

Design, Synthesis, and Evaluation of Biological Activity of Ferrocene-Ispinesib Hybrids: Impact of a Ferrocenyl Group on the Antiproliferative and Kinesin Spindle Protein Inhibitory Activity

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With the aim to combine more than one biologically-active component in a single molecule, derivatives of ispinesib and its (S) analogue were prepared that featured ferrocenyl moieties or bulky organic substituents. Inspired by the strong kinesin spindle protein (KSP) inhibitory activity of ispinesib, the compounds were investigated for their antiproliferative activity. Among these compounds, several derivatives demonstrated significantly higher antiproliferative activity than ispinesib with nanomolar IC₅₀ values against cell lines. Further evaluation indicated that the antiproliferative activity is not directly

correlated with their KSP inhibitory activity while docking suggested that several of the derivatives may bind in a manner similar to ispinesib. In order to investigate the mode of action further, cell cycle analysis and reactive oxygen species formation were investigated. The improved antiproliferative activity of the most active compounds may be assigned to synergic effects of various factors such as KSP inhibitory activity due to the ispinesib core and ability to generate ROS and induce mitotic arrest.

Introduction

Antimitotic agents are widely used for cancer chemotherapy.^[1] The most important of them are taxanes and *Vinca* alkaloids, disrupting the normal dynamics of microtubules – structures playing a crucial role in mitosis – and consequently leading to cell death.^[2] Due to the critical role of microtubules in many processes, also in non-dividing cells, the treatment with microtubule poisons is associated with many undesirable side effects. Therefore, compounds capable of affecting cancer cell division that will specifically inhibit the activity of other key players in mitosis are highly sought after.^[3] Among various mitotic proteins, the motor proteins – kinesins – have attracted particular interest, with kinesin spindle protein (KSP, also known as Eg5 and KIF11) being a particularly promising target.^[4]

KSP plays a crucial role in forming the bipolar spindle in dividing cells and is virtually absent in non-dividing cells. KSP is required for transition from prophase to prometaphase and, therefore, its inhibition disrupts mitosis leading to mitotic arrest and apoptosis.^[4a] The discovery of the first low-molecular-weight KSP inhibitors, such as S-trityl-L-cysteine^[5] and monastrol,^[6] initiated an extensive search for other Eg5 inhibitors.^[7] KSP modulators with a variety of structures, for example, ispinesib, SB-743921,^[8] MK-0731,^[9] ARY-520,^[10] K858^[11] and others, have been reported.^[12] Ispinesib is a small-molecule inhibitor of KSP ATPase activity, exhibiting high selectivity for KSP over other kinesins.^[13] Ispinesib has been extensively studied in 16 phase I/II clinical trials, both as monotherapy and in combination therapies.^[12a] Although none of the kinesin inhibitors has been approved for clinical use yet, searching for new, selective and effective antimitotic compounds is an important area of research.

One method of developing new drug candidates involves the structural modification of known active compounds which can improve or alter their biological activities. The incorporation of an organometallic scaffold to tune the bioactivity of known drugs is often more beneficial than use of purely organic substituents.^[14] The introduction of an organometallic motif into an organic molecule allows, for example, to utilize unusual geometries and/or reactivity of the metal center. An example of this approach involves coupling a biologically active molecule to an organometallic group, particularly a sandwich^[14c,i,15] or a half-sandwich complex.^[14f,h,16] Previous studies have shown that introducing a ferrocenyl substituent into an antimitotic molecule increases the antitumor activity of the obtained conjugates.^[14e,17] Moreover, in the case of ferrocenyl analogues of paclitaxel, a significantly increased ability to induce tubulin

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polymerization was observed compared to the parent compound.^[18] The presence of an organometallic group in podophyllotoxin aminoconjugates was responsible for revealing dual inhibitory properties affecting both tubulin polymerization and topoisomerase II activity.^[19] Furthermore, replacing a phenyl group in cytotoxic plinabulin with a ferrocenyl moiety led to compounds capable of circumventing multidrug resistance caused by overexpression of ABCB1 and ABCG2 proteins.^[20] We have shown that conjugating a ferrocenyl substituent to monastrol resulted in higher antiproliferative and KSP inhibitory activity.^[21] We also found that half-sandwich complexes of Ru, Rh, Ir and Os featuring a 2-(1-amino-2-methylpropyl)-3-benzyl-7-chloroquinazolin-4(3H)-one ligand showed high cytotoxicity and the ability to inhibit KSP activity.^[22]

These results encouraged us to investigate the effect of a ferrocenyl organometallic group on the antiproliferative activity of ispinesib and its (*S*)-enantiomer in comparison to related organic derivatives (Figure 1). We report structure-activity relationship (SAR) studies based on antiproliferative activity, impact on cell cycle, induction of reactive-oxygen species formation, molecular docking and KSP inhibitory activity.

Results and Discussion

Three types of ispinesib analogues, i.e., (*S*)- and (*R*)-**2a–f**, **3a–e** and **4a–f** (Figure 1) were synthesized starting from enantiomerically pure (*S*)- and (*R*)-**5**^[23] or (*S*)- and (*R*)-**1**.^[23] The precursors *o*-, *m*- and *p*-ferrocenylbenzaldehyde (**6b–d**) were obtained by oxidation of the readily available benzyl alcohols with MnO₂,^[18a] while the 1-adamantanecarboxaldehyde **6f** was prepared by oxidation of 1-adamantylmethanol with pyridine chlorochromate.^[24] The type I compounds (*S*)- and (*R*)-**2a–f** were synthesized in a reaction of amines (*S*)- or (*R*)-**5** with the corresponding aldehydes **6a–f** and sodium triacetoxyborohydride in 1,2-dichloroethane. We found that the reductive amination conducted at room temperature for 24 h (Procedure A) or under microwave conditions with two cycles at 120 °C for 15 min (Procedure B) led to the desired products with comparable yields (Table S1). Unfortunately, partial racemization occurred (see Figure S1 for an example of chiral HPLC analysis) when conducting the reaction in the microwave. Therefore, all compounds of type I were synthesized according to procedure A, which prevented racemization (Figure S1).

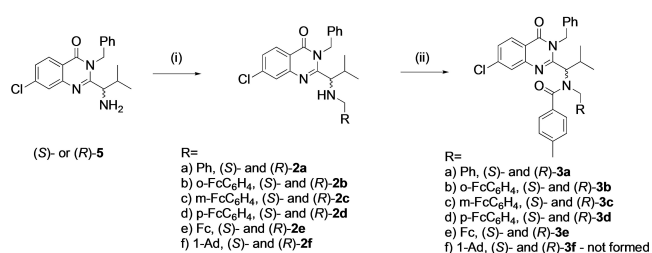
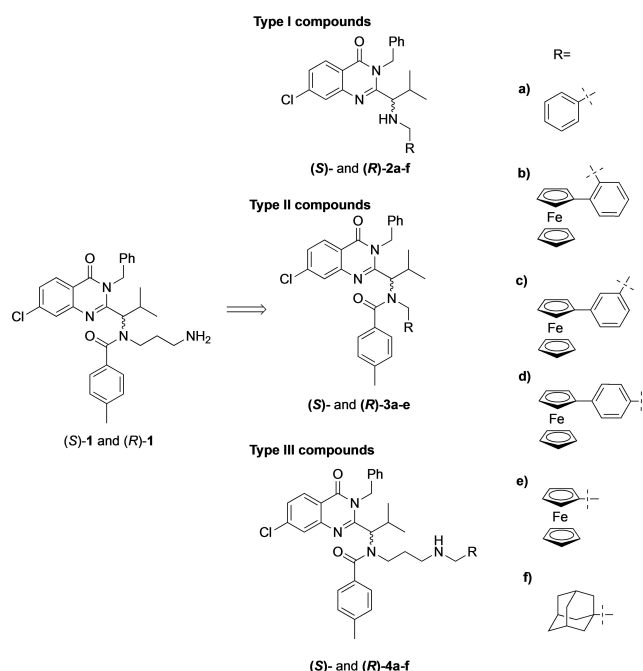


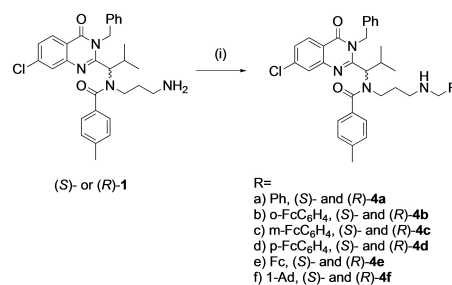
Figure 1. Three types of ispinesib analogues (**2a–f**, **3a–e** and **4a–f**) were designed to investigate the impact of different substituents and chirality on the biological activity.

Compounds **2a–f** were *N*-acylated in a reaction with *p*-toluoyl chloride in dichloromethane and DIPEA and afforded the compounds of type II, i.e., (*S*)- and (*R*)-**3a–e** in 20–99 % yield (Scheme 1). However, the adamantyl analogues (*S*)- and (*R*)-**3f** were not formed, presumably due to the bulkiness of the adamantyl moiety.

The type III compounds (*S*)- and (*R*)-**4a–f** were prepared in two steps from enantiomerically pure ispinesib (*R*)-**1** and its (*S*)-enantiomer (*S*)-**1**^[23] by reductive amination with aldehydes **6a–f** in 1,2-dichloroethane (Condition B) at 120 °C in the microwave for 15 min. This reaction was followed by treatment with sodium triacetoxyborohydride at 120 °C for another 15 min in the microwave (Scheme 2). The obtained products were fully characterized by 1D and 2D NMR, MS and elemental and HPLC-MS analysis and no racemization was observed.



Scheme 1. Synthesis of ispinesib analogues type I (**2a–f**) and type II (**3a–e**). Reagents and conditions: (i) 1.12 equiv RCHO (**6a–f**), 1.44 equiv NaBH(OAc)₃, 1,2-dichloroethane, RT, 24 h; (ii) 1.40 equiv *p*-toluoyl chloride, 1.2 equiv DIPEA, DCM, RT, 24 h.



Scheme 2. Synthesis of ispinesib analogues type III (**4a–f**). Reagents and conditions: (i) 1.12 equiv RCHO (**6a–f**), 1.44 equiv NaBH(OAc)₃, 1,2-dichloroethane, MW, 120 °C, 15 min;

Cytotoxic activity

Using a two-step approach described previously,^[19] we evaluated the antiproliferative activity of both the (*R*)- and (*S*)-enantiomers at a fixed concentration, i.e., equal to the IC₅₀ value for ispinesib (*R*)-1 and (*S*)-1 for the respective cell lines (Figure 2).

To focus on a general pattern of activity for the investigated compounds and to avoid bias resulting from using a single cell line of specific background and often unusual biology, we decided to employ a panel of cell lines originating from different human tumors: lung carcinoma – A549, hepatocellular carcinoma – Hep G2, breast carcinoma – MCF7, and colorectal carcinoma cell lines of different staging: HCT116 (Dukes' A), SW620 (Dukes' C) and Colo 205 (Dukes' D).

Of the (*R*)-enantiomers of the type I compounds, only (*R*)-2c containing a *m*-ferrocenylbenzyl group demonstrated in the case of A549 and Colo 205 cells the same survival rate, a higher survival rate of about 44% in case of Hep G2 cells, or a significantly higher survival rate in the range of 18–30% for HCT116, MCF7 and SW620 cell lines compared to ispinesib. Further *N*-acylation of type I compounds (*R*)-2a–e led to the formation of type II molecules (*R*)-3a–e and they either showed reduced or slightly increased activity. Notably, only (*R*)-3a exhibited a significantly higher cytotoxicity compared to the analogous amine (*R*)-2a, whereas the transformation in the case of (*R*)-3c practically abolished its antiproliferative activity. It should be noted that all type II compounds bearing *o*-, *m*- or *p*-ferrocenylbenzyl substituents only exhibited activity comparable to that of ispinesib with survival rates in the range of 48.2–49.7% against SW620 cells. Further modification of the terminal amino group of ispinesib (*R*)-1 (Type III compounds (*R*)-4a–f) resulted in substantially increased cytotoxicity in all cases, with kill rates exceeding 99%.

The (*S*)-enantiomers were significantly less bioactive than their (*R*) counterparts, with a few exceptions such as (*S*)-3b and (*S*)-4e in some cell lines (Figure 2). Some of the type III (*S*)-enantiomers demonstrated significantly higher activity than (*S*)-1, with (*S*)-4a, (*S*)-4e, and (*S*)-4f being the most active.

Based on the screening phase data, we selected the most active compounds, i.e., (*R*)-2c, (*R*)-3c, (*R*)-4a–4f, (*S*)-4a, (*S*)-4e, and (*S*)-4f for more detailed studies in which we determined their IC₅₀ values towards a set of colon cancer cell lines, i.e., Colo 205, HCT116 and SW620, extended with a multidrug resistant (MDR), ABCB1-overexpressing variant of the latter – SW620E^[25] (Table 1). The overall resistance of the cell lines towards the investigated ispinesib analogues followed the pattern Colo 205 < SW620 < HCT116 ≪ SW620E. Except for (*R*)-3c, all compounds exhibited higher antiproliferative activity than the corresponding ispinesib enantiomer toward non-MDR cancer cell lines. The response of the cells to the modifications of the parent compound was diverse. For example, the replacement of the 3-aminopropyl and *p*-tolyl substituents in ispinesib (*R*)-1 with a *m*-ferrocenylbenzyl moiety in (*R*)-2c resulted in a 4.4-fold increased cytotoxicity (IC₅₀ = 177 nM) towards HCT116 cells compared to (*R*)-1 (IC₅₀ = 848 nM) while the modification did not affect its activity in the other cell lines. However, *N*-acylation of (*R*)-2c, which led to (*R*)-3c, resulted in loss of activity towards Colo 205 and HCT116 cells while the compound remained cytotoxic towards SW620 cells (IC₅₀ = 162 nM). All type III compounds derived from (*R*)-1 were more active than the parent compound with the *p*-ferrocenylbenzyl analogue (*R*)-4d being the most potent derivative followed by *m*-ferrocenylbenzyl analogue (*R*)-4c. The antiproliferative potency of the *o*-ferrocenylbenzyl ((*R*)-4b), ferrocenyl ((*R*)-4e) and benzyl ((*R*)-4a) derivatives was similar. The introduction of an adamantylmethyl moiety as a bulky organic group in (*R*)-4f increased the cytotoxic activity compared to ispinesib, however, the effect was less pronounced than for the ferrocenyl

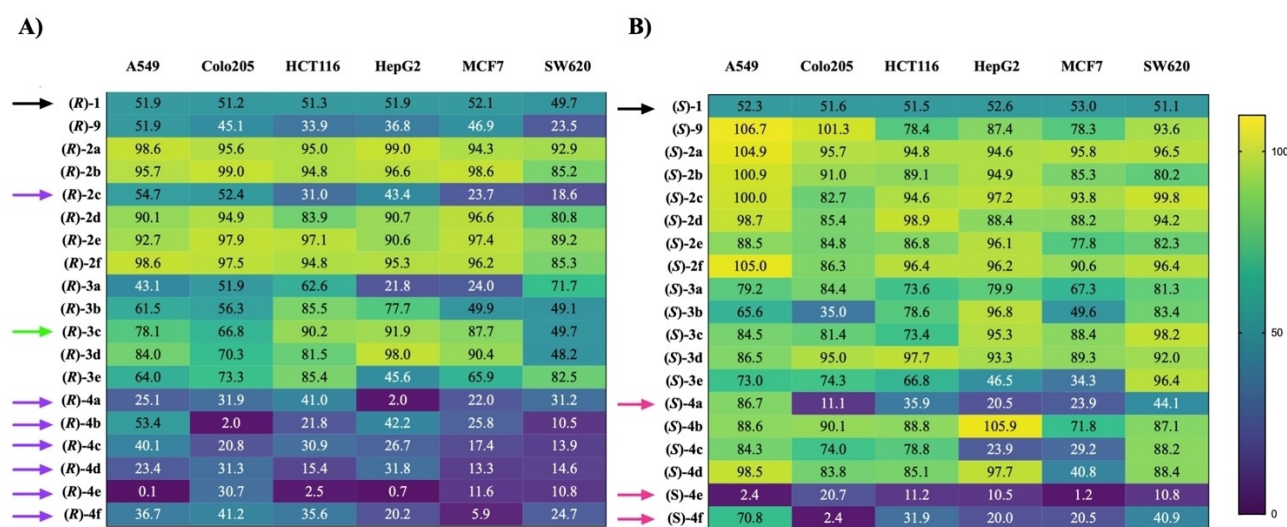


Figure 2. Averaged viability of cancer cells treated with the investigated compounds at concentrations equal to the IC₅₀ values for (*R*)-1 or (*S*)-1, respectively (0.20/0.76 μM for A549, 0.12/0.30 for Colo 205, 0.85/0.77 for HCT116, 0.17/0.25 for Hep G2, 0.21/0.42 for MCF7 and 0.18/0.36 for SW620), n = 3. Dispersion measures were omitted for the clarity of presentation. A) Data for (*R*)-isomers and B) for (*S*)-isomers.

Table 1. Activity (nM) of (*R*)- and (*S*)-1 and their organic and organometallic analogues in human colorectal cancer cell lines (Exposure time 72 h; IC₅₀ values and corresponding 95 % confidence intervals in parentheses).

Compound	Colo 205	HCT116	SW620	SW620E
<i>(R)</i> -1	121	848	185	1,562
	(106–137)	(729–986)	(146–235)	(1,412–1,728)
<i>(R)</i> -2 c	122	177	189	1,254
	(102–148)	(154–202)	(149–242)	(1,090–1,445)
<i>(R)</i> -3 c	14,380	75,710	163	12,930
	(11,940–17,460)	(67,560–84,460)	(121–219)	(9,371–18,910)
<i>(R)</i> -4 a	47	135	111	1,597
	(40–54)	(116–157)	(88–139)	(1,273–2,007)
<i>(R)</i> -4 b	43	54	103	6,864
	(38–48)	(39–75)	(75–145)	(5,113–9,333)
<i>(R)</i> -4 c	84	108	44	11,200
	(58–121)	(77–156)	(37–54)	(8,719–14,550)
<i>(R)</i> -4 d	49	139	23	2,170
	(39–60)	(86–234)	(15–34)	(1,852–2,543)
<i>(R)</i> -4 e	43	73	132	1,472
	(37–51)	(61–88)	(109–159)	(1,260–1,720)
<i>(R)</i> -4 f	76	240	136	17,260
	(57–102)	(163–356)	(100–185)	(15,080–19,860)
<i>(S)</i> -1	305	774	355	2,734
	(251–370)	(637–943)	(317–397)	(2,269–3,311)
<i>(S)</i> -4 a	200	691	292	918
	(164–246)	(630–759)	(260–328)	(779–1,085)
<i>(S)</i> -4 e	122	671	127	2,084
	(103–144)	(529–857)	(108–148)	(1,806–2,406)
<i>(S)</i> -4 f	164	704	662	7,495
	(128–212)	(564–882)	(450–985)	(6,442–8,738)

analogues. The positive effect of *N*-benzylation of ispinesib was also observed in the series of the (*S*)-enantiomers, however the impact of introducing phenyl, ferrocenyl or adamantyl substituents was lower than observed for their (*R*)-counterparts.

Unfortunately, none of the novel compounds was able to overcome the multidrug resistance of SW620E cells. The resistance index (RI, ratio of IC₅₀ for SW620E to IC₅₀ for SW620 for a selected compound) was usually higher for the investigated compounds than for ispinesib and its (*S*)-enantiomer (RI for (*R*)-1 was 8.45 while it was 7.70 for (*S*)-1). A notable exception was the type III benzyl analogue (*S*)-4 a, which exhibited marked activity towards the MDR cancer cell line (IC₅₀ = 918 nM for (*S*)-4 a compared to 2,734 nM for (*S*)-1, RI of 3.15).

Impact on the cell cycle

We investigated the compounds for their effects on the cell cycle as a coarse measure of their mechanisms of action (Figures 3 and S2 and Table 2). It was reported that ispinesib induces G₂/M arrest in cancer cells.^[26] All investigated (*S*)-

enantiomers exhibited a similar pattern in cell cycle distribution as (*S*)-1 and ispinesib (*R*)-1. However, for the (*R*)-series of compounds, i.e. (*R*)-3 c, 4 c–f, we observed a significantly higher reduction of the G₁ (up to 40%) and increased G₂/M (up to 46%) fractions of SW620 cells compared to ispinesib (*R*)-1. These results can be correlated with the elevated antiproliferative potential of the studied compounds toward SW620 cells, for example, the most antiproliferative active (*R*)-4 c–e also induced G₂/M arrest. As expected, this effect was not observed in etoposide-resistance SW620E cells, which are significantly less sensitive toward the studied compounds, all of which caused a similar pattern of cell cycle phase distribution in SW620E cancer cells.

KSP inhibitory activity

The primary mechanism of biological activity of ispinesib is associated with its ability to inhibit KSP. Therefore, we evaluated the ability of the synthesized compounds to inhibit the KSP activity at 10 μM (Figure 4). Neither (*S*)-1 nor its analogues demonstrated inhibitory activity at that concentration, while

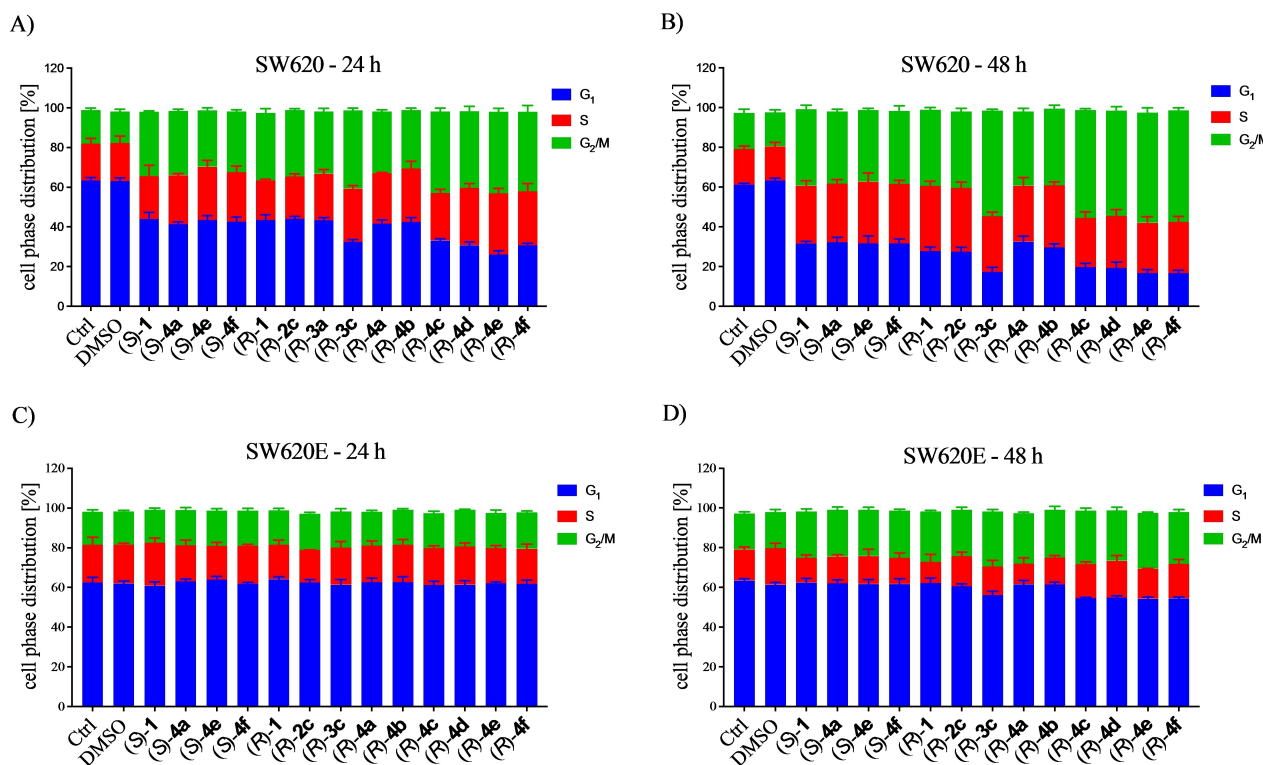


Figure 3. Cell cycle phase distribution in SW620 cells (A and B) and SW620E (C and D) exposed to (S)-1 or ispinesib (R)-1 and their analogues at concentrations equal to the IC_{75} values for 24 h (A and C) and 48 h (B and D). Data are presented as mean $\pm$ SEM, $n = 3$.

Table 2. Cell cycle phase distribution in SW620 cells exposed to (S)-1 or ispinesib (R)-1 and their analogues at concentrations equal to the IC_{75} values for 24 h and 48 h. Data are presented as mean $\pm$ SEM, $n = 3$.

Compound	24 h			48 h		
	G ₁	S	G ₂ /M	G ₁	S	G ₂ /M
Ctrl	63.4 $\pm$ 1.5	18.6 $\pm$ 2.6	16.9 $\pm$ 0.9	61.2 $\pm$ 0.8	18.1 $\pm$ 1.4	18.2 $\pm$ 1.8
DMSO	63.2 $\pm$ 1.6	19.1 $\pm$ 3.5	15.9 $\pm$ 1.0	63.3 $\pm$ 1.2	17.2 $\pm$ 2.0	17.1 $\pm$ 1.2
(S)-1	44.0 $\pm$ 3.2	21.6 $\pm$ 5.4	32.4 $\pm$ 0.9	31.5 $\pm$ 1.2	29.2 $\pm$ 2.4	38.5 $\pm$ 2.0
(S)-4a	41.4 $\pm$ 1.2	24.7 $\pm$ 0.7	32.3 $\pm$ 0.9	32.2 $\pm$ 2.5	29.5 $\pm$ 2.0	36.3 $\pm$ 1.2
(S)-4e	43.6 $\pm$ 2.0	26.7 $\pm$ 3.2	28.4 $\pm$ 1.3	31.8 $\pm$ 3.7	30.9 $\pm$ 4.5	36.1 $\pm$ 0.9
(S)-4f	42.6 $\pm$ 2.3	25.0 $\pm$ 3.0	30.6 $\pm$ 0.9	31.7 $\pm$ 2.1	29.9 $\pm$ 1.7	36.6 $\pm$ 2.6
(R)-1	43.6 $\pm$ 2.5	19.8 $\pm$ 0.5	34.1 $\pm$ 2.1	27.8 $\pm$ 2.0	32.8 $\pm$ 2.3	38.3 $\pm$ 1.1
(R)-2c	44.1 $\pm$ 1.2	21.3 $\pm$ 1.3	33.4 $\pm$ 0.7	27.6 $\pm$ 2.2	32.1 $\pm$ 2.8	38.5 $\pm$ 1.5
(R)-3c	32.4 $\pm$ 1.2	26.9 $\pm$ 1.5	39.5 $\pm$ 1.0	17.3 $\pm$ 2.3	28.1 $\pm$ 2.0	53.1 $\pm$ 0.7
(R)-4a	41.7 $\pm$ 1.7	25.4 $\pm$ 0.3	31.1 $\pm$ 0.9	32.5 $\pm$ 2.7	28.3 $\pm$ 4.1	37.4 $\pm$ 1.6
(R)-4b	42.5 $\pm$ 2.2	26.9 $\pm$ 3.7	29.5 $\pm$ 1.0	29.7 $\pm$ 1.7	31.2 $\pm$ 1.8	38.6 $\pm$ 1.7
(R)-4c	33.1 $\pm$ 0.9	24.0 $\pm$ 1.8	41.2 $\pm$ 1.6	19.8 $\pm$ 1.8	24.7 $\pm$ 3.1	54.3 $\pm$ 0.6
(R)-4d	30.9 $\pm$ 1.5	28.8 $\pm$ 2.0	38.6 $\pm$ 2.5	19.3 $\pm$ 2.8	26.2 $\pm$ 3.1	52.9 $\pm$ 2.0
(R)-4e	26.1 $\pm$ 1.9	30.9 $\pm$ 2.5	41.1 $\pm$ 1.8	16.7 $\pm$ 1.8	25.2 $\pm$ 3.2	55.6 $\pm$ 2.4
(R)-4f	30.7 $\pm$ 1.0	27.3 $\pm$ 3.7	39.9 $\pm$ 3.3	16.7 $\pm$ 1.4	25.8 $\pm$ 2.7	56.1 $\pm$ 1.3

(R)-1 exhibited high inhibitory potency. Interestingly, among the (R)-enantiomers, (R)-2c, (R)-3a and (R)-3c did not affect KSP activity, while (R)-4b, (R)-4c and (R)-4d demonstrated some minor effects. They reduced the KSP activity to 75–84% which was surprising in view of their high antiproliferative potential.

The benzyl ((R)-4a), ferrocenylmethyl ((R)-4e), and 1-adamantylmethyl ((R)-4f) ispinesib derivatives retained the ability to inhibit KSP significantly, while *o*-, *m*- or *p*-ferrocenyl-benzyl ((R)-4b–d) were significantly less potent KSP inhibitors than ispinesib.

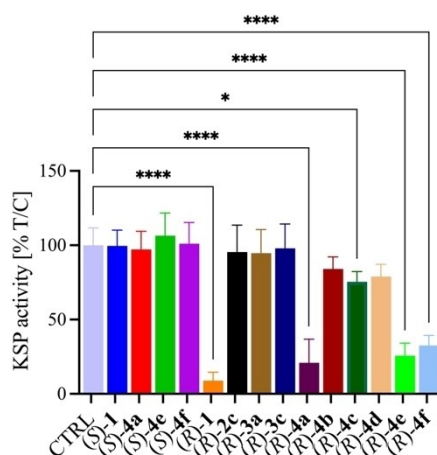


Figure 4. KSP inhibitory activity of (S)- and (R)-1 and their analogues (10 μ M). Data are presented as mean $\pm$ SEM, $n = 3$.

To further analyze the effects of the (R)-enantiomers of the type III compounds on kinesin activity, we examined their specificity towards a set of kinesins and kinesin-like proteins (Figure 5). Similarly to the parent compound, (R)-4a, (R)-4e and

(R)-4f were highly specific for KSP while none of the investigated compounds affected the ATPase activity of other motor proteins.

For the most active compounds from the screening at 10 μ M, we determined the KSP inhibitory activity expressed as the IC_{50} values, i.e., the concentration required to inhibit the KSP activity by 50 % (Table 3). Compared to (R)-1, the investigated conjugates were 2–4 times less potent as KSP inhibitors, with the organometallic hybrid (R)-4e bearing the ferrocenylmethyl moiety exhibiting the highest KSP inhibitory activity.

All three compounds were among the most active in terms of antiproliferative potential, therefore one can infer that KSP inhibition is their dominant mode of action. However, other mechanisms are also of importance for the biological activity of these ispinesib analogues, as some poor KSP inhibitors were also highly cytotoxic.

Molecular docking

Molecules that showed promising biological activity were docked into the ispinesib binding site of KSP and compared to

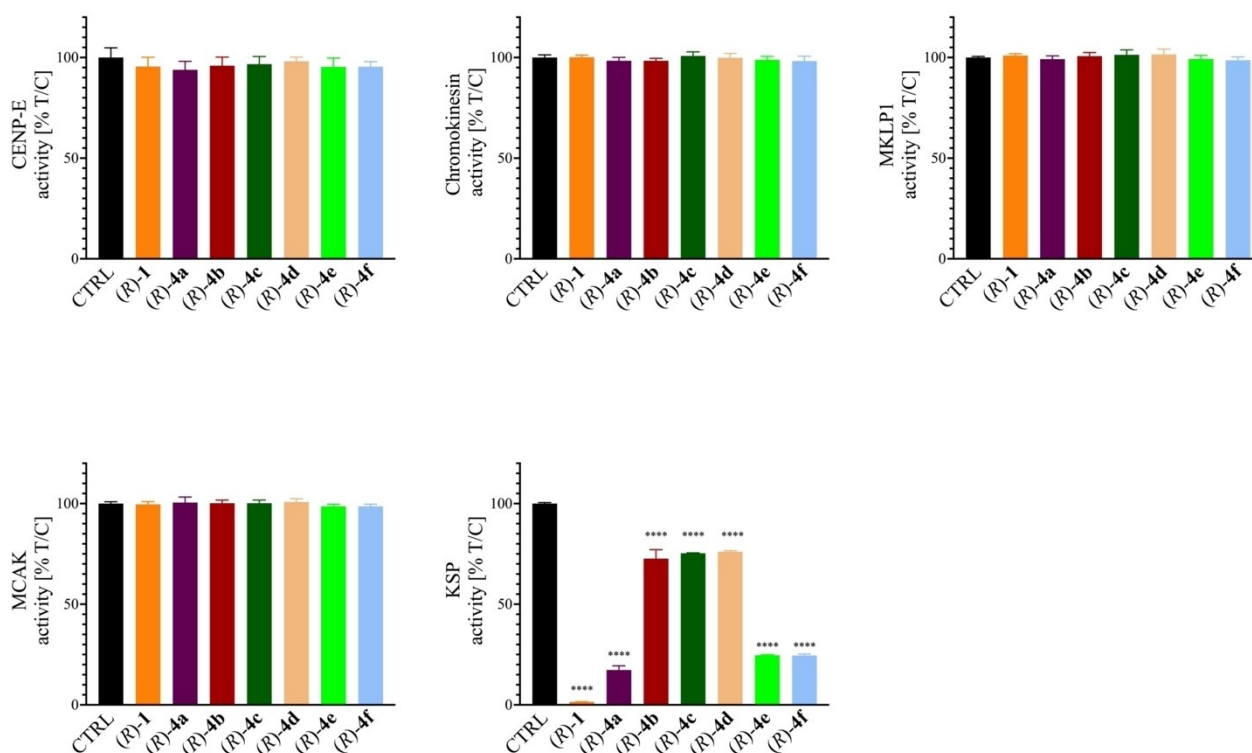


Figure 5. Inhibitory activity of (S)- and (R)-1 and (R)-4a–f (10 μ M) of kinesins and kinesin-like proteins. Data are presented as mean $\pm$ SEM, $n = 3$.

Table 3. IC_{50} values for the most active KSP inhibitors (R)-1, (R)-4a, (R)-4e and (R)-4f. Data presented as mean and 95 % confidence interval.

Compound	(R)-1	(R)-4a	(R)-4e	(R)-4f
IC_{50} [μ M]	0.44 (0.33–0.60)	1.53 (0.91–2.65)	0.85 (0.54–1.37)	1.84 (1.22–2.95)

the results for ispinesib (R)-1 and its (S)-enantiomer (S)-1. The docking was validated by comparing the configurations of ispinesib with the co-crystallized molecule in the crystal structures using GoldScore (GS),^[27] ChemScore (CS),^[28] ChemPLP,^[29] and Astex statistical potential (ASP)^[30] scoring

functions (Table S2). The modelling showed that the molecules can bind in a variety of manners into the pocket depending on the chirality of the ispinesib backbone and the substituents introduced synthetically. For the (*R*)-enantiomers derived from (*R*)-1 and in consequence of (*R*)-4a, configurations were among the highest-scoring poses that showed overlaps with the ispinesib core structure in an inner hydrophobic region in the binding pocket whilst the various substituents occupied a peripheral, more hydrophilic region. In general, the highest scores for all the docking functions were found for molecules derived from (*R*)-4a (Table S3). For example, the binding mode of (*R*)-4e is shown in Figure 5 and features hydrogen bonds of the carbonyl group of the benzamide moiety with the side chain amine moiety of Arg119 and the hydroxyl group of Tyr211. The benzyl group of the organometallic moiety also formed π -stacking interactions with the side chain indene of Trp127 and the phenolic group of Tyr211 (Figure 6). Compared to ispinesib, its (*S*)-enantiomer (*S*)-1 gave lower values for 3 of the 4 scoring functions, and only GoldScore (GS) resulted in a higher result than ispinesib. However, GS scored ispinesib among the lowest structures upon docking and with all the other methods, the (*S*)-enantiomers gave lower scores than the equivalent (*R*)-enantiomers. These results reflect to some extent the studies on the KSP inhibitory activity of the compounds and the cytotoxicity of the compounds. It appears as the inhibition of KSP may not be the only mode of action relevant to the biological activity of the compounds.

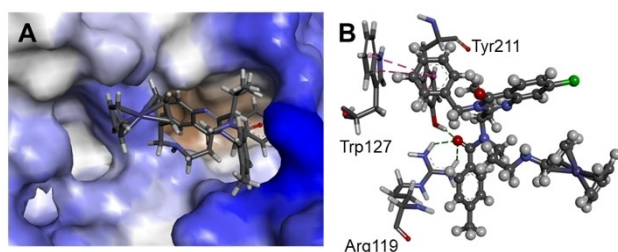


Figure 6. The highest scoring configuration of (*R*)-4e docked into the ispinesib binding site of KSP as predicted by ChemScore. (A) The molecule occupies the same binding pocket as ispinesib with the backbone largely overlapping with that of co-crystallized ispinesib. The protein surface is rendered where blue depicts a hydrophilic region on the surface, brown a hydrophobic region and grey shows neutral areas. (B) Hydrogen bonds are shown as green dotted lines between (*R*)-4e and the amino acids Tyr211 and Arg119. π -Stacking interactions are shown as purple dotted lines between (*R*)-4e and the amino acids Trp127 and Tyr211

ROS generation

To explain the high antiproliferative activity of the ferrocenyl-benzyl moiety-substituted compounds, which was not reflected in the KSP inhibition data, we investigated the induction of reactive oxygen species (ROS) formation in SW620 cells after exposure to the ispinesib analogues with the dihydrorhodamine 123 (DHR123) oxidation assay (Table 4). Neither ispinesib nor its (*S*)-enantiomer affected the ROS level in cells and neither did their benzyl analogues (*S*)-4a and (*R*)-4a. However, upon exposure to all the other compounds, the ROS generation increased by 15–29%. Despite a lack of significant correlation between the antiproliferative potential and ROS generation, the data suggests that the presence of a ferrocenyl moiety may induce oxidative stress within the cell. Interestingly, a similar effect was observed for the adamantyl-functionalized (*S*)-4f and (*R*)-4f, suggesting that the bulkiness of the conjugate may be more important than the ferrocenyl moiety for inducing oxidative stress within the cell. However, the observed level of ROS generation is comparatively small and its contribution to the antiproliferative potential of the compounds needs further investigation.

Conclusions

We have designed organometallic and organic analogues of ispinesib, resulting in a series of highly antiproliferative ferrocenyl ispinesib counterparts, which allowed to derive structure-activity relationships. The obtained analogues can be categorized in three types of compounds – those that lack the toluoyl moiety of ispinesib (type I), those that are functionalized on their tertiary amine group (type II) and the third group that was obtained by derivatization of its primary amine (type III). The cytotoxicity of the synthesized compounds was strongly dependent on their structure, however, was not found to be clearly correlated to the ability to inhibit KSP activity. Only the type I compound (*R*)-2c exhibited cytotoxic activity comparable to that of ispinesib with IC_{50} values in the nM range but it was not a KSP inhibitor, while it was able to induce more ROS formation than ispinesib. Among the type III compounds, the *N*-alkylated ispinesib derivatives exhibited similar or higher cytotoxicity than ispinesib with the most active being (*R*)-4b-e. However, only (*R*)-4a, (*R*)-4e and (*R*)-4f inhibited KSP activity in the sub- to low- μ M concentration range. Interestingly, these were also the compounds that scored highly in many of the docking experiments relative to ispinesib. Although the con-

Table 4. ROS generation in SW620 cells exposed to the ispinesib analogues (1 μ M) for 4 h. Data are presented as mean $\pm$ SEM, $n = 3$. Verapamil (10 μ M) was added to all samples to inhibit any residual activity of the ABCB1 protein which eliminates rhodamine 123 from the cells. Data presented as mean $\pm$ SEM, $n = 3$.

Compound	Verapamil	(<i>S</i>)-1	(<i>S</i>)-4a	(<i>S</i>)-4f	(<i>R</i>)-1	(<i>R</i>)-2c	(<i>R</i>)-3c
ROS [%] vs control	103.7 $\pm$ 1.6	103.7 $\pm$ 1.9	103.7 $\pm$ 2.7	128.1 $\pm$ 3.8	102.1 $\pm$ 1.1	129.1 $\pm$ 2.0	116.7 $\pm$ 1.3
Compound	Verapamil	(<i>R</i>)-4a	(<i>R</i>)-4b	(<i>R</i>)-4c	(<i>R</i>)-4d	(<i>R</i>)-4e	(<i>R</i>)-4f
ROS [%] vs control	103.7 $\pm$ 1.6	102.5 $\pm$ 1.2	115.2 $\pm$ 1.4	125.9 $\pm$ 5.6	128.2 $\pm$ 3.1	120.8 $\pm$ 2.5	129.5 $\pm$ 4.0

jugation of a ferrocenylbenzyl moiety to ispinesib decreased the KSP inhibitory activity of the investigated conjugates, the presence of the ferrocenyl group but also of a bulky adamantyl moiety significantly increased the level of G₂/M fraction of cells. Also, the presence of either a ferrocenyl or adamantyl moiety increased ROS production in cancer cells. As the latter effects are small, oxidative stress will unlikely play an important role in their mode of action which warrants further investigation given the potent biological activity of many of the compounds.

Experimental Section

All reactions were carried out under Ar atmosphere. Commercially available chemicals and solvents were used as received unless stated otherwise. 1,2-Dichloroethane (1,2-DCE) and dichloromethane (DCM) for reactions were dried by distillation from calcium hydride prior to use. Column chromatography was performed on silica gel (Silicycle, 0.040–0.063 mm, 230–400 mesh). Thin-layer chromatography was performed on aluminum sheets precoated with silica (Silicycle). The purity of the synthesized compounds was determined by HPLC-MS analysis performed on a Phenomenex XB-C18 column (50×2.1 mm, 1.7 μm) with a mobile phase flow of 0.3 mL min⁻¹ using a Shimadzu Nexera XR system equipped with SPD-M40 and LCMS-2020 detectors (see Supporting Information for details). The enantiomer ratios for (S)- and (R)-**2a–f** were determined on a Lux Cellulose-2 column (250×4.6 mm) using IPA–n-heptane 1:9 as the mobile phase with a flow rate of 1 mL min⁻¹ using a Shimadzu HPLC system equipped with a PDA detector. ¹H and ¹³C{¹H} NMR spectra were recorded on a Bruker ARX 600 MHz spectrometer (600.3 MHz for ¹H and 150.0 MHz for ¹³C) at 294 K. Chemical shifts were calibrated on the residual solvent signals: [D₆]DMSO 2.51 ppm for ¹H and 39.5 ppm for ¹³C. Synthesis of compounds along with atom numbering for the NMR assignment is presented in the Supporting Information.

General procedure A. Reductive amination at RT. Synthesis of amines (S)- and (R)-**2a–f**

1.12 equiv of aldehyde was added to a solution of (S)- or (R)-**5** in 1,2-DCE (c=0.1 M) and the resulting mixture was stirred at RT for 15 min. 1.44 equiv of sodium triacetoxyborohydride was added and the obtained mixture was stirred at RT for 24 h. After completion, the reaction mixture was diluted with DCM, washed with sodium bicarbonate and brine. The organic solution was dried over sodium sulfate and evaporated to dryness. The products were isolated by chromatography on silica gel using cyclohexane-ethyl acetate 7:3 as the eluent. If required, additional purification on silica gel using DCM or DCM-ethyl acetate 9:1 was performed. Synthesis of all reagents and target compounds and atom numbering for NMR assignment is presented in Supporting Information.

(S)-3-benzyl-2-(1-(benzylamino)-2-methylpropyl)-7-chloroquinazolin-4(3H)-one (S)-2a**:** This compound was obtained in 56% yield using General procedure A starting from 200 mg of (S)-**5**, and 70 mg of benzaldehyde **6a**. ¹H NMR (600 MHz, [D₆]DMSO) δ 8.18 (d, J=8.6 Hz, 1H, H-5), 7.77 (d, J=2.0 Hz, 1H, H-8), 7.59 (dd, J=2.1, 8.6 Hz, 1H, H-6), 7.31 (t, J=7.4 Hz, 2H, Ph-2), 7.26 (t, J=7.3 Hz, 1H, Ph-2), 7.20 (t, J=7.3 Hz, 2H, Ph-2), 7.16–7.11 (m, 5H, Ph-1), 5.48 (d, J=16.4 Hz, 1H, CH₂), 5.19 (d, J=16.1 Hz, 1H, CH₂), 3.55 (s, 1H), 3.51 (m, 2H, CH₂ and H-1'), 3.11 (d, J=12.8 Hz, 1H, CH₂) 2.64 (br s, 1H, NH), 1.97–1.91 (m, 1H, H-2'), 0.88 (d, J=6.7 Hz, 3H, H-3'), 0.73 (d, J=6.4 Hz, 3H, H-3'). ¹³C{¹H} NMR (151 MHz, [D₆]DMSO) δ 161.2, 161.1, 147.7, 140.3, 139.3, 136.5, 128.7 (CH), 128.7 (CH), 128.0 (CH), 127.7 (CH), 127.4 (CH), 127.1 (CH), 126.5 (CH), 126.4 (CH), 126.2 (CH),

118.8, 50.5 (CH₂), 45.3 (CH₂), 31.7 (CH), 20.1 (CH), 17.1 (CH); Elemental analysis calculated for C₂₆H₂₆ClN₃O C 72.29, H 6.07, N 9.73, found C 72.21, H 5.98, N 9.94; HPLC-MS (see Supporting Information) analysis, R_f (HPLC) τ=2.53 min, m/z calculated for C₂₆H₂₇³⁵ClN₃O [M+H]⁺ 432.2 found 432.4 [M+H]⁺.

General procedure B. Reductive amination in a microwave reactor. Synthesis of (S)- and (R)-**4a–f**

A mixture of 1.12 equiv of aldehyde and 1.0 equiv of (S)- or (R)-**1** in 1,2-DCE (c=0.1 M) were placed in a reaction vessel, and the resulting mixture was stirred under MW at 120 °C for 15 min. After cooling to RT, 1.44 equiv of sodium triacetoxyborohydride was added and stirred for an additional 15 min at 120 °C under MW. The reaction mixture was diluted with DCM, washed with sodium bicarbonate and brine. The organic solution was dried over sodium sulfate and evaporated to dryness. The products were isolated by chromatography on silica gel using cyclohexane-ethyl acetate 7:3 as the eluent.

(S)-N-(1-(3-benzyl-7-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)-2-methylpropyl)-N-(3-(benzylamino)propyl)-4-methylbenzamide (S)-**4a**:

This compound was obtained in 59% yield using General procedure B starting from 100 mg of (S)-**1** and 23 mg of **6a**. ¹H NMR (600 MHz, [D₆]DMSO) δ 8.24 (d, J=8.6 Hz, 1H, H-5), 7.76 (d, J=2.0 Hz, 1H, H-8), 7.66 (dd, J=2.0, 8.6 Hz, 1H, H-6), 7.36 (t, J=7.5 Hz, 2H, CH_{Ar}), 7.30 (t, J=7.3 Hz, 1H, CH_{Ar}), 7.27–7.21 (m, 8H, CH_{Ar}), 7.16 (t, J=7.3 Hz, 1H, CH_{Ar}), 6.99 (d, J=7.2 Hz, 2H, CH_{Ar}), 5.89 (d, J=16.3 Hz, 1H, CH₂Ph), 5.52 (d, J=10.5 Hz, 1H, H-1'), 5.07 (d, J=16.3 Hz, 1H, CH₂Ph), 3.30 (t, J=8.1 Hz, 2H, H-1'), 3.15 (s, 2H), 2.77–2.73 (m, 1H, H-2'), 2.29 (s, 3H, CH₃C₆H₄CO), 1.93–1.85 (m, 2H), 1.23–1.20 (m, 1H), 0.90 (d, J=6.8 Hz, 3H, H-3'), 0.90–0.68 (m, 1H), 0.48 (d, J=6.4 Hz, 3H, H-3'). ¹³C{¹H} (151 MHz, [D₆]DMSO) δ 171.9, 161.1, 155.4, 147.2, 140.6, 139.5, 138.6, 136.7, 133.9, 128.8 (CH), 128.7 (CH), 128.6 (CH), 128.1 (CH), 127.9 (CH), 127.5 (CH), 127.4 (CH), 126.5 (CH), 126.4 (CH), 126.3 (CH), 125.9 (CH), 119.1, 58.9 (CH), 51.9 (CH₂), 45.6 (CH₂), 45.1 (CH₂), 42.4 (CH₂), 29.5 (CH₂), 28.0 (CH), 20.8 (CH), 19.4 (CH), 18.1 (CH). Elemental analysis calculated for C₃₇H₃₉ClN₄O₂ C 73.19, H 6.47, N 9.23, found C 73.31, H 6.59, N 9.00. HPLC-MS (see Supporting Information) analysis, R_f (HPLC) τ=2.06 min, m/z calculated for C₃₇H₄₀³⁵ClN₄O₂ [M+H]⁺ 607.3 found 607.7 [M+H]⁺.

General procedure C. Synthesis of compounds (S)- and (R)-**3a–e**

1.4 equiv of p-toluoyl chloride was added to a solution of 1.0 equiv of amine (S)- or (R)-**2a–f** and 1.2 equiv of diisopropylethylamine in anhydrous DCM (10 mL per 1 mmol of **2**). The resulting mixture was stirred at RT for 24 h, quenched by adding sodium bicarbonate and the product was extracted with DCM. The organic phase was washed with brine, dried over sodium sulfate and evaporated. The product was isolated by chromatography on silica using DCM-ethyl acetate 9:1 with 0.1 % of ammonia.

(S)-N-benzyl-N-(1-(3-benzyl-7-chloro-4-oxo-3,4-dihydroquinazolin-2-yl)-2-methylpropyl)-4-methylbenzamide (S)-**3a**:

This compound was obtained in 20% yield using General procedure C starting from 200 mg of (S)-**2a**. ¹H NMR (600 MHz, [D₆]DMSO) δ 7.88 (d, J=8.3 Hz, 1H, H-5), 7.48–7.47 (m, 2H, CH_{Ar}), 7.41 (d, J=7.9 Hz, 2H, CH_{Ar}), 7.38 (t, J=7.6 Hz, 2H, CH_{Ar}), 7.32 (d, J=7.1 Hz, 1H, CH_{Ar}), 7.29 (d, J=7.7 Hz, 2H, CH_{Ar}), 7.21 (d, J=7.9 Hz, 2H, CH_{Ar}), 6.77–6.75 (m, 4H, CH_{Ar}), 6.77–6.66 (m, 1H, CH_{Ar}), 5.92 (d, J=16.2 Hz, 1H, CH₂Ph), 5.66 (d, J=10.4 Hz, 1H, H-1'), 5.17 (d, J=16.2 Hz, 1H, CH₂Ph), 4.72 (d, J=17.2 Hz, 1H, CH₂Ph), 4.54 (d, J=17.2 Hz, 1H, CH₂Ph), 2.95–2.91 (m, 1H, H-2'), 2.28 (s, 3H, CH₃C₆H₄CO), 0.98 (d, J=6.8 Hz, 3H, H-3'), 0.45 (d, J=6.4 Hz, 3H, H-3'). ¹³C{¹H} (151 MHz, [D₆]DMSO) δ 172.6,

160.1, 154.1, 146.8, 139.1, 138.6, 137.2, 136.8, 133.1, 128.9 (CH), 128.7 (CH), 128.0 (CH), 127.5 (CH), 127.4 (CH), 127.3 (CH), 126.5 (CH), 126.3 (CH), 126.2 (CH), 125.7 (CH), 124.8 (CH), 118.5, 58.9 (CH), 47.6 (CH₂), 44.8 (CH₂), 27.3 (CH), 20.8 (CH), 19.3 (CH), 17.9 (CH). Elemental analysis calculated for C₃₄H₃₂ClN₃O C 74.24, H 5.86, N 7.64, found C 74.02, H 5.97, N 7.76. HPLC-MS (see Supporting Information) analysis, R_f (HPLC) τ = 2.40 min, m/z calculated for C₃₄H₃₃³⁵ClN₃O₂ [M + H]⁺ 550.2 found 550.4 [M + H]⁺.

Cell viability assay – uptake of neutral red

10,000 cells were seeded per well of 96-well plate and left for 24 h to attach to the substrate. The test compounds were dissolved in DMSO, diluted in a complete culture medium and added to the cells in a 1:1 volume ratio. DMSO concentrations were kept to the same level and did not exceed 0.1%. After 70 h, neutral red was added to yield a final concentration of 0.033%, and kept in 37 °C for 2 h. Afterwards, the medium was discarded, the cells were washed two times with ice-cold PBS (138 mM NaCl, 5 mM KCl, 7.5 mM phosphate buffer, pH 7.4), and the incorporated dye was solubilized using 1% acetic acid in 50% ethanol for 10 min at an orbital platform shaker. The absorbance of the solution was measured 540 nm using an EnVision plate reader. All values were calculated as a percentage of control. IC₅₀ values were calculated using GraphPad Prism Software ver. 9.

Cell cycle

100,000 cells per well were seeded in 6-well plates, and left for 24 h to attach to the substrate. The test compounds were added at the IC₇₅ concentrations of (S)-1 or (R)-1 calculated from viability curves for the SW620 cell line. After 24 h and 48 h, the cells were trypsinized, washed once with ice cold PBS and fixed using 70% cold ethanol. The cells were then rehydrated with PBS and stained with 75 μ M propidium iodide and 50 Kunitz units/mL of RNase A in PBS for 30 min at 37 °C. The samples were analyzed on an LSRII (Becton Dickinson) instrument (excitation 488 nm, emission 575/26 nm) and the cell cycle phase distribution was determined with FlowJo 7.6.1 software (FlowJo, LLC) using a built-in cell cycle analysis module (Watson pragmatic algorithm).

ROS generation

100,000 cells per well were seeded in 6 well plates for 24 h. The cells were treated with the test compounds (1 μ M), dihydrorhodamine 123 (1 μ M) and verapamil (10 μ M) for 4 h. The cells were then harvested and suspended in a complete medium. The samples were then collected on an LSRII (Becton Dickinson) instrument and median fluorescence (excitation 488 nm, emission 530/30 nm) was analyzed. Results were calculated as a percentage of control.

Kinesin ATPase inhibition assay

The kinesin modulatory activity was determined using Kinesin ATPase Endpoint Biochem Kit (Cytoskeleton Inc) according to the manufacturer's protocol. Phosphate release was measured at 650 nm (EnVision Plate Reader – Perkin Elmer, USA). The DMSO concentration in the samples was 0.1 %.

Molecular Modelling

The compounds were docked to the crystal structure of human kinesin KSP (PDB ID: 4AP0, resolution 2.59 Å)^[31] which was obtained from the Protein Data Bank (PDB).^[32] Scigress version FJ 2.6^[33] was

used to prepare the crystal structure for docking, i.e., hydrogen atoms were added and co-crystallized ispinesib and adenosine-5'-diphosphate (ADP) were removed. The center of the binding pocket was defined as the nitrogen in the ring close to the carbonyl group. ($x=41.663$, $y=0.113$, $z=11.931$) with a radius of 10 Å. The Gold-Score (GS),^[27] ChemScore (CS),^[28] ChemPLP,^[29] and Astex statistical potential (ASP)^[30] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the GOLD v5.4 software suite. The co-crystallized ligand ispinesib was first docked and root mean square deviation (RMSD) values were calculated for the heavy atoms. ASP obtained average RMSDs of 0.9255, PLP of 0.7299, CS of 0.7265 and for GS a value of 0.8090 was found which show the strong prediction power of the scoring functions. The RMSD values and binding scores are given in Tables S1 and S2 in the Supporting Information.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

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

Keywords: antiproliferative agents • enzyme inhibitors • ferrocene • ispinesib • organometallic compounds

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