the IC_{50} of the substances that were examined. All other details were provided in the supplementary data.

Cell cycle analysis

To predict the effect of the enaminone-sulphonamide **3c** on cell cycle progression in MCF-7, cell cycle analysis assay was accomplished, and the experimental assay was discussed in the supplementary data.

Apoptotic assay

Enaminone-sulphonamide **3c** was assayed using Annexin V-FITC/PI apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ) with propidium iodide double staining method, more details in the supplementary data.
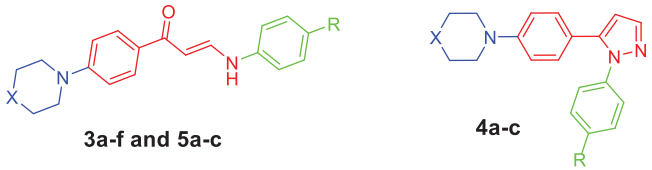
Results and discussion

Chemistry

The synthetic pathways used to synthesise the new target derivatives are represented in Scheme 1. The starting point refluxed the substituted acetophenones **1a–c** with the modified Vilsmeier–Haack reagent; dimethylformamide dimethyl acetal (DMF-DMA) in xylene to obtain the reported enaminoketones **2a–c**[38–40]. Reaction of compounds **2a–c** with *p*-aminobenzenesulfonamides namely sulphanilamide and sulfaguanidine in glacial acetic acid furnished

the desired sulphonamide compounds **3a–f**[30,41]. Their IR spectra revealed the appearance of the NH absorption bands of the amino benzene sulphonamide moiety in the region 3564–3155 cm^{-1} besides prominent two SO_2 bands at 1369–1315 and 1153–1134 cm^{-1}. ^{1}H NMR spectra of the 4-substituted enaminones **3a–f** confirmed their presence as Z/E geometric isomers around their olefinic bond $CH_a{=}CH_b$[30,41]. The up-field doublet signal of olefinic hydrogen Z-$\underline{H}_a$ appeared at δ 6.16–6.19 ppm with $J_{Z\text{-}Ha=Hb} = 7.2$–8.6 Hz whereas the down-field E-$\underline{H}_a$ appeared at δ 6.55 ppm with $J_{E\text{-}Ha=Hb} = 12.4$ and 12.6 Hz. Another characteristic feature was the presence of two sets of doublet signals assigned to the NH proton at δ 10.12–10.20 and 12.03–12.04 ppm with J values 11.7–12.8 and 11.6–12.3 Hz, respectively (Figure 3).

On the other hand, the obtained enaminoketones **2a–c** were reacted with 4-sulphanilamide hydrazine hydrochloride via an amine exchange heterocyclisation reaction[42,43] to afford exclusively the diarylpyrazoles **4a–c**. These compounds revealed new absorption bands corresponding to the NH and SO_2 groups at 3424–3379 and 1327–1315, 1161–1157 cm^{-1}, respectively, along with the disappearance of the C=O group characterising the parent enaminones **2a–c**. Interestingly, ^{1}H NMR of these pyrazole derivatives demonstrated two signals at δ 5.83–6.61 and 6.90–7.79 ppm, respectively, corresponding to pyrazole protons.

Finally, the reaction of enaminoketones **2a–c** with 4-aminobenzoic acid yielded the final compounds **5a–c**. IR spectra of **5a–c** exhibited the NH band at 3465–3425 cm^{-1} in addition to the OH broadband at 2669–2453 cm^{-1} and C=O band at 1635–1678 cm^{-1}. ^{1}H NMR of the derivatives **5a–c** exhibited the Z/E geometric isomers around the olefinic bond $CH_a{=}CH_b$ with two sets of up-field doublets of Z-H_a proton at δ 6.16–6.18 ppm and E-H_b δ 6.55–

Table 1. Inhibition data of human CA isoforms I, II, IX, and XII for the target compounds (**3a–f**, **4a–c**, and **5a–c**), using (AAZ) as a standard drug.

3a-f and 5a-c

4a-c

			K_I (nM)[a]			
Compound	R	X	hCA I	hCA II	hCA IX	hCA XII
3a	SO_2NH_2	CH_2	289.1	65.3	42.1	43.5
3b	SO_2NH_2	O	211.2	54.8	63.7	38.9
3c	SO_2NH_2	Nme	275.4	49.6	55.5	26.2
3d	$SO_2NHC{=}NHNH_2$	CH_2	822.3	172.5	149.2	116.1
3e	$SO_2NHC{=}NHNH_2$	O	607.9	166.3	141.8	137.4
3f	$SO_2NHC{=}NHNH_2$	Nme	643.0	161.9	125.3	102.6
4a	SO_2NH_2	CH_2	>100 000	689.2	226.7	298.3
4b	SO_2NH_2	O	>100 000	623.8	245.4	343.7
4c	SO_2NH_2	Nme	>100 000	586.7	287.6	404.5
5a	COOH	CH_2	>100 000	61 550	14 790	19 840
5b	COOH	O	>100 000	57 840	19 160	18 510
5c	COOH	Nme	>100 000	52 370	27 460	12 090
AAZ	–	–	250.0	12.0	25.0	5.7

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

6.75 ppm with J = 8.1–8.2 and 12.6 Hz, respectively, along with two exchangeable doublets for the NH proton at δ 10.12–10.21 and 12.05–12.06 ppm with J = 12.6–12.7 and 11.9–12.05 Hz.

The additional signal for the OH group in all these series was remarked at 12.16–13.80 ppm. All the target compounds **3a–f**, **4a–c**, and **5a–c** demonstrated the expected aliphatic signals assigned for the piperidine, morpholine, and methyl piperazine rings. ^{13}C NMR of all the synthesised compounds was in full agreement with their predicted carbon skeleton.

Biological evaluation

Carbonic anhydrase inhibition

Acetazolamide (AAZ) was used as a standard inhibitor for the measurement of the CA inhibitory activities of sulphonamide derivatives **3a–3f**, **4a–4c**, and carboxylic acid derivatives **5a–5c** against the isoforms hCA I, and II (cytosolic) and hCA IX and XII (transmembrane, tumour-associated isoform) using a stopped-flow CO_2 hydrase assay[44] (Table 1).

i. The ubiquitous off-target hCA I was moderately inhibited by the enaminone-sulphonamide analogues **3a–3c** with inhibition constants between 211.2 and 289.1 nM while the enaminone-sulfaguanidine analogues **3d–3f** possessed slightly inferior inhibitory activities with inhibition constants between 643.0 and 822.3. The enaminone-benzene carboxylic acid derivatives **5a–5c** failed to produce detectable inhibition properties. It is noteworthy, the presence of sulphonamide functionality is optimal for activity while substitution with COOH completely abolished activity. On the other hand, the substitution of the heterocycloalkyl moiety was detrimental to the activity of pyrazole-sulphonamide analogues **4a–4c** probably causing unfavourable steric clashes with the active site.

ii. The ubiquitous and physiologically predominant isoform hCA II was significantly inhibited by the enaminone-sulphonamide analogues **3a–3c** with inhibition constants between 49.6 and 65.3 nM. The replacement of sulphonamide by sulfaguanidine group as in analogues **3d–3f**, significantly reduced the

Table 2. Selectivity ratios for the inhibition of hCA IX and XII over hCA I and II for compounds (**3a–f**, **4a–c**, and **5a–c**) and acetazolamide.

Compound	I/IX	II/IX	I/XII	II/XII
3a	6.87	1.55	6.65	1.50
3b	3.32	0.86	5.43	1.41
3c	4.96	0.89	10.51	1.89
3d	5.51	1.16	7.08	1.49
3e	4.29	1.17	4.24	1.21
3f	5.13	1.29	6.27	1.58
4a	>441.11	3.04	>335.23	2.31
4b	>407.50	2.54	>290.95	1.81
4c	>347.71	2.04	>247.22	1.45
5a	>6.76	4.16	>5.04	3.10
5b	>5.22	3.02	>5.40	3.12
5c	>3.64	1.91	>8.27	4.33
AAZ	10.0	0.5	43.9	2.2

Table 3. In vitro anti-proliferative activity of **3a** and **3c** towards breast MCF-7 and MDA-MB-231 cancer cell lines.

	IC_{50} (μM) Normoxia		IC_{50} (μM)a Hypoxia	
Compound	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
3a	21.09 ± 0.98	35.04 ± 1.28	16.73 ± 0.92	26.68 ± 1.27
3c	4.918 ± 0.23	12.27 ± 0.45	1.689 ± 0.22	5.898 ± 0.45
Doxorubicin	3.386 ± 0.16	4.269 ± 0.16	1.368 ± 0.08	2.62 ± 0.18

[a]IC_{50} values are the mean ± SD of three separate experiments.

activity with inhibition constants between 607.9 and 822.3 nM while replacement with COOH as in analogues **5a–5c** completely abolished the activity. The heterocycloalkyl substituents in compounds **4a–4c** exhibited moderate inhibitory activity with inhibition constants between 586.7 and 689.2 nM probably due to unfavourable steric clashes.

iii. The target tumour-associated isoforms hCA IX and hCA XII are potently inhibited by the enamine-sulphonamide derivatives **3a–3c** (K_Is 26.2–63.7 nM). On the other hand, the guanidino group of **3d–3f** decreased the activity (102.6–149.2 nM) probably due to less efficient zinc binding in the active sites of hCA IX and hCA XII. The COOH substituted derivatives **5a–5c** were the least

potent inhibitors (12 090–27 460 nM) probably because of the lack of zinc binding to the active site. Heterocycloalkyl substituted **4a**–**4c** gave moderate inhibitory effects.

iv. The selectivity index (SI) (Table 2) shows that, while actually all compounds **3a**–**f** are selective CAIs for the tumour-associated isoforms over hCA I (SI in the range 3.32–6.87 for hCA IX and 4.24–10.51 for hCA XII), significant selectivity for the target CAs over hCA II exists within this series **3a**–**f** ranging from 0.86 to 1.55 for hCA IX and 1.21 to 1.89 for hCA XII, which indicates that these targets could represent interesting candidates for further anti-tumour assessments.

Anti-proliferative activity towards hormonal breast MCF-7 and non-hormonal breast MDA-MB-231 cancer cell lines

The *in vitro* cytotoxic IC_{50} assessment against two hormonal and non-hormonal breast cancer cell lines (MCF-7 and *MDA-MB-231*)

Table 4. Cell cycle analysis of the treated MCF-7 cells with **3c**.

Comp.	%G0–G1	%S	%G2/M
3c	51.44	25.04	23.52
Control	56.49	28.61	14.9

Table 5. Apoptotic cells sub-population percentage in treated MCF-7 cells with **3c**.

Comp.	Total	Early	Late	Necrosis
3c/MCF-7	46.32	13.91	26.38	6.03
Control/MCF-7	2.15	0.34	0.19	1.62

was performed for the best two derivatives **3a** and **3c**, in terms of the CA inhibition assay under both normoxic and hypoxic conditions. Derivative **3c** showed comparable potency against both MCF-7 and MDA-MB-231 cancer cell lines under both normoxic (IC_{50} = 4.918 and 12.27 μM, respectively) and hypoxic (IC_{50} = 1.689 and 5.898 μM, respectively) conditions compared to the reference drug doxorubicin under normoxic (IC_{50} = 3.386 and 4.269 μM, respectively) and hypoxic conditions (IC_{50} = 1.368 and 2.62 μM, respectively). Compound **3a**, on the other hand, exhibited weaker inhibitory activity against both MCF-7 and MDA-MB-231 cancer cell lines under both normoxic (IC_{50} = 21.09 and 35.04 μM, respectively), and hypoxic conditions (IC_{50} = 16.73 and 26.68 μM, respectively) (Table 3). Therefore, it was found that the MCF-7 cancer cell line was the most affected cell to compound **3c** under both normoxic and hypoxic conditions.

Cell cycle analysis

For more investigation, cell cycle analysis was achieved for MCF-7 cells treated with **3c** derivative at IC_{50} concentration using untreated MCF-7 cells as a negative control. It shows slight decrease in G0–G1 and S phases (51.44 and 25.04%, respectively) compared to the control cells (56.49 and 28.61%, respectively) whereas it increase G2/M phase, 23.52% compared to the control cells, 14.9% (Table 4 and Figure 4).

Annexin V-FITC apoptosis assay

Annexin V-FITC and propidium iodide double staining methods were performed to reinforce the assumption that **3c** may act as a
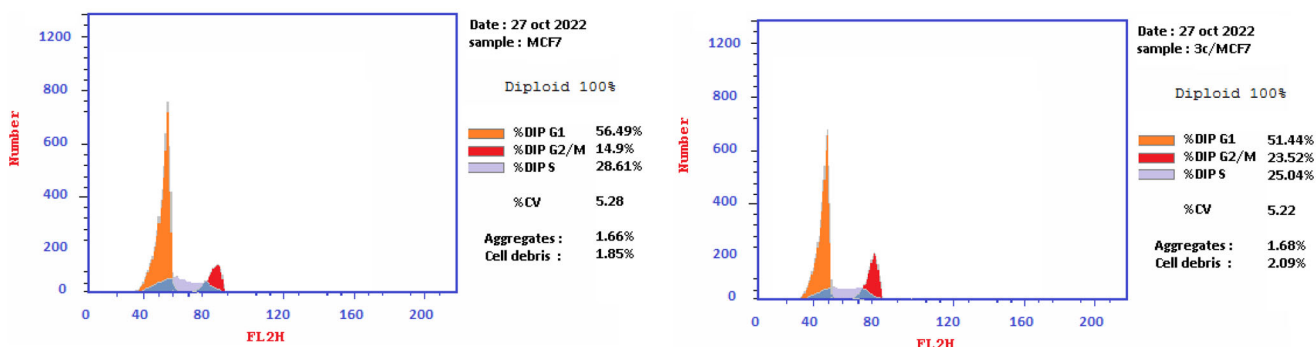

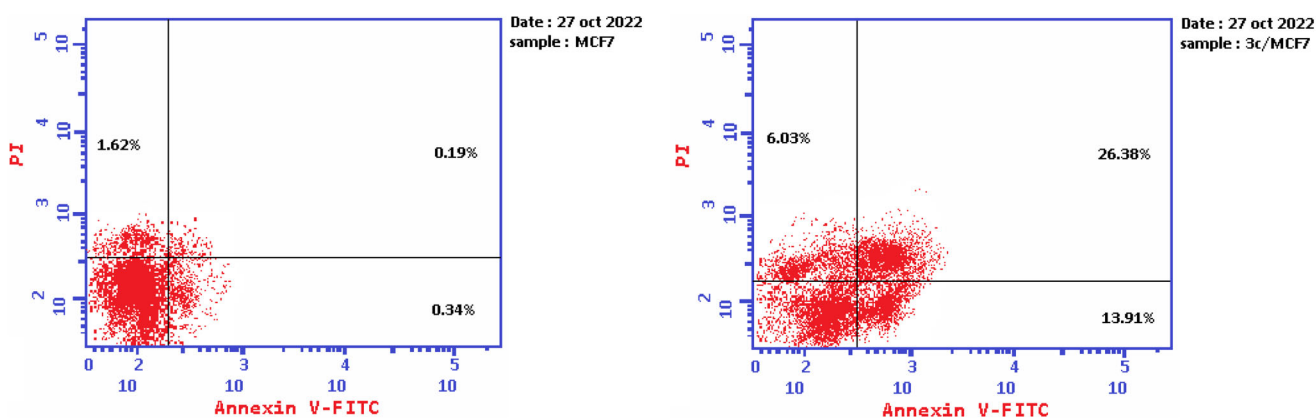**Figure 4.** The cell cycle of treated MCF-7 cells with **3c**.

Figure 5. Effect of compound **3c** on the percentage of annexin V-FITC-positive staining in MCF-7 cells.

cytotoxic agent through apoptotic induction in MCF-7 cancer cells. There was a significant increase in both early and late population percentages of **3c** treated MCF-7 cancer cells (13.91% and 26.38%, respectively) compared to the negative control MCF-7 cells (0.34% and 0.19%, respectively). This result supports the proposed **3c** antiproliferative mechanism of action as an apoptotic inducer (Table 5 and Figure 5).

Conclusions

Novel series of aryl enaminones (**3a**–**f** and **5a**–**c**) and pyrazole (**4a**–**c**) linked compounds with sulphonamides, sulfaguanidine, or carboxylic acid functionalities as human carbonic anhydrases inhibitors (hCAIs) were designed, synthesised, and screened for their inhibitory activity against the isoforms hCA I, and II (cytosolic) and hCA IX and XII (transmembrane, tumour-associated isoform) using a stopped-flow CO_2 hydrase assay. The target tumour-associated isoforms hCA IX and hCA XII are potently inhibited by the enamine-sulphonamide derivatives **3a**–**c** (KIs 26.2–63.7 nM). Moreover, the most selective hCA IX and XII inhibitors **3a** and **3c** were assessed for their potential anti-cancer against (MCF-7 and MDA-MB-231) cancer cell lines. Derivative **3c** showed comparable potency against both MCF-7 and MDA-MB-231 cancer cell lines under both normoxic (IC_{50} = 4.918 and 12.27 μM, respectively) and hypoxic (IC_{50} = 1.689 and 5.898 μM, respectively) conditions. Therefore, compound **3c** was additionally subjected to cell cycle analysis and apoptotic assay at IC_{50} concentration using untreated MCF-7 cells as a negative control. It shows slight decrease in G0–G1 and S phases (51.44 and 25.04%, respectively) compared to the control cells (56.49 and 28.61%, respectively), while, it increased G2/M phase, 23.52% compared to the control cells, 14.9%. Whereas, there were significant increases in both early and late apoptotic percentages of **3c** treated MCF-7 cancer cells (13.91% and 26.38%, respectively) compared to the negative control MCF-7 cells (0.34% and 0.19%, respectively), which supports the proposed **3c** antiproliferative mechanism of action as an apoptotic inducer.

Disclosure statement

Funding

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