Short Communication

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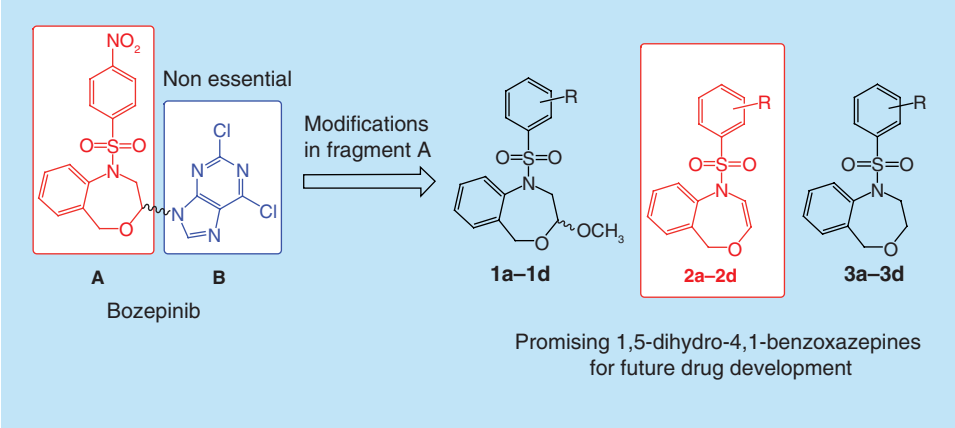
Future Medicinal Chemistry

1-(Benzenesulfonyl)-1,5-dihydro-4,1-benzoxazepine as a new scaffold for the design of antitumor compounds

Aim: Bozepinib is a potent and selective anticancer compound which chemical structure is made up of a benzofused seven-membered ring and a purine moiety. We previously demonstrated that the purine fragment does not exert antiproliferative effect *per se*. **Methodology:** A series of 1-(benzenesulfonyl)-4,1-benzoxazepine derivatives were synthesized in order to study the influence of the benzofused seven-membered ring in the biological activity of bozepinib by means of antiproliferative, cell cycle and apoptosis studies. **Results & conclusion:** Our results show that the methyleneoxy enamine sulfonyl function is essential in the antitumor activity of the structures and thus, it is a scaffold suitable for further modification with a view to obtain more potent antitumor compounds.



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Keywords: antitumor • apoptosis • benzoxazepine • bozepinib • cell cycle • methyleneoxy enamine sulfonyl

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Cancer is a leading cause of disease worldwide. In 2012, 14.1 million new cases of cancer were estimated in the world [1]. We previously described a series of benzofused seven-membered *O,N*-acetals as potent antitumor compounds [2–4]. Among them, (*RS*)-2,6-dichloro-9-[1-(*p*-nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-yl]-9*H*-purine (bozepinib, Figure 1) is the most potent and selective antitumor compound that is able to induce apoptosis in breast cancer cells [5]. We demonstrated the role of the PKR as a biological target of bozepinib involved in the apoptosis of breast and colon cancer cells. In addition, the specific HER2, JNK and ERKs inhibition, the antiangiogenic and antimigration activity together with the *in vivo* antitumor and antimetastatic

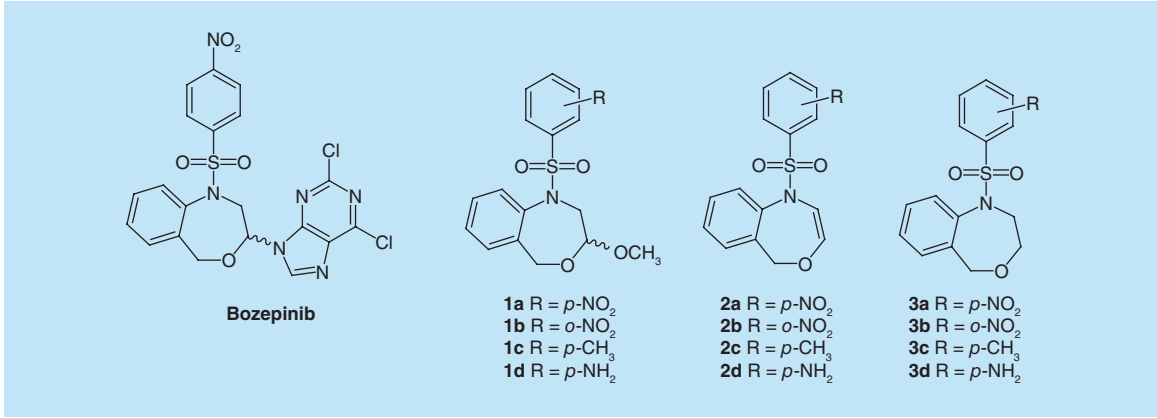

Figure 1. Chemical structure of the target compound.

effect and the nonsystemic toxicity of bozepinib was reported [5–7].

The chemical structure of bozepinib is made up by the benzofused seven-membered ring and the purine moiety. We demonstrated that the purine fragment does not exert an antiproliferative effect *per se* [8]. Moreover, several 2,6-disubstituted- and 9-ethylpurine derivatives showed moderate antiproliferative activities against several cancerous cell lines [9,10]. We have now decided to study the influence of the benzofused seven-membered ring in the biological activity of bozepinib. Thus, we have carried out the synthesis of a series of 1-(benzenesulfonyl)-4,1-benzoxazepine derivatives (Figure 1).

We previously reported **1a** and **1b** as promising intermediates in the synthesis of anticancer agents [11]. Compounds **2a** and **2b** were obtained along with bozepinib in the Vorbrüggen reaction of purines with **1a** and **1b**, respectively [5]. We also envisioned the saturated analogs **3a** and **3b** for their close relationship with the same fragment of bozepinib. Finally, methyl and amine groups were chosen as substituent (series **c** and **d**) to complete the target structures of the present study. The antiproliferative effect of the amine analog of bozepinib remains the same against the human breast cancer cell lines MCF-7 and MDA-MB-231 [5]. The methyl group may be oxidized to form an alcohol and further to a carboxylic acid favoring its quickly elimination from the body [12].

Consequently, compounds **1a**–**1d**, **2a**–**2d** and **3a**–**3d** were synthesized in order to study the structure–activity relationships of the 1-(benz enesulfonyl)-4,1-benzoxazepine series.

Materials & methods

Chemistry

Melting points were taken in open capillaries on a Stuart Scientific SMP3 electrothermal melting point apparatus and are uncorrected. Elemental analyses were performed on the Thermo Scientific Flash 2000 analyzer and the measured values indicated by the symbols of the elements or functions were within ± 0.4% of the theoretical values. Analytical TLC was performed using Merck Kieselgel 60 F_{254} aluminium plates and visualized by UV light or iodine. All evaporations were carried out *in vacuo* in a Büchi rotary evaporator and the pressure controlled by a Vacuubrand CVCII apparatus. Merck silicagel 60 with a particle size of 0.040–0.063 mm (230–400 mesh ASTM) was used for flash chromatography. NMR spectra were recorded on a 500 MHz ^{1}H and 125 MHz ^{13}C NMR Varian Direct Drive spectrometer at ambient temperature. Chemical shifts (δ) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. Spin multiplicities are given as s (singlet), bs (broad singlet), d (doublet), dd (double doublet), ddd (double double doublet), pt (pseudo triplet) and m (multiplet). J values are given in Hz. High-resolution nano-assisted laser desorption/ionization (NALDI-TOF) or ESI (ESI-TOF) mass spectra were carried out on a Bruker Autoflex or a Waters LCT Premier Mass Spectrometer, respectively. Anhydrous CH_2Cl_2 and CH_3CN were purchased from VWR International Eurolab. Anhydrous conditions were performed under argon. All reagents were purchased from Sigma-Aldrich.

(RS)-3-Methoxy-1-(p-methylbenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (1c)

A solution of **4** [11] (1.0 equiv) and p-nitrobenzenesulfonyl chloride (2.0 equiv) in anhydrous CH_2Cl_2 (3 ml/mmol) was prepared under an argon atmosphere and cooled to 0°C and at this temperature TEA (3.0 equiv) was added. The mixture was microwave-irradiated at 110°C for 30 min. Then, the reaction mixture was poured into water and extracted with CH_2Cl_2. The combined organic layers were washed with brine, dried (Na_2SO_4) and evaporated. The residue was purified by flash chromatography

using EtOAc/hexane 1/7 as eluent. White solid, 40%, mp: 106–107°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.56 (d, J = 8.3 Hz, 2H), 7.50 (d, J = 7.7 Hz, 1H), 7.30–7.14 (m, 5H), 4.66 (pt, J = 3.9, J = 3.8 Hz, 1H), 4.43 (d, J = 13.6 Hz, 1H), 4.10 (d, J = 13.6 Hz, 1H), 3.94–3.63 (bs, 2H), 3.38 (s, 3H), 2.40 (s, 3H). ^{13}C NMR ($CDCl_3$): 144.35 (C), 140.53 (C), 138.64 (C), 138.20 (C), 130.15 (CH) (×2), 130.04 (CH), 129.72 (CH), 129.66 (CH), 128.69 (CH), 128.51 (CH) (×2), 101.68 (CH), 65.11 (CH_2), 56.18 (CH_3), 54.37 (CH_2), 22.54 (CH_3). High-resolution mass spectrometry (HRMS) (ESI-TOF) (m/z) calcd. for $C_{17}H_{19}NO_4SNa$ $(M + Na)^+$ 356.0933, found 356.0932. Anal. Calc. for $C_{17}H_{19}NO_4S$: C, 61.24; H, 5.74; N, 4.20. Found: C, 61.30; H, 5.58; N, 4.01.

(RS)-3-methoxy-1-(p-aminobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (1d)

$SnCl_2.2H_2O$ (5 equiv) was added to a suspension of **1a** [11] (1.0 equiv) in EtOH (5 ml/mmol) and heated at the reflux temperature for 1 h. The mixture was then cooled at 0°C and the pH was fixed to 7–8 with saturated $NaHCO_3$ solution. The aqueous layer was extracted with CH_2Cl_2 and the combined organic layers were washed with brine, dried (Na_2SO_4), filtered and evaporated. The residue was purified by flash chromatography using EtOAc/hexane 1/3 as eluent. Viscous oil, 60%. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.48 (d, J = 8.2 Hz, 1H), 7.44 (d, J = 8.7 Hz, 2H), 7.25 (ddd, J = 7.5 × 2, 1.1 Hz, 1H), 7.19 (ddd, J = 7.3 × 2, 1.5 Hz, 1H), 7.14 (dd, J = 7.4, 2.1 Hz, 1H), 6.56 (d, J = 8.8 Hz, 2H), 4.67 (dd, J = 5.8, 2.4 Hz, 1H), 4.49 (d, J = 13.7 Hz, 1H), 4.15 (d, J = 13.7 Hz, 1H), 4.10 (bs, 2H), 3.89 (d, J = 14.6 Hz, 1H), 3.63 (bs, 1H), 3.39 (s. 3H). ^{13}C NMR ($CDCl_3$): 150.61 (C), 139.89 (C), 137.33 (C), 128.95 (C), 129.70 (CH) (×2), 129.05 (CH), 128.70 (CH), 128.68 (CH), 127.53 (CH), 113.70 (CH) (×2), 101.16 (CH), 64.68 (CH_2), 54.43 (CH_3), 53.34 (CH_2). HRMS (ESI-TOF) (m/z) calcd. for $C_{16}H_{18}N_2O_4SNa$ $(M + Na)^+$ 357.0885, found 357.0883. Anal. Calc. for $C_{16}H_{18}N_2O_4S$: C, 57.47; H, 5.43; N, 8.38. Found: C, 57.35; H, 5.60; N, 8.31.

1-(p-Methylbenzenesulfonyl)-1,5-dihydro-4,1-benzoxazepine (2c)

A suspension of **1c** (1.0 equiv) and 6-bromopurine (2.5 equiv) in anhydrous CH_3CN (7 ml/mmol) was prepared under an argon atmosphere and cooled to 0°C. At this temperature, TMSCl (4.0 equiv), HMDS (4.0 equiv) and a 1.0 M solution of $SnCl_4$ in CH_2Cl_2 (4.0 equiv) were added subsequently. The mixture was irradiated by microwave at 160°C for 5 min. The reactions were quenched by the addition of distilled water and the pH was fixed to 7–8 with a saturated $NaHCO_3$ solution. The mixture was filtered over Celite and the filtrate was extracted with CH_2Cl_2. The combined organic layers were dried (Na_2SO_4), filtered and evaporated. The residue was purified by flash chromatography using EtOAc/hexane 1/3 as eluent. White solid, 66.5%, mp: 85–86°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.60 (d, J = 7.9 Hz, 1H), 7.50 (d, J = 8.2 Hz, 2H), 7.40 (ddd, J = 1.5, 7.9 × 2 Hz, 1H), 7.29–7.26 (m, 3H), 7.10 (dd, J = 1.4, 7.5 Hz, 1H), 5.90 (d, J = 5.9 Hz, 1H), 5.76 (d, J = 5.9 Hz, 1H), 4.00 (s, 2H), 2.43 (s, 3H). ^{13}C NMR ($CDCl_3$): 143.96 (C), 141.78 (C), 137.86 (CH), 134.34 (C), 132.52 (C), 129.96 (CH), 129.61 (CH) (×2), 129.48 (CH), 129.46 (CH), 128.10 (CH), 127.48 (CH) (×2), 104.80 (CH), 70.07 (CH_2), 21.68 (CH_3). HRMS (ESI-TOF) (m/z) calcd. for $C_{16}H_{15}NO_3SNa$ $(M + Na)^+$ 324.0671, found 324.0670. Anal. Calc. for $C_{16}H_{15}NO_3S$: C, 63.77; H, 5.02; N, 4.65. Found: C, 63.84; H, 4.89; N, 4.75.

1-(p-Aminobenzenesulfonyl)-1,5-dihydro-4,1-benzoxazepine (2d)

A solution of **2a** [5] in MeOH (100 ml/mmol) was hydrogenated in a Parr apparatus (50 psi) for 4 h using 10% Pd/C as catalyst. The mixture was filtered over Celite and the filtrate was concentrated *in vacuo* to give an oil that was purified by flash chromatography using EtOAc/hexane 1/2 as eluent. Yellow solid, 42%, mp: 153–154°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.58 (dd, J = 0.8, 7.8 Hz, 1H), 7.40–7.35 (m, 3H), 7.27–7.22 (m, 1H), 7.10 (dd, J = 1.6, 7.8 Hz, 1H), 6.61 (d, J = 8.6 Hz, 2H), 5.89 (d, J = 5.8 Hz, 1H), 5.73 (d, J = 5.8 Hz, 1H), 4.12 (s, 4H). ^{13}C NMR ($CDCl_3$): 151.02 (C), 142.98 (C), 137.92 (CH), 132.93 (C), 130.34 (CH), 129.85 (CH) (×2), 129.64 (CH), 129.62 (CH), 128.18 (CH), 126.05 (C), 114.13 (CH) (×2), 105.37 (CH), 70.48 (CH_2). HRMS (ESI-TOF) (m/z) calcd. for $C_{15}H_{15}N_2O_3S$ $(M + H)^+$ 303.0803, found 303.0801. Anal. Calc. for $C_{15}H_{14}N_2O_3S$: C, 59.59; H, 4.67; N, 9.27. Found: C, 59.63; H, 4.46; N, 9.09.

1-(p-Nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (3a)

To a degassed solution of **2a** in a 1:0.2 mixture of THF and *tert*-BuOH (6 ml/mmol) and saturated by hydrogen was added 4% Wilkinson's catalyst. The solution was stirred under a hydrogen atmosphere at room temperature for 3 h. The solvent was evaporated and the residue was purified by flash chromatography using EtOAc/hexane 1/4 as eluent. Beige solid, 64%, mp: 177–178°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.33 (d, J = 8.8 Hz, 2H), 7.9 (d, J = 8.8 Hz, 2H), 7.36–7.27 (m, 3H), 7.24–7.22 (m, 1H), 4.26 (bs, 3H), 3.86 (s, 3H). ^{13}C NMR ($CDCl_3$): 150.05 (C), 146.99 (C), 139.05

(C), 137.92 (C), 130.01 (CH) 129.09 (CH), 128.68 (CH), 128.53 (CH), 128.31 (CH) (×2), 124.40 (CH) (x2), 73.46 (CH_2), 71.24 (CH_2), 52.57 (CH_2). HRMS (NALDI-TOF) (m/z) calcd. for $C_{15}H_{14}N_2O_5SNa$ $(M + Na)^+$ 357.0521, found 357.0523. Anal. Calc. for $C_{15}H_{14}N_2O_5S$: C, 53.88; H, 4.22; N, 8.38. Found: C, 53.99; H, 4.23; N, 8.32.

1-(o-Nitrobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (3b)

To a degassed solution of **2b** in a 1:0.2 mixture of THF and *tert*-BuOH (6 ml/mmol) and saturated by hydrogen was added 4% Wilkinson's catalyst. The solution was stirred under a hydrogen atmosphere at room temperature for 3 h. The solvent was evaporated and the residue was purified by flash chromatography using EtOAc/hexane 1/4 as eluent. White solid, 46%, mp: 142–143°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 8.84 (dd, J = 7.9, 1.0 Hz, 1H), 7.76–7.70 (m, 2H), 7.6 (ddd, J = 6.9 × 2, 1.9 Mz, 1H), 7.32–7.25 (m, 2H), 7.17 (ddd, J = 7.7, 7.4, 1.9 Hz, 1H), 6.69 (d, J = 7.8 Hz, 1H), 4.64 (s, 2H), 3.99 (s, 2H), 3.90 (bs, 2H).^{13}C NMR ($CDCl_3$): 147.82 (C), 139.49 (C), 139.14 (C), 134.34 (C), 133.89 (CH) 131.67 (CH), 131.45 (CH), 130.15 (CH), 128.73 (CH), 128.59 (CH), 128.11 (CH), 124.36 (CH), 73.71 (CH_2), 72.86 (CH_2), 52.98 (CH_2). HRMS (ESI-TOF) (m/z) calcd. for $C_{15}H_{15}N_2O_5S$ $(M + H)^+$ 335.0701, found 335.0703. Anal. Calc. for $C_{15}H_{14}N_2O_5S$: C, 53.88; H, 4.22; N, 8.38. Found: C, 54.06; H, 4.05; N, 8.24.

1-(p-Methylbenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (3c)

A solution of **2c** in MeOH (100 ml/mmol) was hydrogenated in a Parr apparatus (50 psi) for 5 h using 10% Pd/C as catalyst. The mixture was filtered over Celite and the filtrate was concentrated *in vacuo* to give an oil that was purified by flash chromatography using EtOAc/hexane 1/2 as eluent. Viscous oil, 85%. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.60 (d, J = 8.3 Hz, 2H), 7.40 (dd, J = 1.2, 7.7 Hz, 1H), 7.30–7.18 (m, 5H), 4.23 (bs, 3H), 3.81 (s, 3H), 2.42 (s, 3H). ^{13}C NMR ($CDCl_3$): 143.63 (C), 139.95 (C), 138.59 (C), 138.02 (C), 129.76 (CH) (×2), 129.72 (CH), 129.02 (CH), 128.80 (CH), 127.84 (CH), 127.07 (CH) (×2), 73.53 (CH_2), 71.05 (CH_2), 52.21 (CH_2), 21.55 (CH_3). HRMS (ESI-TOF) (m/z) calcd. for $C_{16}H_{18}NO_3S$ $(M + H)^+$ 304.1007, found 304.1009. Anal. Calc. for $C_{16}H_{17}NO_3S$: C, 63.34; H, 5.65; N, 4.62. Found: C, 63.39; H, 5.55; N, 4.72.

1-(p-Aminobenzenesulfonyl)-1,2,3,5-tetrahydro-4,1-benzoxazepine (3d)

A solution of **2a** in MeOH (100 ml/mmol) was hydrogenated in a Parr apparatus (50 psi) for 8 h using 10%

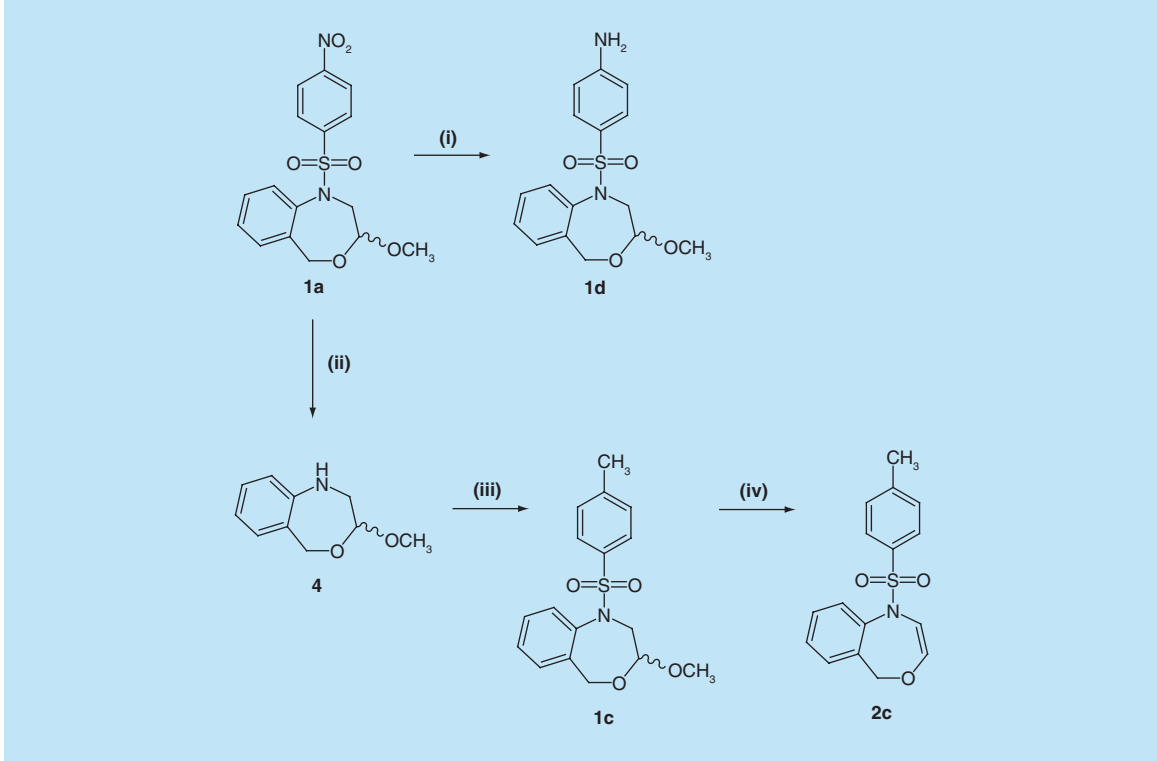

Figure 2. Reagents and conditions. (i) $SnCl_2{\cdot}2H_2O$, EtOH; **(ii)** PhSH, K_2CO_3, DMF; **(iii)** *p*-CH_3PhSO_2Cl, CH_2Cl_2, TEA, microwave irradiation; **(iv)** 6-bromopurine, TMSCl, HMDS, $SnCl_4$ (1 M solution in CH_2Cl_2), microwave irradiation.

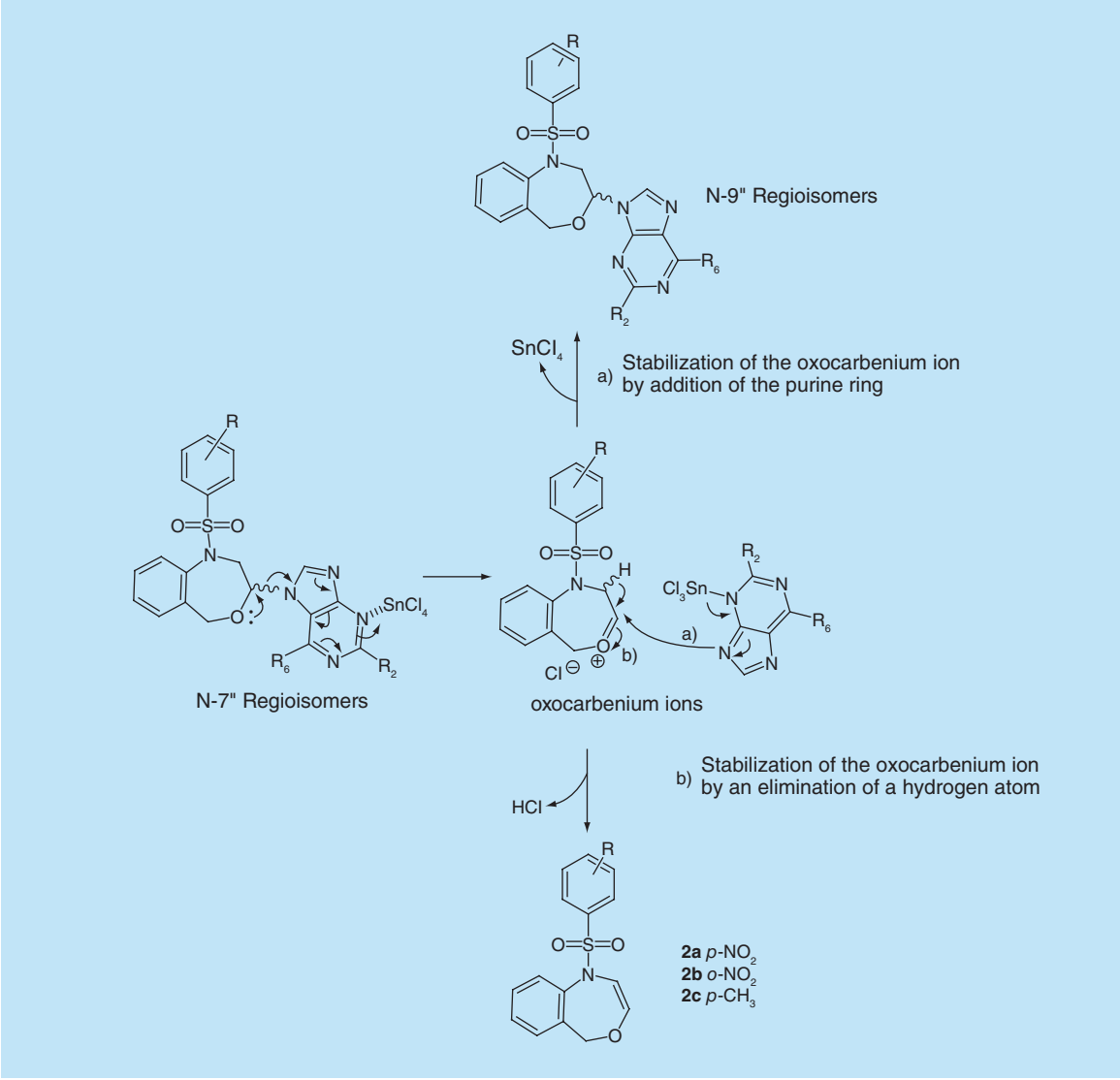

Figure 3. Proposed reaction mechanism for the formation of 2a–2c.

Pd/C as catalyst. The mixture was filtered over Celite and the filtrate was concentrated *in vacuo* to give an oil that was purified by flash chromatography using EtOAc/hexane 1/2 as eluent. White solid, 94%, mp: 146–147°C. ^{1}H NMR ($CDCl_3$): δ (ppm) 7.47 (d, J = 8.6 Hz, 2H), 7.42 (d, J = 7.8 Hz, 1H), 7.29–7.25 (m, 1H), 7.23–7.17 (m, 2H), 6.62 (d, J = 8.6 Hz, 2H), 4.29 (bs, 3H), 4.13 (bs, *2*H), 3.82 (s, 3H). ^{13}C NMR ($CDCl_3$): 150.62 (C), 140.34 (C), 138.15 (C), 129.84 (C), 129.74 (CH) 129.33 (CH) (×2), 129.27 (CH), 128.78 (CH), 127.71 (C), 114.14 (CH) (×2), 73.69 (CH_2), 71.13 (CH_2), 52.13 (CH_2). HRMS (NALDI-TOF) (m/z) calcd. for $C_{15}H_{16}N_2O_3SNa$ $(M + Na)^+$ 327.0780, found 327.0780. HRMS (ESI-TOF) (m/z) calcd. for $C_{15}H_{17}N_2O_3S$ $(M + H)^+$ 305.0960, found 305.0959. Anal. Calc. for $C_{15}H_{16}N_2O_3S$: C, 59.19; H, 5.30; N, 9.20. Found: C, 49.98; H, 5.33; N, 9.16.

Biology

Cell culture

MCF-7, HCT-116 and A-375 cells were grown at 37°C in an atmosphere containing 5% CO_2, with Dulbecco's modified Eagle medium (Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2% L-glutamine, 2.7% sodium bicarbonate, 1% Hepes buffer, 40 mg/l gentamicin and 500 mg/l ampicillin.

Drug treatment

Compounds were dissolved in DMSO and stored at -20°C. For each experiment, the stock solutions were further diluted in medium to obtain the desired concentrations. The final solvent concentration in cell culture was ≤0.1% v/v of DMSO, a concentration without any effect on cell replication. Parallel cultures

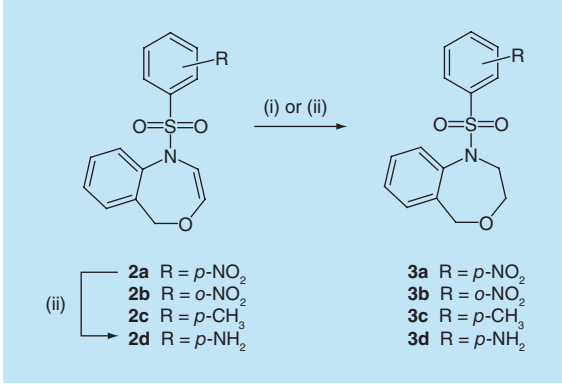

Figure 4. Reagents and conditions. (i) H_2, Wilkinson's catalyst, THF/*t*BuOH for **3a** and **3b**; **(ii)** H_2, Pd/C, MeOH for **2d**, **3c** and **3d**.

of MCF-7, HCT-116 and A-375 cells in medium with DMSO were used as controls.

Proliferation assays

The effect of the compounds on cell viability was assessed using the sulforhodamine-B colorimetric assay. Cells suspension (1×10^3 cells/well) was seeded onto 24-well plates and incubated for 24 h. The cells were then treated with different concentrations of drugs in their respective culture medium and maintained with the treatment for 3 days. Three days later, wells were aspirated and fresh medium and drug were added. Then cell cultures were maintained for 3 additional days. Thereafter, we used a Titertek Multiscan (Flow, CA, USA) at 492 nm. We evaluated the linearity of the sulforhodamine-B assay with a cell number for each cell stock before each cell growth experiment. The IC_{50} values were calculated from semi-logarithmic dose-response curves by linear interpolation. All the experiments were plated in triplicate wells and were carried out twice.

Cell cycle distribution analysis

The cells at 70% confluence were treated with either DMSO alone or with concentrations of the compounds determined at their $2 \times IC_{50}$ dose values. Flow cytometry was performed after 24 h of treatment as described [13]. All the experiments were plated in triplicate wells and were carried out twice.

Apoptosis detection by staining with annexin V-FITC & propidium iodide

The Ann V apoptosis detection kit I (Pharmingen, CA, USA) was used to detect apoptosis by flow cytometry according to our previous published protocol [6,7]. Apoptosis inductions in the MCF-7, HCT-116 and A-375 human cancer cell lines after treatment for 24 h were determined for the compounds at doses of their corresponding $2 \times IC_{50}$ values. All the experiments were plated in triplicate wells and were carried out twice.

Western blot analysis

Cells were plated on 6-well plates in their respective medium. After treatment, medium was removed and the cells were lysed in Laemmli buffer. The protein sample was subjected to electrophoresis, transferred

Table 1. Antiproliferative activities for compounds 1–3 against the cancerous cell lines MCF-7, HCT-116 and A-375.

Compounds	IC_{50} (μM) MCF-7	IC_{50} (μM) HCT-116	IC_{50} (μM) A-375
Bozepinib	0.78 ± 0.06[†]	0.48 ± 0.08[†]	–
1a	62.30 ± 0.05	24.10 ± 0.02	2.66 ± 0.05
1b	51.78 ± 0.21	24.20 ± 0.07	8.19 ± 0.05
1c	7.08 ± 0.02	11.57 ± 0.05	6.64 ± 0.02
1d	73.84 ± 0.06	53.15 ± 0.05	20.15 ± 0.07
2a	3.91 ± 0.05	27.78 ± 0.05	8.70 ± 0.05
2b	7.16 ± 0.06	34.24 ± 0.06	6.79 ± 0.03
2c	9.05 ± 0.06	6.74 ± 0.09	3.88 ± 0.06
2d	23.03 ± 0.04	18.82 ± 0.06	13.64 ± 0.04
3a	31.10 ± 0.08	51.01 ± 0.07	17.69 ± 0.03
3b	33.03 ± 0.01	26.07 ± 0.01	26.16 ± 0.01
3c	27.02 ± 0.05	35.48 ± 0.06	7.10 ± 0.02
3d	46.10 ± 0.02	33.40 ± 0.04	10.12 ± 0.03

[†]All experiments were conducted in duplicate and gave similar results. The data are mean ± SEM of three independent determinations.
A-375: Human melanoma cancer cell line; HCT: Human colon cancer cell line; MCF-7: Human breast cancer cell line.
Data taken with permission from [6].

Table 2. Cell cycle distribution in the MCF-7, HCT-116 and A-375 cancer cell lines after treatment for 24 h with the most active compounds 2a–2d as anti-proliferative agents.

Compound	Cell cycle		
	G_0/G_1	S	G_2/M
MCF-7 breast cancer cell line			
Control	89.88 ± 1.73	1.57 ± 2.12	8.55 ± 0.40
Bozepinib†	87.2 ± 2.69	1.57 ± 1.12	8.55 ± 0.40
Control	34.58 ± 0.09	14.56 ± 1.06	50.91 ± 1,06
2a	36.96 ± 1.06	10.46 ± 1.97	52.55 ± 1.30
2b	37.57 ± 1.42	36.97 ± 0.87	27.14 ± 0.55
2c	33.88 ± 1.09	14.80 ± 0.52	51.31 ± 0.57
2d	31.07 ± 0.30	12.11 ± 2.10	56.81 ± 1.84
HCT-116 colon cancer cell line			
Control	76,38 ± 0.85	0.00 ± 0.00	23.63 ± 0.85
2a	76.10 ± 2.35	1.30 ± 0.16	22.60 ± 1.39
2b	76.47 ± 2.16	1.37 ± 0.18	22.15 ± 0.34
2c	75.90 ± 2.47	0.40 ± 0.22	23.70 ± 1.52
2d	76.90 ± 1.58	0.70 ± 0.24	22.40 ± 2.12
A-375 melanoma cancer cell line			
Control	57.97 ± 0.09	20.99 ± 0.78	12.42 ± 1,70
2a	58.33 ± 0.21	28.25 ± 0.52	13.42 ± 0.31
2b	70.22 ± 1.42	3.64 ± 0.87	26.14 ± 0.55
2c	58.37± 1.55	3.17 ± 1.49	38.50 ± 1.92
2d	56.41 ± 0.42	29.26 ± 0.65	14.32 ± 0.23

†All experiments were conducted in duplicate and gave similar results. The data are means ± SEM of three independent determinations. Data taken with permission from [5].

onto nitrocellulose membranes (Bio-Rad, CA, USA, 162-0115), and blocked in PBS containing 5% non-fat dry milk for 1 h at room temperature. Primary antibodies used included a polyclonal phospho-PKR (phospho T446 antibody ab131447, Abcam, Cambridge, UK), a polyclonal antibody to phospho-eIF2a (Ser 52; Invitrogen CA, USA, 44–728, G) and a monoclonal antibody to b-actin (Sigma-Aldrich, MO, USA, A2228). Secondary antibodies used included anti-rabbit IgG-peroxidase conjugate (Sigma-Aldrich, A0545) and anti-mouse IgGperoxidase conjugate (Sigma-Aldrich, A9044). Bands were visualized using the ECL system (Amersham Pharmacia Biotech, Little Chalfont, UK).

Results & discussion

Compounds **1a** and **1b** were synthesized as previously described by our group [11]. The following modifications were carried out on **1a** (Figure 2): treatment with thiophenol (PhSH) to produce the deprotected intermediate **4** [11] that was subsequently substituted to afford **1c**; reduction of the nitro group with $SnCl_2$ to give rise to **1d**.

Compounds **2a** and **2b** were synthesized as previously described by our group [5]. The Vorbrüggen reaction was employed to give **2c** using the same conditions that we have previously reported for the synthesis of bozepinib. Following our proposed reaction mechanism [5] the presence of the purine is necessary to form compounds **2a**–**2c** (Figure 3). According to an elimination mechanism, loss of the purine ring would lead to oxocarbenium ions, which could either attack the nucleophile positions of the purine giving rise to the formation of the *N*-9″ *O,N*-acetals, or eliminate one β proton in relation to the O^+ atom, with formation of a double bond to give **2a**–**2c**. The direct elimination of methanol in an acidic medium from **1a**–**1c** did not proceed.

Compound **2d** was obtained by Pd/C catalytic reduction of the nitro group of **2a**. These reagents were also employed to reduce the double bond of **2c** and **2d** to produce **3c** and **3d**, respectively. Finally **3a** and **3b** were synthesized from **2a** and **2b** using the Wilkinson's catalyst (chlorotris[triphenylphosphine]rhodium]I]) in order to reduce the double bond without affecting the nitro group [14] (Figure 4).

The effects on cell proliferation were investigated on the human breast cancer cell line MCF-7, human colon carcinoma cell line HCT-116 and the human malignant melanoma cell line A-375. The antiproliferative activities for the target compounds (**1a**–**1d**, **2a**–**2d** and **3a**–**3d**) against the three tumor cell lines are shown in Table 1 and Supplementary Figures 1–3. We have included our previously reported data of bozepinib as reference drug [6].

In general, compounds **2** with double bond present better activity than their corresponding analogs **1** and **3** against the MCF-7 cell line (**2a > 3a > 1a**, **2b > 3b > 1b** and **2d > 3d > 1d**), with the only exception being compound **1c** that presents a similar activity to that of **2c** (Table 1). On the contrary, unsaturated compounds **2** exert antiproliferative effects similar to the acetals **1** against HCT-116 and A-375. The saturated derivatives **3a**–**3d** are the less active compounds as antiproliferative agents against the three cancer cell lines (Table 1). It should be pointed out that all the target compounds show better antiproliferative activity against the human melanoma cell line.

Finally, whereas the presence of the amino group leads to a decrease in the antiproliferative activity of the acetals **1** and the unsaturated **2** molecules, the nitro and methyl groups maintain the biological effect of both series against MCF-7, HCT-116 and A-375 cells. Nevertheless, the effect of the substituent of the aromatic ring of the sulfonamide in the biological activity of saturated compounds **3** is different in each cell line and does not allow us to determine a clear structure–activity relationship (Table 1).

Although the values of antiproliferative activities are lower than those of bozepinib, some of the results obtained are sufficiently relevant to continue the biological studies of these derivatives.

To determine whether the antiproliferative effect of these molecules involves changes in cell-cycle distribution, MCF-7, HCT-116, A-375 cells were treated with compounds **2a**–**2d** for 24 h and then analyzed by flow cytometry (Table 2). No changes in MCF-7 cell-cycle distribution were detected after treating the cells with compounds **2a**, **2c** and **2d** in comparison

Table 3. Apoptosis induction in the MCF-7, HCT-116 and A-375 cancer cell lines after treatment for 24 h with the most active compounds 2a–2d as anti-proliferative agents.

Compound	Apoptosis			
	Viable cells	Early	Late	Necrosis
MCF-7 breast cancer cell line				
Control	90.42+/–0.63	0.00	0.03+/–0.01	9.55+/–0.21
Bozepinib†	94.16+/–0.64	0.66+/–0.22	0.23+/–0.20	4.93+/–0.23
Control	86.50 ± 1.78	4.45 ± 0.21	6.35 ± 1.06	2.70 ± 0.99
2a	32.53 ± 1.13	45.17 ± 1.29	20.67 ± 2.45	1.63 ± 0.67
2b	61.24 ± 1.06	20.72 ± 1.48	9.37 ± 1.20	8.67 ± 1.63
2c	68.67 ± 2.34	14.97 ± 0.98	13.43 ± 1.25	2.93 ± 0.85
2d	76.70 ± 1.09	10.90 ± 1.56	9.95 ± 0.64	2.45 ± 0.21
HCT-116 colon cancer cell line				
Control	83.16 ± 1.44	4.40 ± 0.60	9.86 ± 0.80	2.65 ± 0.50
2a	21.20 ± 0.69	23.10 ± 1.94	47.93 ± 2.55	7.76 ± 0.66
2b	82.34 ± 2.84	7.85 ± 0.84	8.09 ± 0.98	1.72 ± 0.54
2c	67.63 ± 0.99	12.00 ± 1.47	17.40 ± 1.21	2.86 ± 0.90
2d	63.10 ± 2.10	10.03 ± 1.56	19.99 ± 1.90	6.96 ± 0.66
A-375 melanoma cancer cell line				
Control	91.90 ± 0.34	3.13 ± 0.50	3.70 ± 0.60	1.26 ± 0.21
2a	86.56 ± 0.98	7.96 ± 0.99	4.60 ± 0.61	0.86 ± 0.11
2b	90.50 ± 0.35	7.27 ± 0.42	1.33 ± 0.44	0.90 ± 0.14
2c	75.73 ± 1.11	11.56 ± 0.60	10.83 ± 1.04	1.86 ± 0.30
2d	90.10 ± 1.47	4.15 ± 0.56	4.30 ± 0.14	1.45 ± 0.21

All experiments were conducted in duplicate and gave similar results. The data are mean ± SEM of three independent determinations.
† Data taken with permission from [5].

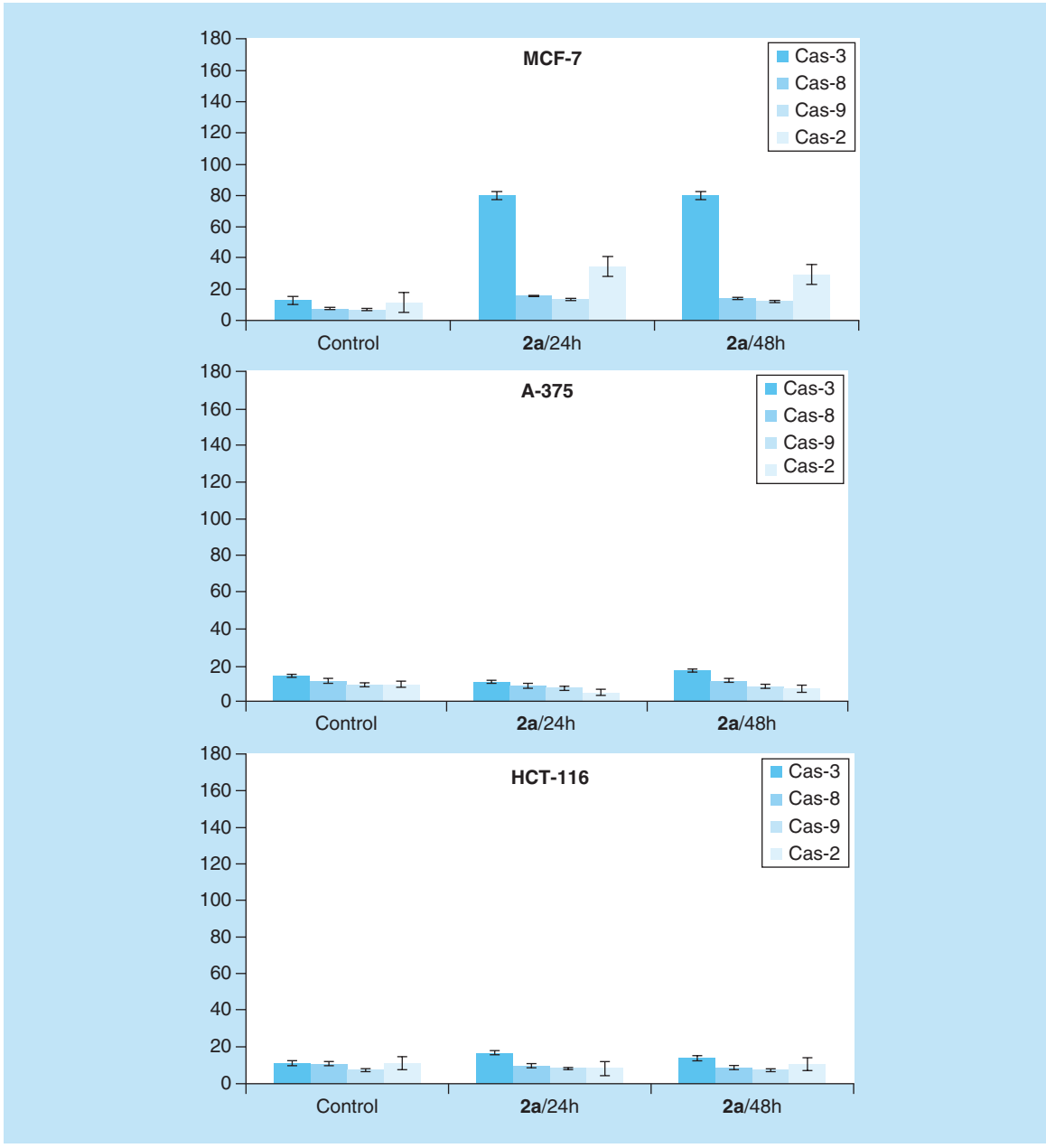

Figure 5. Apoptosis studies by activation of caspases.

with control-DMSO-treated cells similarly to bozepinib (Table 2). Analogous results were obtained in the HCT-116 cells using the same compounds. For the melanoma cell line A-375, **2a** and **2d** did not modify the cell cycle phases and only treatment with **2c** led to a cell-cycle arrest in G2/M phase (38.50 vs 12.42%) at the expense of the S-phase population (3.17 vs 20.99%).

In the case of compound **2b** it is worth pointing out that the accumulation in specific phases was detected during treatment with this drug in the MCF-7 and A-375 cell lines, contrary to what is observed against HCT-116 cells (Table 2). MCF-7 cells treated with **2b** display an important increase in the S phase population (36.97% vs 14.56%), associated with a decrease in the G2/M phase (27.14% vs 50.91%). On the contrary, accumulation in the G0/G1 and G2/M phases (70.22% vs 57.97% and 26.14% vs 12.42, respectively) was detected in the A-375 **2b**-treated cells with the consequent significant decrease in the S phase (3.64% vs 20.99%).

In order to analyze if the observed growth inhibition was due to an apoptotic or cytotoxic effect, MCF-7, HCT-116, A-375 cells were treated with compounds **2a**–**2d** and stained using Annexin V-FITC (Ann V) and propidium

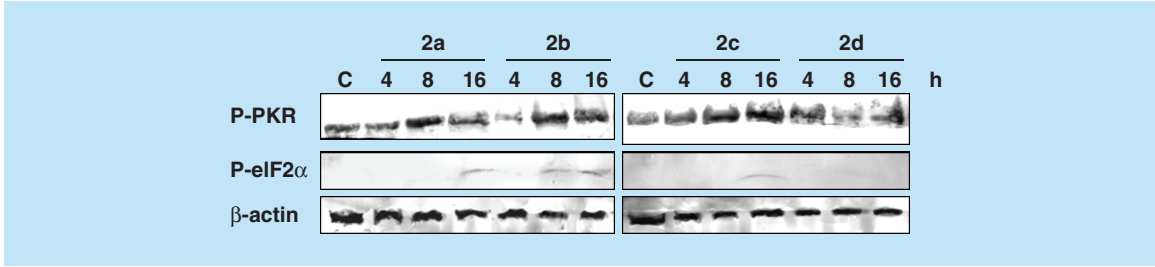

Figure 6. PKR activation. MCF-7 cells were treated with compounds **2a–d** (2x IC_{50}) during 4, 8 and 16 h. Total proteins were extracted for western blot analysis using anti-phospho PKR, antiphospho eIF2-α, and anti-ß-actin antibodies.

iodide (PI) at 24 h post-drug treatment and analyzed by flow cytometry (Table 3). Our previous studies of bozepinib showed no induction of apoptosis in MCF-7 cells after 24 h of treatment [5]. However, compounds **2a, 2c** and **2d** exhibited in general a significant increase in apoptotic cells in comparison with control DMSO-treated cells for the three lines studied with compound **2a** being the most apoptotic agent (early and late apoptosis) from 10% and 14% in MCF-7 and HCT-116 control cells to 66% and 71%, respectively (Table 3). On the contrary, compound **2b**, which induced significant changes in the cell cycle for MCF-7 and A-375 cells, did not increase the apoptotic cells. This fact could suggest the cytotoxic effect is more important that the apoptotic mechanism of action for this drug. Moreover, **2b** modified neither the cell cycle nor the apoptosis in the HCT-116 cell line due to its low antiproliferative activity. The effect of **2c** on G2/M phase at A375 melanoma cell line could be explained by a lineage-selective activity of this compound. Moreover, this phase arrest was accompanied by a high apoptosis induction. In fact, it has been demonstrated that compounds such as thiosemicarbazone derivatives induced G2/M cell cycle arrest and tumor selective apoptosis as consequence of growth inhibition in human leukemia cell lines [15]. These results provide information about the main mechanisms for the compounds and may indicate that they are lineage-selective for breast cancer and melanoma. However, further studies are necessary to determine the molecular targets responsible for this antitumor activity.

Thus, we decided to carry out studies of apoptosis by activation of caspases to determine which metabolic pathway activated these drugs. Compound **2a** not only activates the canonical intrinsic caspase-8/caspase-3 apoptotic pathway on the MCF-7 cell line but also induces slightly caspase-2 activation (Figure 5). However, this compound does not activate significantly any caspase in HCT-116 and A-375 cell lines (Figure 5). It can be explained by the ability of **2a** to activate PKR and consequently to induce the phosphorylation of the initiation translation factor eIF2-α. It has been demonstrated that a prolonged induction of eIF2α, finally triggers the apoptosis phenomena [16,17], activating both the canonical intrinsic caspase-8/caspase-3 apoptotic pathway and caspase-2 on the MCF-7 cell line.

The caspase independent apoptosis in cells exposed to different drugs with diverse cellular effects has been previously described. Indeed, potent antitumor apoptotic drugs predominantly act through the

Executive summary

Design & synthesis of the target structures

- Bozepinib is a potent and selective anticancer drug that is able to induce apoptosis in breast cancer cells.
- The chemical structure of bozepinib is made up by a benzofused seven-membered ring and a purine moiety.
- We previously demonstrated that the purine fragment does not exert an antiproliferative effect *per se*.
- In the present paper, we have carried out the synthesis of a series of 1-(benzenesulfonyl)-4,1-benzoxazepine derivatives in order to study the influence of the benzofused seven-membered ring in the biological activity of bozepinib.

Biological studies

- We have investigated the effects of the target molecules on cell proliferation, cell cycle progression and apoptosis on the human breast cancer cell line MCF-7, human colon carcinoma cell line HCT-116 and the human malignant melanoma cell line A-375.
- In addition, we carried out studies of apoptosis by activation of caspases to determine which metabolic pathway activated these drugs.
- Our results show that the methyleneoxy enamine sulfonyl function is essential in the antitumor activity of the structures and thus, it is a scaffold suitable for further modification with a view to obtain more potent antitumor compounds.

noncaspase-mediated pathway [18–22]. While caspase-2 activation could induce cell death through cytochrome c/mitochondria damage [23], noncaspase-mediated increase in phosphatidylserine externalization can occur in response to high intracellular $Ca2^{+}$ levels, which alters scramblase and translocase [24].

Therefore, to evaluate the ability of compounds **2a–2d** to induce the activation of PKR kinase and consequently the phosphorylation of eIF2-α, MCF-7 cells were treated and analyzed by western blot as shown in Figure 6. According to apoptosis induction showed in Table 3, compounds **2a**, **2b** and **2c** were able to activate PKR and induce the phosphorylation of eIF2-α; however, low levels of apoptosis were induced by compound **2d**, according to the absence of PKR activation and eIF2-α phosphorylation detected. These results suggest the importance of PKR kinase as a molecular target involved in the cell death by induction of apoptosis in response to the compounds, as has been described previously for 5-fluorouracil, bozepinib and other antitumor drugs [6,25].

Conclusion & future perspective

We have demonstrated the influence of the benzo-fused seven-membered ring in the biological activity of bozepinib, the 1-(benzenesulfonyl)-1,5-dihydro-4,1-benzoxazepine being an essential fragment in the antiproliferative effect against the breast (MCF-7) and melanoma (A-375) cancerous cell lines. Although the values of antiproliferative activities of **2a–2d** are lower than those of bozepinib, these compounds show in general better values of induction of apoptosis in MCF-7 cells. Moreover, we have identified PKR kinase as a molecular target involved in the cancer cell death by induction of apoptosis in response to the compounds. This is the first biological study of such a pioneering functionality. We are now carrying out the *de novo* synthesis of compounds **2a–2c**, and the study of the reactivity of the pioneering methyleneoxy enamine sulfonyl functionality, a π–electron rich olefin that is a useful scaffold for the preparation of complex nitrogen molecules. Efficient routes toward new molecules are always desirable, particularly if they expand the scope of the chemical methodology for the generation of molecule libraries for biological screening by means of more effective pathways to complex substitution patterns. These results encourage further studies on the mechanism of action and the therapeutic potential of these novel synthetic compounds.

Financial & competing interests disclosure

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