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Dihydropyrimidine-2-thiones as Eg5 inhibitors and L-type calcium channel blockers: potential antitumour dual agents†

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The use of multitarget drugs has evolved as an alternative to “magic bullets” for the treatment of complex diseases such as cancer, in order to affect simultaneously several targets relevant to the disease. We have designed and synthesized a series of dual agents as both Eg5 inhibitors and calcium channel blockers, bearing a 4-aryldihydropyrimidine core. Compound 2 (aryl: 3-nitrophenyl) was selected as potential dual agent due to displaying both activities: it is a vasorelaxant agent (>90% relaxation at 10⁻⁵ M in KCl-precontracted aorta rings), it decreases the response to calcium and it is cytotoxic to MCF-7 (breast), HCT-116 (colon) and A-549 (lung) cancer cell lines. The dual mechanism of action was confirmed by blocking (–)-BAY K8644-induced vascular contraction and production of monopolar spindles, typical of Eg5 inhibition. Docking suggests that both (*R*) and (*S*)-enantiomers could bind Eg5.

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Introduction

The aim of pharmacological treatment of a disease has usually been the use of a single, highly selective “magic bullet” that targets a biological macromolecule responsible for the disease. However, in the case of complex diseases, such as cancer, this strategy, although it has produced some successful drugs, has often failed due to resistance arising after an initial response to treatment. In this respect, a balanced modulation of several targets related to a multifactorial disease can provide a superior therapeutic effect and side effect profile compared to the action of a selective ligand. Furthermore, the probability of developing resistance is exponentially lower when inhibiting two or more targets than if aiming just at one. This approach, often named polypharmacology,^{1,2} has

been explored in CNS diseases,^{3–6} inflammation and pain,⁷ HIV therapy,⁸ cardiovascular disease,⁹ protozoan infections¹⁰ or antibacterial agents,¹¹ among others. In cancer, most examples aim at the inhibition of several kinases.^{12,13}

The use of a single agent instead of a cocktail of drugs (in different or in the same pill) implies some important advantages, such as the more predictable pharmacokinetic and pharmacodynamic profiles, among others. Such agents are frequently called “designed multiple ligands” or “multitarget drugs”.¹⁴ They can be conjugates (the pharmacophores are linked together by a metabolically cleavable or non-cleavable linker) or overlapped pharmacophores, which in turn range from slightly overlapped (fused) to highly integrated (merged) systems. Such merged systems are more likely to feature drug-like size and lipophilicity, appropriate for oral administration according to Lipinski's rules.¹⁵ Multitarget drugs are often low-affinity binders, with dissociation constants in the higher micromolar or even close to the millimolar range.^{16,17} Low affinity does not necessarily mean low efficiency, and it might allow for a lower prevalence of side effects.¹⁸

Kinesin spindle protein (KSP, also known as Eg5, KIF11, KNSL1 or kinesin-5) is a mitotic kinesin that contributes to the proper establishment of a bipolar mitotic spindle during mitosis, using ATP hydrolysis as the driving force for sliding cross-bound microtubules apart.^{19,20} Eg5 does not play any other role known so far, and therefore it is considered a promising target to be addressed at for generating new anti-mitotic drugs that overcome the neurotoxicity produced by antitubulin drugs (due to additional cellular functions of tubulin apart from building up the mitotic spindle during

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mitosis).^{21,22} There are several classes of Eg5 inhibitors reported so far. Among them, dihydropyrimidines, *S*-trityl-L-cysteine and its analogues, quinazolines and pyrrole/pirazole/thiadiazole derivatives are main representative chemotypes²³ (Chart 1). Some of them have reached clinical trials, being one of them (filanesib, ARRY-520) in phase III.^{24,25} Most Eg5 inhibitors bind to the allosteric L5 binding site in the motor domain, being ATP non-competitive²⁶ (including all the above mentioned). Additionally, they are remarkably selective for this kinesin.^{27,28} Dihydropyrimidine monastrol was the first reported Eg5 inhibitor.²⁹

Among the dihydropyrimidine class of Eg5 inhibitors, which can exist as two separate enantiomers, one of them usually binds preferentially to its target ((*R*) or (*S*), depending on the particular compound); however, in most cases activity assays are carried out on the racemic mixture.²³

Calcium channels have been recently identified as potential targets for cancer therapy, playing a role in all the six hallmarks of cancer: self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis.^{30–33} Abnormally elevated levels of calcium channels have been found in several cancer tissues.^{34,35} L-type calcium channel blockers (CCB) have been since long used for the treatment of hypertension. Among them, 1,4-dihydropyridines (1,4-DHP, *e.g.* nifedipine, nitrendipine, nimodipine, nicardipine) (Chart 2) are one of the largest and more studied class. Nevertheless, the calcium channel blocking activity of 1,4-dihydropyridines has not been exploited so far as anticancer.

The structural similarity between CCB 1,4-dihydropyridines and the dihydropyrimidine class of Eg5 inhibitors has prompted us to consider the combination of both structural frameworks in order to obtain potential antitumour agents that would act at both targets (Eg5 and L-type calcium channels) simultaneously. In this way, the antiproliferative effect would be reinforced by addressing two of the biological macromolecules involved in tumour development.

The target compounds have been designed as highly merged multitarget ligands (Chart 3). The common core is formed by the dihydropyridine/dihydropyrimidine ring and the aryl ring at position 4. Both of them are needed for the

R: Me, R¹: 2-NO₂, R²: Me	Nifedipine
R: Me, R¹: 3-NO₂, R²: Et	Nitrendipine
R: CH₂CH₂OMe, R¹: 3-NO₂, R²: <i>i</i> Pr	Nimodipine
R: CH₂CH₂N(Me)Bn, R¹: 3-NO₂, R²: Et	Nicardipine

Chart 2 Structure of some 1,4-dihydropyridines, L-type calcium channel blockers.

separate activities, and it has been shown that dihydropyrimidines may adopt in solid state a molecular conformation similar to that reported for 1,4-DHP.³⁶ Modifications have been addressed at the structure of the aryl system.

A few compounds with dihydropyrimidine structure have been described as displaying antihypertensive activity through blocking of Ca²⁺ channels.^{36–39} However, they have neither been designed nor evaluated simultaneously as antimitotic agents.

Results and discussion

Chemistry

The designed compounds 1–8 have been prepared by means of the Biginelli reaction.⁴⁰ It is a multicomponent reaction involving an aldehyde, a β -dicarbonyl compound and a urea. In our case, the aldehyde is chosen so that it supplies the variable aryl system required. Ethyl acetoacetate is employed as β -dicarbonyl compound, and thiourea is used in the reaction, so the resulting compounds bear a 4-aryl-3,4-dihydropyrimidine-2(1*H*)-thione structure, as in the reference compound monastrol (Table 1). The reaction is carried out under microwave irradiation for a variable time, in the presence of HCl as catalyst. Compounds are obtained as racemic mixtures.

The synthesized compounds were isolated by direct crystallization from methanol or methanol/diethyl ether of the crude mixture (although for compounds 1 and 5 previous column chromatography was necessary). The structure of all compounds was confirmed by ¹H and ¹³C NMR, IR and HRMS.

Chart 1 Structure of some Eg5 inhibitors binding at the allosteric L5 site: monastrol (dihydropyrimidine), STLC, ispinesib (quinazoline) and filanesib (thiadiazole).

Chart 3 Designed compounds as multitarget ligands.

Calcium channel blocking activity in aortic rings

The endothelium integrity was verified by relaxation to acetylcholine (ACh, 10^{-6} M) in rings precontracted by phenylephrine (PE, 10^{-6} M). The vascular endothelium was considered intact when a relaxant response above 60% was observed, whereas the endothelium-denuded ring was confirmed from absence of vasorelaxation. Fig. 1 shows that all the assayed analogues exhibited vasorelaxant activity against potassium chloride and PE, being 1, 2 and 8 the most potent compounds. Compound 2, like nifedipine, showed an $E_{\max}$ close to 100% when the rings were precontracted with potassium chloride (Table 2). These three compounds were then selected for subsequent studies. Their relaxing activity was independent of the presence of endothelium (data not shown).

After incubation with the selected compounds in calcium-free Krebs, the response to calcium was reduced, as shown in the concentration-response curves to CaCl_2 (Fig. 2). Compound 2 showed activity at concentrations 100-fold lower than compounds 1 or 8. The response to PE after incubation of rings under the same conditions was decreased significantly in presence of compound 1 ($81.8 \pm 6.4\%$, $p < 0.05$) and compound 8 ($41.8 \pm 8.3\%$, $p < 0.05$) and remain almost unchanged with compound 2 ($18.1 \pm 11.5\%$, $p > 0.05$) (Fig. 3). These results could indicate that the inhibition of the release of calcium from sarcoplasmic reticulum was not significantly modified by compound 2.

Table 1 Designed multiple ligands and general scheme of synthesis

Compound	Ar	Reaction time (min)	Yield (%)
1	3-Hydroxyphenyl	45	33 ^{a,b}
2	3-Nitrophenyl	35	42 ^b
3	4-Hydroxy-3-nitrophenyl	40	56 ^b
4	4-Hydroxy-3-methoxyphenyl	45	33 ^b
5	3-Hydroxy-4-methoxyphenyl	35	31 ^{a,b}
6	Pyridin-3-yl	40	29 ^b
7	1 <i>H</i> -Indazol-6-yl	35	27 ^b
8	2,3-Dihydro-[1,4]benzodioxin-6-yl	45	42 ^b

^a After column chromatography. ^b After crystallization.

To confirm the effect on the Ca^{2+} influx through L-type channels, (–)-Bay K8644 response was evaluated after incubation with compounds 1, 2 and 8. Our results showed that contractions induced by this calcium channel agonist were drastically reduced with a concentration of 10^{-4} M. Compound 2, the most active in blocking calcium response in calcium free-Krebs without affecting intracellular calcium release, was also assayed at 10^{-5} M (Fig. 4). This lower concentration was able to decrease significantly the contraction evoked by (–)-Bay K8644, demonstrating that the vasodilating action is caused by L-type calcium channels blockade.

Cytotoxicity

Compounds 2–8 were assayed as cytotoxic agents. Compound 1, monastrol, was also evaluated as reference. Results are shown in Table 3. Compounds displaying IC_{50} values higher than 100 μM are considered to be inactive.

Fig. 1 Vasorelaxant activity on phenylephrine (left) or potassium chloride (right)-precontracted rings. The values are expressed as mean $\pm$ SEM ($n = 5-7$). * $p < 0.05$ vs. DMSO.

Table 2 IC_{50} and E_{max} values for vasorelaxation in phenylephrine and KCl-precontracted rings

Compound	Phenylephrine		KCl	
	IC_{50} (M)	E_{max} (%)	IC_{50} (M)	E_{max} (%)
1	6.9×10^{-6}	95.2 ± 2.3	1.2×10^{-6}	89.5 ± 2.7
2	3.2×10^{-6}	84.9 ± 4.5	1.6×10^{-5}	95.4 ± 1.7
3	2.6×10^{-5}	62.4 ± 4.9	3.8×10^{-5}	57.1 ± 7.7
4	2.9×10^{-5}	78.0 ± 4.8	9.1×10^{-5}	78.3 ± 2.8
5	2.5×10^{-5}	88.4 ± 2.3	3.7×10^{-5}	77.0 ± 4.8
6	4.6×10^{-5}	80.9 ± 4.7	5.2×10^{-5}	78.4 ± 3.6
7	4.2×10^{-5}	61.2 ± 4.9	3.6×10^{-5}	48.5 ± 3.5
8	5.6×10^{-6}	83.3 ± 3.8	1.2×10^{-5}	71.7 ± 4.9
Nifedipine	1.9×10^{-8}	75.1 ± 5.8	2.9×10^{-9}	98.7 ± 0.5

Fig. 4 Concentration–response curves to (–)-Bay K 8644 in absence or presence of compounds 1, 2, 8. The values are expressed as mean $\pm$ SEM ($n = 5-7$). * $p < 0.05$ vs. DMSO. **Fig. 2** Concentration–response curves to $CaCl_2$ in calcium-free Krebs solution in absence or presence of compounds 1, 2, 8 and nifedipine (NIF). The values are expressed as mean $\pm$ SEM ($n = 5-7$). * $p < 0.05$ vs. DMSO.

3-Nitrophenyl analogue 2 shows cytotoxicity against colon (HCT-116), lung (A-549) and breast (MCF-7) tumour cell lines, being about two-fold more potent than monastrol in the two latter. The presence of an additional hydroxyl group at position 4 of the phenyl ring (compound 3) decreases potency. Addition of a methoxy group at position 4 of the phenyl ring of monastrol (compound 5) abolishes cytotoxicity. This could mean that *para*-methoxy group is too big to fit into the binding site, despite the fact that there is a hydrogen forming moiety at the same position as in the reference ligand. Swapping positions of hydroxy and methoxy substituents (compound 4) does not regain cytotoxicity. However, the binding site would accept larger moieties than monosubstituted phenyl rings, as the indazol-6-yl system (compound 7). Such system can hydrogen bond Eg5 (as donor) through its NH, in the same way as monastrol does. Nevertheless, the presence of such hydrogen bond donor is not required for cytotoxicity. For example, the benzodioxol-5-yl derivative, which cannot act as hydrogen bond donor, has proved to be more cytotoxic than monastrol in some tumour cell lines.⁴¹ In this respect, the inactivity of the benzodioxine derivative 8 might be

Fig. 3 Response to phenylephrine after incubation of rings in calcium free Krebs in the presence of compounds 1, 2 and 8. The values are expressed as mean $\pm$ SEM ($n = 5-7$). * $p < 0.05$ vs. DMSO.

caused by the increased size of the ring system (6 + 6 vs. 6 + 5), which would prevent it from fitting into the binding site.

Cytotoxic effect of nifedipine was also evaluated. Nifedipine has shown to be non-cytotoxic in three of the four tumour cell lines tested (A-549, HCT-116 and MCF-7), in which compound 2 has proved to be cytotoxic at micromolar level. This would mean that the cytotoxic effect of compound 2 in those tumour cells must be caused by an additional mechanism to calcium channel blocking, *i.e.* Eg5 inhibition (see below). However, nifedipine has displayed some cytotoxicity in the myeloma cell line MM.1S. As calcium channels are likely to play a role in tumour development, cytotoxic properties of nifedipine could be due to its ability to block such channels in MM.1S cells. Therefore, the effect of compound 2 in this cell line, although weaker than that of nifedipine, could be due to calcium channel blocking or a combination of both mechanisms.

In order to ascertain whether the displayed cytotoxicity is selective against tumour cells, compounds 2, 7, 8 and nifedipine have also been assayed against primary bone marrow mesenchymal stromal cells (MSCs) as representatives of healthy cells. The tested compounds showed no significant effect on the viability of these cells (Table 3), so cell death seems to be selectively promoted in cancer cells.

Effect on spindle bipolarity

Next, we tested whether our compounds were effectively inhibiting the Eg5 protein. One characteristic sequela of this

Table 3 Cytotoxicity IC_{50} values (μ M) for compounds 1–8 and nifedipine in myeloma (MM.1S), lung (A-549), colon (HCT-116) and breast (MCF-7) tumour cell lines, and bone marrow mesenchymal stromal cells (MSCs) (nd: not determined)

Compound	MM.1S	A-549	HCT-116	MCF-7	MSCs
1	8.7	>100	9.0	73.0	n.d
2	96.5	44.9	58.0	32.2	>100
3	>100	>100	>100	>100	n.d
4	>100	>100	>100	>100	n.d
5	>100	>100	>100	>100	n.d
6	>100	>100	>100	>100	n.d
7	63.1	>100	>100	>100	>100
8	>100	>100	>100	>100	>100
Nifedipine	57.9	>100	>100	>100	>100

inhibition is the formation of monopolar spindle mitosis instead of the typical bipolar spindle ones. For this purpose, we treated the tumor cell lines for 24 hours with 100 μM of each compound and compared the number of monopolar spindle mitosis to that observed in the same cell line without treatment. A higher percentage of mitosis with monopolar spindles were observed after treatment of cells with compounds 2, 4 and 8 when compared to untreated cell lines. Specifically, compound 2 induced a significant increase in the number of monopolar spindles (Fig. 5 and 6).

Although some monopolar-spindle mitotic figures may in fact correspond to a polar view of normal bipolar ones, proof of concept of Eg5 inhibition was obtained when rotating some confocal maximum z-projections, since only one spindle was observed (see video at supplementary Fig. S1, ESI[†]).

Binding to Eg5 L5 allosteric site: molecular modelling

Compound 2 was selected as the best candidate for dual activity as cytotoxic agent and calcium channel blocker. Therefore, it was docked into Eg5 in order to evaluate whether it actually could fit into the allosteric L5 site. The X-ray structure of monastrol-bound Eg5 (PDB ID 1Q0B) was used as the input structure. In the first place, the (*S*)-enantiomer of compound 2 was used for docking, for that is the configuration of the monastrol enantiomer that preferably binds its target.⁴² It was found that compound 2 could fit into the binding site. The top scoring pose (Fig. 7A) ($-8.4 \text{ kcal mol}^{-1}$ vs. $-7.9 \text{ kcal mol}^{-1}$ for monastrol) shows the ligand interacting with Arg211, Glu116, Ile136, Tyr211 and Leu214, all of them lining the L5 binding site. In particular, a hydrogen bond is formed between the carboxylate group of Glu116 (as acceptor) and the hydrogen bound to N3 of the dihydropyrimidine ring of the ligand. Additionally, a further interaction is established between the nitro group and physiologically protonated Arg 221 side chain.

Binding of monastrol to Eg5 implies interaction between the 3-hydroxyphenyl group of the inhibitor and Glu118 (through hydrogen bonding) and Arg119.^{42,43} Therefore, and

Fig. 5 Quantitative analysis of monopolar vs. bipolar spindle mitosis observed in the MCF-7 cell line after treatment with compounds 2 and 8 at 100 μM for 24 hours. The number of bipolar spindles in control cells was arbitrarily considered as 100 (**, $p < 0.01$ vs. control; n.s.: not significant).

Fig. 6 A. Representative micrographs of MCF-7 cells after treatment with compound 2 (100 μM) for 24 h. Bar = 10 μm . B. Detail of monopolar spindle mitosis in MCF-7 cells after treatment with compound 2. Bar = 7.5 μm .

in order to determine more accurately whether those amino-acids could be also involved in binding of compound (*S*)-2, the docking procedure was re-run allowing side chains of both residues to be flexible. In this case, the best delivered pose ($-7.9 \text{ kcal mol}^{-1}$) shows two potential hydrogen bonds, from N(3)H to Glu116 carboxylate and from Arg119 (flexible side chain) to non-carbonyl oxygen of the ester moiety, respectively, although the protein-ligand interaction through the aromatic nitro group is missing (Fig. 7B).

Unlike monastrol, other reported dihydropyrimidine-based ligands bind their target preferentially as the (*R*)-enantiomer,^{44,45} thus belonging to the so-called class II inhibitors. Therefore, the (*R*)-enantiomer of compound 2 was docked into Eg5, in order to investigate whether this enantiomer could also fit into the binding site. As for the (*S*) counterpart, docking was performed with both rigid protein and allowing flexibility for Glu118 and Arg119 side chains. In the first case, two poses displaying the same overall binding energy ($-8.2 \text{ kcal mol}^{-1}$) are produced, and in both of them a main polar interaction is seen: between the nitro group and Arg221 side chain in one of them (not shown) and a hydrogen bond between N(3)H of the dihydropyrimidine ring and backbone carbonyl oxygen of Gly117 in the other one (Fig. 8A). The nitrophenyl moiety is placed in the same pocket of the binding site as for the (*S*)-enantiomer, and the thiocarbonyl group points to the outer part of the site in both cases, but the dihydropyrimidine ring is placed perpendicularly to each other, in order to accommodate the phenyl ring attached to carbon atoms bearing opposite configurations. If Glu118 and Arg 119 side chains are allowed to be flexible in the docking of the (*R*)-enantiomer, the best pose ($-8.3 \text{ kcal mol}^{-1}$) (Fig. 8B) shows interaction between the nitro group and Arg221, very similarly to that produced by interaction with the rigid protein.

Experimental

Chemistry

Reagents were used as purchased without further purification. Chromatographic separations were performed on silica gel (Kieselgel 40, 0.040–0.063 mm, Merck) column chromatography. TLC was performed on precoated silica gel

Fig. 7 A. Compound (S)-2 docked into rigid Eg5. B. Compound (S)-2 docked into Eg5 with flexible Glu118 and Arg119 side chains.

polyester plates (0.25 mm thickness, with UV 254 fluorescent indicator, Polychrom SI F254) and stained with sulphuric acid (10% in ethanol) or phosphomolybdic acid (10% in ethanol). IR spectra were recorded on a FTIR Nicolet Impact 410 spectrophotometer (as KBr pellets), and ν values are given in cm^{-1} . ^1H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury spectrometer at 200 (^1H) / 50 (^{13}C) MHz or at 400 (^1H) / 100 (^{13}C) MHz, using CD_3OD or $\text{DMSO}-d_6$ as solvent. Chemical shifts (δ) are given in ppm from solvent residual peaks, and coupling constants (J values) are in Hertz. HRMS analyses were performed on an Applied Biosystems QSTAR XL mass spectrometer, using electrospray as ionization mode (55 V) and a TOF detector. Microwave irradiation was performed on an Anton Paar Monowave 300 monomode reactor.

General procedure for the synthesis of dihydropyrimidines 1–8. A mixture of aldehyde (3.00 mmol), ethyl acetoacetate (4.50 mmol), thiourea (3.00 mmol) and HCl 12 N (one drop) in ethanol (5.0 mL) was irradiated for a variable time at 120 °C. The crude mixture was either directly crystallized or chromatographed on silica gel prior to crystallization.

Ethyl 4-(3-hydroxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (1). After irradiation for 45 min, the crude mixture was subjected to column chromatography using CH_2Cl_2 /methanol (95:5) as eluent, obtaining 421 mg (48%) of 1 as white foam. After crystallization from methanol at room temperature pure compound was isolated (293 mg, 33%) as white crystals.

^1H NMR (CD_3OD). δ 1.06 (3 H, t, J = 7.2 Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.23 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 3.97 (2 H, q, J = 7.2 Hz, $\text{CH}_3\text{-}$

$\text{CH}_2\text{-O}$), 5.15 (1 H, s, H4), 7.02 (1 H, t, J = 7.5 Hz, H5'), 6.55–6.70 (3 H, m, H2'/H4'/H6').

^{13}C NMR (CD_3OD). δ 14.5 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.7 ($\text{CH}_3\text{-pyrimidine}$), 56.3 (C4), 61.3 ($\text{CH}_3\text{-CH}_2\text{-O}$), 103.2 (C5), 114.5 (CH, aromatic), 115.9 (CH, aromatic), 119.0 (CH, aromatic), 130.7 (CH, aromatic), 145.7 (C, aromatic), 146.0 (C, aromatic), 158.7 (C6), 167.3 (C=O), 175.9 (C=S).

IR (film). 3240 (OH, NH), 1687 (C=O), 1666 (aromatic), 1572 (C=S), 1461, 1187, 1108, 1017.

HRMS. m/z 293.0945 ($\text{M} + \text{H}$). Calculated for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_3\text{S}$: 293.0954.

Ethyl 4-(3-nitrophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (2). After irradiation for 35 min, direct crystallization of the crude product from ethanol at room temperature afforded compound 2 (407 mg, 42%), as white crystals.

^1H NMR (CD_3OD). δ 1.09 (3 H, t, J = 7.2 Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.27 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 4.00 (2 H, q, J = 7.2 Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.35 (1 H, s, H4), 7.50 (1 H, t, J = 7.9 Hz, H5'), 7.62 (1 H, dt, J_1 = 7.9 Hz, J_2 = 1.6 Hz, H6'), 8.02–8.09 (2 H, m, H2'/H4').

^{13}C NMR ($\text{DMSO}-d_6$). δ 14.0 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.3 ($\text{CH}_3\text{-pyrimidine}$), 53.5 (C4), 59.8 ($\text{CH}_3\text{-CH}_2\text{-O}$), 99.8 (C5), 121.2 (CH, aromatic), 122.8 (CH, aromatic), 130.5 (CH, aromatic), 133.1 (CH, aromatic), 145.5 (C, aromatic or C6), 146.0 (C, aromatic or C6), 147.8 (C, aromatic or C6), 164.9 (C=O), 174.5 (C=S).

IR (KBr). 3190 (NH), 1709 (C=O), 1658 (aromatic), 1589 (C=S), 1471 (NO_2), 1344 (NO_2), 1274, 1191, 1103.

HRMS. m/z 322.0871 ($\text{M} + \text{H}$). Calculated for $\text{C}_{14}\text{H}_{16}\text{N}_3\text{O}_4\text{S}$: 322.0856.

Ethyl 4-(4-hydroxy-3-nitrophenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (3). After irradiation for 40 min, direct crystallization of the crude product from methanol at room temperature yielded compound 3 (566 mg, 56%), as yellow needles.

^1H NMR (CD_3OD). δ 1.10 (3 H, t, J = 7.2 Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.26 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 4.00 (2 H, q, J = 7.2 Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.22 (1 H, s, H4), 7.04 (1 H, d, J = 9.0 Hz, H5'), 7.46 (1 H, dd, J_1 = 9.0 Hz, J_2 = 2.2 Hz, H6'), 7.88 (1 H, d, J = 2.2 Hz, H2').

^{13}C NMR ($\text{CD}_3\text{OD} + \text{DMSO}-d_6$). δ 15.6 ($\text{CH}_3\text{-CH}_2\text{-O}$), 18.8 ($\text{CH}_3\text{-pyrimidine}$), 55.8 (C4), 62.1 ($\text{CH}_3\text{-CH}_2\text{-O}$), 102.9 (C5), 122.1 (CH, aromatic), 125.2 (CH, aromatic), 136.8 (CH, aromatic), 136.9 (C, aromatic), 137.8 (C, aromatic), 147.4 (C4'), 155.1 (C6), 167.5 (C=O), 176.9 (C=S).

IR (KBr). 3412 (OH), 3162 (NH), 1653 (C=O), 1631 (aromatic), 1593 (C=S), 1475 (NO_2), 1321 (NO_2), 1191 (C-O), 1103.

HRMS. m/z 360.0632 ($\text{M} + \text{Na}$). Calculated for $\text{C}_{14}\text{H}_{15}\text{N}_3\text{O}_5\text{SNa}$: 360.0625.

Ethyl 4-(4-hydroxy-3-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (4). After irradiation for 45 min, direct crystallization of the crude product from methanol at 4 °C afforded compound 4 (295 mg, 33%), as pale yellow crystals.

Fig. 8 A. Compound (R)-2 docked into rigid Eg5. B. Compound (R)-2 docked into Eg5 with flexible Glu118 and Arg119 side chains.

^1H NMR (CD_3OD). δ 1.08 (3 H, t, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.23 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 3.72 (3 H, s, $\text{CH}_3\text{-O}$), 3.99 (2 H, q, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.13 (1 H, s, H4), 6.62 (2 H, s, aromatic), 6.74 (1 H, aromatic).

^{13}C NMR (CD_3OD). δ 14.5 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.6 ($\text{CH}_3\text{-pyrimidine}$), 56.1 (C4), 56.3 ($\text{CH}_3\text{-O}$), 61.2 ($\text{CH}_3\text{-CH}_2\text{-O}$), 103.4 (C5), 111.4 (CH, aromatic), 116.1 (CH, aromatic), 120.3 (CH, aromatic), 136.3 (C1'), 145.4 (C, aromatic or C6), 147.4 (C, aromatic or C6), 148.9 (C, aromatic or C6), 167.4 (C=O), 175.9 (C=S).

IR (KBr). 3478 (OH), 3322 (NH), 3176 (NH), 1671 (C=O), 1573 (aromatic), 1459 (C=S), 1275, 1187 (C-O), 1111.

HRMS. m/z 323.1073 ($\text{M} + \text{H}$). Calculated for $\text{C}_{15}\text{H}_{19}\text{N}_2\text{O}_4\text{S}$: 323.1060.

Ethyl 4-(3-hydroxy-4-methoxyphenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (5). After irradiation for 35 min, the crude product was chromatographed on silica gel, using hexane/ethyl acetate (4:6) as eluent, and compound 5 was isolated as yellow oil. Crystallization from methanol at room temperature afforded pure 5 (300 mg, 31%), as pale yellow crystals.

^1H NMR (CD_3OD). δ 1.07 (3 H, t, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.23 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 3.71 (3 H, s, $\text{CH}_3\text{-O}$), 3.97 (2 H, q, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.10 (1 H, s, H4), 6.58–6.68 (2 H, m, H2'/H6'), 6.74 (1 H, d, $J = 7.9$ Hz, H5').

^{13}C NMR (CD_3OD). δ 14.5 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.6 ($\text{CH}_3\text{-pyrimidine}$), 56.1 (C4), 56.4 ($\text{CH}_3\text{-O}$), 61.2 ($\text{CH}_3\text{-CH}_2\text{-O}$), 103.4 (C5), 112.5 (CH, aromatic), 114.7 (CH, aromatic), 119.1 (CH, aromatic), 137.7 (C1'), 145.4 (C, aromatic or C6), 147.6 (C, aromatic or C6), 148.8 (C, aromatic or C6), 167.4 (C=O), 175.8 (C=S).

IR (KBr). 3529 (OH), 3310 (NH), 3170 (NH), 1671 (C=O), 1575 (aromatic), 1507 (aromatic), 1457 (C=S), 1275, 1192, 1111, 1027 (C-O).

HRMS. m/z 323.1067 ($\text{M} + \text{H}$). Calculated for $\text{C}_{15}\text{H}_{19}\text{N}_2\text{O}_4\text{S}$: 323.1060.

Ethyl 4-(3-pyridyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (6). After irradiation for 40 min, direct crystallization of the crude product from methanol/diethyl ether at 4 °C yielded compound 6 (229 mg, 29%), as orange crystals.

^1H NMR (CD_3OD). δ 1.07 (3 H, t, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.26 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 4.00 (2 H, q, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.28 (1 H, s, H4), 7.35 (1 H, dd, $J_1 = 7.9$ Hz, $J_2 = 4.3$ Hz, H5'), 7.69 (1 H, d, $J = 7.9$ Hz, H6'), 8.16–8.24 (2 H, m, H2'/H4').

^{13}C NMR ($\text{CD}_3\text{OD} + \text{DMSO-}d_6$). δ 14.7 ($\text{CH}_3\text{-CH}_2\text{-O}$), 18.0 ($\text{CH}_3\text{-pyrimidine}$), 54.1 (C4), 61.4 ($\text{CH}_3\text{-CH}_2\text{-O}$), 101.9 (C5), 125.8 (C5'), 137.0 (C6'), 147.0 (C2' or C4'), 148.7 (C4' or C2'), 141.3 (C1' or C6), 147.0 (C6 or C1'), 166.7 (C=O), 176.6 (C=S).

IR (KBr). 3249 (NH), 1712 (C=O), 1651 (aromatic), 1566 (aromatic), 1473 (C=S), 1310, 1192 (C-O), 1093.

HRMS. m/z 278.0975 ($\text{M} + \text{H}$). Calculated for $\text{C}_{13}\text{H}_{16}\text{N}_3\text{O}_2\text{S}$: 278.0958.

Ethyl 4-(1H-indazole-6-yl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (7). After irradiation for 35 min, crystallization of the crude mixture from methanol at 4 °C afforded pure 7 (256 mg, 27%), as pale yellow crystals.

^1H NMR (CD_3OD). δ 1.06 (3 H, t, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.26 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 3.98 (2 H, q, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 5.36 (1 H, s, H4), 7.04 (1 H, dd, $J_1 = 8.3$ Hz, $J_2 = 1.4$ Hz, H6'), 7.33 (1 H, s, H2'), 7.63 (1 H, d, $J = 8.3$ Hz, H5'), 7.90 (1 H, s, H7').

^{13}C NMR (CD_3OD). δ 14.4 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.7 ($\text{CH}_3\text{-pyrimidine}$), 56.7 (C4), 61.3 ($\text{CH}_3\text{-CH}_2\text{-O}$), 103.0 (C5), 109.1 (CH, aromatic), 121.1 (CH, aromatic), 122.3 (CH, aromatic), 123.8 (C, aromatic), 134.5 (CH, aromatic), 134.6 (C, aromatic or C6), 143.6 (C, aromatic or C6), 145.9 (C, aromatic or C6), 167.3 (C=O), 176.2 (C=S).

IR (KBr). 3178 (NH), 1703 (C=O), 1647 (indazole), 1583 (aromatic), 1473 (C=S), 1315, 1191, 1098, 943.

HRMS. m/z 317.1084 ($\text{M} + \text{H}$). Calculated for $\text{C}_{15}\text{H}_{17}\text{N}_4\text{O}_2\text{S}$: 317.1067.

Ethyl 4-(2,3-dihydro[1,4]benzodioxan-6-yl)-6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (8). After irradiation for 45 min, direct crystallization of the crude product from methanol at 4 °C afforded pure 8 (426 mg, 42%), as orange crystals.

^1H NMR (CD_3OD). δ 1.08 (3 H, t, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 2.23 (3 H, s, $\text{CH}_3\text{-pyrimidine}$), 3.98 (2 H, q, $J = 7.2$ Hz, $\text{CH}_3\text{-CH}_2\text{-O}$), 4.09 (4 H, s, H7'/H8'), 5.08 (1 H, s, H4), 6.63 (3 H, s, H2'/H5'/H6').

^{13}C NMR (CD_3OD). δ 14.4 ($\text{CH}_3\text{-CH}_2\text{-O}$), 17.6 ($\text{CH}_3\text{-pyrimidine}$), 55.7 (C4), 61.2 ($\text{CH}_3\text{-CH}_2\text{-O}$), 65.5 ($\times 2$, $\text{O-CH}_2\text{-CH}_2\text{-O}$), 103.2 (C5), 116.5 (CH, aromatic), 118.2 (CH, aromatic), 120.6 (CH, aromatic), 137.9 (C1'), 144.8 ($\times 2$, C3'/C4'), 145.6 (C6), 167.3 (C=O), 176.1 (C=S).

IR (KBr). 3305 (NH), 3173 (NH), 1666 (C=O), 1575 (aromatic), 1505 (aromatic), 1461 (C=S), 1282 (thiourea), 1191, 1126 (C=O), 1113 (C=O), 1067 (C-O).

HRMS. m/z 335.1057 ($\text{M} + \text{H}$). Calculated for $\text{C}_{16}\text{H}_{19}\text{N}_2\text{O}_4\text{S}$: 335.1060.

Vascular reactivity assays

Male Wistar rats (200–250 g) were obtained from the Animal Experimentation Service of Salamanca University. All animal procedures were approved by the Bioethics Committee of Salamanca University and performed in accordance with the European Union guidelines and Spanish legislation for the care and use of laboratory animals (2010/63/UE; RD 53/2013). Pharmacological studies were carried out using descending thoracic aortic rings. Animals were exsanguinated and the thoracic aorta was immediately dissected and placed in chilled Krebs solution (composition in mM: NaCl, 118, KCl, 4.7, NaHCO_3 , 25, MgSO_4 , 1.2, CaCl_2 , 2.5, KH_2PO_4 , 1.2 and glucose, 11). The pH of the solution after saturation with carbogen (95% O_2 and 5% CO_2) was 7.4. After cleaning the connective tissue and fat, the aorta was cut into rings (3 mm in length), mounted under the basal tension of 2 g in organ baths containing Krebs solution and attached to force-displacement transducers to measure isometric contractile force. The tissue bath was maintained at 37 °C and bubbled with carbogen. Each preparation was allowed to equilibrate at least 60 min prior to initiation of experimental procedures. Some experiments were conducted in endothelium-denuded

rings by mechanically removing the endothelial layer by gentle rubbing around the internal vascular surface.

All compounds were dissolved in dimethyl sulfoxide (DMSO), and control assays were carried out in presence of this solvent. Nifedipine was used as positive control. The following experimental protocols were performed:

Relaxant effect on precontracted aorta. Phenylephrine (PE, 1 μM) or KCl (30 mM) were used to induce steady contraction in endothelium-intact aortic rings, then every compound (10^{-6} – 10^{-4} M) was added cumulatively at 15 min intervals. The relaxant effect was expressed as the percentage inhibition of tension developed by the contracturant agent.

Effect of compounds on response to CaCl_2 in calcium-free Krebs. After 1 hour of incubation in this solution cumulative concentration–response curves to CaCl_2 (0.5–5 mM) were constructed in presence of the assayed compounds.

Effect on sarcoplasmic reticulum calcium release. Response to phenylephrine (10^{-6} M) alone or in presence of DMSO or the assayed compounds were studied after 1 h incubation of rings in calcium-free Krebs.

Role of L-type calcium channels on compounds-induced relaxation. To establish whether the inhibition of the L-type calcium channel could contribute to the vasorelaxant effect, contractions induced by (S)-(–)-Bay K8644 (Tocris) at 10^{-8} to 3×10^{-6} M were examined after 20 min incubation with the compounds or DMSO. Responses to (S)-(–)-Bay K8644 were expressed as percentage of the maximal response of the agonist in control DMSO curves.

Results were expressed as the mean $\pm$ the standard error of the mean (SEM). Concentration–response curves were analyzed by a sigmoidal curve-fitting analysis to calculate IC_{50} values (the drug concentration exhibiting 50% of the maximum relaxation effect) and E_{max} values (the maximal effect). One or two ways analysis of variance were used to compare the effect of each compound with the control. $p < 0.05$ was considered statistically significant. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for fitting and statistical analysis.

Molecular modeling

Monastrol bound KSP (PDB ID 1Q0B) was downloaded from Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). Monastrol was virtually removed and the ligand-free protein was used for docking. The assayed compounds were energy minimized and both ligands and protein (either rigid or with flexible Glu118 and Arg119 side chains) were converted into the appropriate files by using Autodock Tools. Docking studies were performed with Autodock Vina.⁴⁶ A grid of $22 \times 22 \times 22$ Å was generated at the center of the reference ligand binding site and compounds were docked. Docked structures were analyzed with Pymol.⁴⁷

Cytotoxicity assays

Tumour cell lines (MM.1S, A-549, HCT-116 and MCF-7) were purchased from ATCC (LGC Standards S.L.U., Barcelona,

Spain) or DSMZ (Braunschweig, Germany) repositories. Bone marrow samples from subjects undergoing traumatology surgery not related to tumour disease were obtained after signed informed consent from individuals, and used for mesenchymal stromal cell (MSC) isolation and expansion. Briefly, samples were subjected to Ficoll-Paque centrifugation and MSCs were selected by adherence to plasticware and cultured until passage 2; at this time point, hMSCs were checked for appropriate immunophenotypic and differentiation criteria.

Cytotoxicity after treatment with the different compounds was assessed by the MTT assay. Briefly, cells were seeded in 48-well plates (at 5×10^4 cells per well for the MCF-7 and HCT-116 cell lines, at 75×10^4 cells per well for the A549 cell line, and at 25×10^4 cells per well in the case of MM.1S cells), in the presence of different concentrations of each compound (from 10 nM to 100 μM) for 72 hours. A MTT tetrazolium salt solution (Sigma Aldrich, 0.5 mg mL^{-1} final concentration) was added in the last 4 hours of culture. Insoluble formazan products, only produced by metabolically active cells, were solubilized with a 10% SDS and 0.01 M HCl solution and overnight gentle agitation; the relative reduction in cell viability was calculated after absorbance measurement at λ 570 nm (λ 630 nm for reference). Experiments for each compound were carried out three times in quadruplicates. IC_{50} (concentration required for a 50% reduction of cell viability) was calculated with SigmaPlot software. When checking cytotoxicity on bone marrow mesenchymal stromal cells (MSCs) from healthy donors, cells were seeded in 96-well plates at 1×10^4 cells per well, and similarly treated with specific compounds, checking viability after 72 hours of treatment.

Immunofluorescence studies. Tumour cell lines were grown on 1% poly-L-lysine-coated coverslips for 24 hours, and then left untreated (control) or treated with the specified compounds at 100 μM for another 24 hours. Cells were then fixed in cold methanol, blocked and incubated overnight with primary mouse antibody against α -tubulin (Calbiochem), followed by an Alexa Fluor 488-conjugated anti-mouse IgG secondary antibody (Life Technologies); DNA was visualized with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Sigma). Images were captured with a confocal microscope (Leica TCS SP5) using a $\times 63$ oil immersion objective, after excitation at 488 nm for Alexa Fluor 488 and at 405 nm for DAPI. Images are presented as maximum intensity 3D projections from Z-stacks of about 9 μm taken with a step-size of 0.2 μm .

Conclusions

The reported compounds have been designed as dual agents, both Eg5 inhibitors and L-type calcium channel blockers, using a common core formed by an aromatic system bound at position 4 of a dihydropyrimidine ring. Among them, compounds 1 (monastrol), 2 and 8 have shown the most potent vascular relaxant effect, especially marked for 2 when the tissue is precontracted with KCl. Additionally, response to Ca^{2+}

was inhibited most potently by compound 2, although it did not modify Ca^{2+} liberation from sarcoplasmic reticulum. Compound 2 also displayed cytotoxic effect on colon, lung and breast cell lines, without affecting non-tumour cells. The dual mechanism of action of the designed compounds has been proved by blocking of (-)-BAY K8644-induced vascular contraction, a well-known calcium channel agonist, and the generation of monopolar spindles characteristic of Eg5 inhibitors. Docking of compound 2 into Eg5 shows that it could fit into the allosteric L5 binding site (as related dihydropyrimidines), both as its (*S*)- and (*R*)-enantiomers.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

- 1 A. Anighoro, J. Bajorath and G. Rastelli, *J. Med. Chem.*, 2014, 57, 7874–7887.
- 2 J. U. Peters, *J. Med. Chem.*, 2013, 56, 8955–8971.
- 3 K. Nikolic, S. Filipic, D. Agbaba and H. Stark, *CNS Neurosci. Ther.*, 2014, 20, 613–623.
- 4 F. Prati, E. Uliassib and M. L. Bolognesi, *Med. Chem. Commun.*, 2014, 5, 853–861.
- 5 R. León, A. G. Garcia and J. Marco-Contelles, *Med. Res. Rev.*, 2013, 33, 139–189.
- 6 K. S. T. Dias and C. Viegas, *Curr. Neuropharmacol.*, 2014, 12, 239–255.
- 7 S. H. Hwang, A. T. Weeksler, K. Wagner and B. D. Hammock, *Curr. Med. Chem.*, 2013, 20, 1783–1799.
- 8 P. Zhan and X. Liu, *Curr. Med. Chem.*, 2013, 20, 1743–1758.
- 9 A. Bisi, S. Gobbi, F. Belluti and A. Rampa, *Curr. Med. Chem.*, 2013, 20, 1759–1782.
- 10 P. M. Njogu and K. Chibale, *Curr. Med. Chem.*, 2013, 20, 1715–1742.
- 11 S. P. East and L. L. Silver, *Expert Opin. Drug Discovery*, 2013, 8, 143–156.
- 12 D. Melisi, G. Piro, A. Tamburrino, C. Carbone and G. Tortora, *Curr. Opin. Pharmacol.*, 2013, 13, 536–542.
- 13 A. Petrelli, in *Polypharmacology in drug discovery*, ed. J. U. Peters, Wiley, Hoboken (NJ), 2012, pp. 149–165.
- 14 R. Morphy, C. Kay and Z. Rankovic, *Drug Discovery Today*, 2004, 9, 641–651.
- 15 C. A. Lipinski, F. Lombardo, B. W. Dominy and P. J. Feeney, *Adv. Drug Delivery Rev.*, 1997, 23, 3–25.
- 16 M. A. Rogawski, *Amino Acids*, 2000, 19, 133–149.
- 17 J. Mestres and E. Gregori-Puigjané, *Trends Pharmacol. Sci.*, 2009, 30, 470–474.
- 18 T. Korcsmáros, M. S. Szalay, C. Böde, I. A. Kovács and P. Csermely, *Expert Opin. Drug Discovery*, 2007, 2, 1–10.
- 19 K. E. Sawin, K. LeGuellec, M. Philippe and T. J. Mitchison, *Nature*, 1992, 359, 540–543.
- 20 A. Blangy, H. A. Lane, P. d'Herin, M. Harper, M. Kress and E. A. Nigg, *Cell*, 1995, 63, 1159–1169.
- 21 J. J. Lee and S. M. Swain, *J. Clin. Oncol.*, 2006, 24, 1633–1642.
- 22 A. A. Argyriou, J. Bruna, P. Marmioli and G. Cavaletti, *Crit. Rev. Oncol. Hematol.*, 2012, 82, 51–77.
- 23 C. Pérez-Melero, *Curr. Top. Med. Chem.*, 2014, 14, 2286–2311.
- 24 Clinicaltrials.gov, URL: <https://clinicaltrials.gov> (accessed 15/02/2019).
- 25 EU Clinical Trials Register, URL: <https://www.clinicaltrialsregister.eu> (accessed 15/02/2019).
- 26 L. Luo, J. D. Carson, D. Dhanak, J. R. Jackson, P. S. Huang, Y. Lee, R. Sakowicz and R. A. Copeland, *Biochemistry*, 2004, 43, 15258–15266.
- 27 S. DeBonis, J. P. Simorre, I. Crevel, L. Lebeau, D. A. Skoufias, A. Blangy, C. Ebel, P. Gans, R. Cross, D. D. Hackney, R. H. Wade and F. Kozielski, *Biochemistry*, 2003, 42, 338–349.
- 28 J. Turner, R. Anderson, J. Guo, C. Beraud, R. Fletterick and R. Sakowicz, *J. Biol. Chem.*, 2001, 276, 25496–25502.
- 29 T. U. Mayer, T. M. Kapoor, S. J. Haggarty, R. W. King, S. L. Schreiber and T. J. Mitchison, *Science*, 1999, 286, 971–974.
- 30 M. Li and Z. G. Xiong, *Int. J. Physiol., Pathophysiol. Pharmacol.*, 2011, 3, 156–166.
- 31 N. Prevarskaya, R. Skryma and Y. Shuba, *Trends Mol. Med.*, 2010, 16, 107–121.
- 32 N. Prevarskaya, R. Skryma and Y. Shuba, *Nat. Rev. Cancer*, 2011, 11, 609–618.
- 33 J. M. Lee, F. M. Davis, S. J. Roberts-Thomson and G. R. Monteith, *Am. J. Physiol.*, 2011, 301, C969–C976.
- 34 X. T. Wang, Y. Nagaba, H. S. Cross, F. Wrba, L. Zhang and S. E. Guggino, *Am. J. Pathol.*, 2000, 157, 1549–1562.
- 35 M. Oguro-Okano, G. E. Griesmann, E. D. Wieben, S. J. Slaymaker, T. P. Snutch and V. A. Lennon, *Mayo Clin. Proc.*, 1992, 67, 1150–1159.
- 36 G. C. Rovnyak, S. D. Kimball, B. Beyer, G. Cucinotta, J. D. DiMarco, J. Z. Gougoutas, A. Hedberg, M. Malley, J. P. McCarthy, R. Zhang and S. Moreland, *J. Med. Chem.*, 1995, 38, 119–129.
- 37 G. C. Rovnyak, K. S. Atwal, A. Hedberg, S. D. Kimball, S. Moreland, J. Z. Gougoutas, B. C. O'Reilly, J. Schwartz and M. F. Malley, *J. Med. Chem.*, 1992, 35, 3254–3263.
- 38 H. Cernecka, L. Veizerova, L. Mensikova, J. Svetlik and P. Krennek, *J. Pharm. Pharmacol.*, 2012, 64, 735–741.
- 39 P. B. Choudhari, M. S. Bhatia and S. D. Jadhav, *J. Korean Chem. Soc.*, 2013, 57, 99–103.
- 40 C. O. Kappe, *Tetrahedron*, 1993, 49, 6937–6963.
- 41 D. Russowsky, R. F. S. Canto, S. A. A. Sanches, M. G. M. D'Oca, A. Fatima, R. A. Pilli, L. K. Kohn, M. A. Antonio and J. E. Carvalho, *Bioorg. Chem.*, 2006, 34, 173–182.
- 42 Y. Yan, V. Sardana, B. Xu, C. Homnick, W. Halczenko, C. A. Buser, M. Schaber, G. D. Hartman, H. E. Huber and L. C. Kuo, *J. Mol. Biol.*, 2004, 335, 547–554.

- 43 Z. Maliga and T. J. Mitchison, *BMC Chem. Biol.*, 2006, **6**, 2–10.
- 44 H. Y. K. Kaan, V. Ulaganathan, O. Rath, H. Prokopcova, D. Dallinger, C. O. Kappe and F. Kozielski, *J. Med. Chem.*, 2010, **53**, 5676–5683.
- 45 I. Garcia-Saez, S. DeBonis, R. Lopez, F. Trucco, B. Rousseau, P. Thuery and F. Kozielski, *J. Biol. Chem.*, 2007, **282**, 9740–9747.
- 46 O. Trott and A. J. Olson, *J. Comput. Chem.*, 2010, **31**, 455–461.
- 47 The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.