Impact of the ferrocenyl group on cytotoxicity and KSP inhibitory activity of ferrocenyl monastrol conjugates

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ABSTRACT: The incorporation of the ferrocenyl moiety into a bioactive molecule may significantly alter the activity of the resulting conjugate. By applying this strategy, we designed ferrocenyl analogs of monastrol – the first low molecular weight kinesin spindle protein (KSP) inhibitor. The obtained compounds showed low micromolar antiproliferative activity towards a panel of sensitive and ABC-overexpressing cancer cells. Most cytotoxic compounds exhibited also higher KSP modulatory activity and ability to ROS generation compared to monastrol. The increased bioactivity of the studied compounds can be attributed to the presence of the ferrocenyl group.

Microtubule-targeting drugs, taxanes (e.g. paclitaxel, docetaxel, cabazitaxel) and *Vinca* alkaloids (e.g. vincristine, vinblastine, vinflunine, vindesine), are widely used in anticancer therapy.1, 2 As microtubules maintain the cell structure and play a crucial role in numerous cellular processes such as cell motility, intracellular transport and cell division3-5 in all nucleated cells, the use of tubulin-binding drugs results in high systemic toxicity and serious adverse effects.6-8 Thus, a search for novel inhibitors that affect molecular targets involved in cell division and overexpressed in cancer cells, is still highly relevant.9

Kinesins, especially kinesin spindle protein (KSP; also known as Eg5 or kinesin-5), are among the most promising targets for novel anticancer agents. There are over 40 human kinesins classified into 14 families, which are important for cell division and intracellular transport.10 Although hundreds of proteins are associated with mitotic spindle formation,11 KSP plays an essential role in establishing the bipolar spindle.12-15 Additionally, this protein is overexpressed only in neoplastic cells, thus KSP inhibitors arrest only dividing cells16 and are not expected to affect non-proliferating normal cells. Furthermore, it has been reported that overexpression of KSP is correlated with poor clinical outcome in several cancer types.17-20 This makes KSP a good molecular target for anticancer therapy.21-25

The first low molecular weight KSP inhibitor, monastrol **1**, was discovered by Mayer et al. in 1999.26 Despite only moderate anticancer activity of **1**, considerable effort was taken to modify its structure and to design monastrol-derived lead structures as potent KSP inhibitors such as enastron,27 dimethylenastron,27, 28 fluorastrol,27 Mon-97,29 or CPUYL064.30 In addition, other natural and synthetic KSP inhibitors were discovered which are structurally different from monastrol.31, 32

Widespread use of platinum-based anticancer drugs, e.g. cisplatin and its analogs,33, 34 led to the growing interest for other metal-based compounds as prospective antineoplastic agents. The conjugation of a metal-containing structural motif, such as a ferrocene moiety, to bioactive molecules often results in increased bioactivity or even altered mode of action in comparison to the organic parent compound35. In the last three decades, ferrocene derivatives36 were extensively investigated as anticancer,37, 38 antimicrobial,39-42 antiparasitic,43 including antimalarial, drug candidates.44-46 Interestingly, organometallic derivatives of KSP inhibitors have only been studied to a minor extent, while much research was focused on half-sandwich complexes. For example, Al-Masoudi and co-workers reported ruthenium complexes of the dihydropyrimidine (DPH) monastrol, however, the obtained compounds possessed lower activity than monastrol.47, 48 Recently, we reported ruthenium, osmium, iridium and rhodium half-sandwich complexes as KSP inhibitors bearing the 2-(1-aminoalkyl)quinazolin-4(3H)-one moiety as a bidentate ligand.49 Although simple ferrocenyl DPH derivatives have been synthesized,50-53 the biological activity of such compounds has not been investigated in detail, and the impact of the ferrocenyl moiety on KSP activity is unknown.

Continuing our research in organometallic inhibitors of mitosis,54-59 we have prepared ferrocenyl derivatives of monastrol (X = S) and oxo-monastrol (X = O) (Figure 1) and evaluated the influence of the organometallic moiety on the bioactivity of the ferrocenyl derivatives.

**Figure 1.** Structures of monastrol **1** and its ferrocenyl analogs type I and II studied herein

The target compounds of type I and II were prepared in Biginelli reactions. The reaction of ferrocenecarboxaldehyde **2a** with ethyl acetoacetate, thiourea and catalytical amounts of HCl at reflux afforded **3a** in 53% yield. Unfortunately, the same procedure led to an inseparable mixture of several products when using aldehydes **2b–2d**. However, we found that the reaction of ferrocenecarboxaldehyde, o-, m-, or p-ferrocenylbenzaldehydes57 **2a–d** with ethyl acetoacetate and thiourea or urea at RT in anhydrous ethanol in the presence of SbCl3,60, 61 afforded the desired type I compounds **3b–d** and **4a–d** in 23–80% yield (Scheme 1).

To prepare compounds of type II, the corresponding -ketoesters **5a–d** were required. The Friedel-Crafts acylation of ferrocene with mono-ethyl malonate and trifluoroacetic anhydride in the presence of trifluoromethanesulfonic acid in anhydrous dichloromethane gave **5a** in almost quantitative yield (Scheme 2).62 Compounds **5b–d** were prepared in the reaction of **2b–d** with ethyl diazoacetate in the presence of catalytic amounts of NbCl5 in 11–65% yield by adopting a reported procedure (Scheme 2).63 The reaction of **5a–d** with 3-hydroxybenzaldehyde and thiourea or urea under optimized conditions

Scheme 1. Synthesis of the type I ferrocenyl monastrol analogs 3a–4d

(SbCl3 catalyst, 24 h at RT) led to a mixture of undesired and various unidentified products together with small amounts of desired products. When urea was used instead of thiourea, we were able to isolate undesired 2-acyl-3-ureidopropanoates **8a–d** in 10–34% yields which precipitated from the reaction mixture (Scheme 2, condition C).

Therefore, to optimize the reaction conditions, we monitored the progress of the reactions of **5a** with urea or thiourea and 3-hydroxybenzaldehyde in the presence of SbCl3 by HPLC-MS. When urea was used, the HPLC-MS analysis of the crude reaction mixture confirmed that **8a** was the major product (43.8%), while the desired **7a** was only formed in 9.4% yield. Besides, a small amount, 13.1%, of unreacted **5a** remained in the reaction mixture after 24 h. Use of thiourea instead of urea resulted in the formation of the ureido derivative **9a** in 29.0%, while **6a** was only formed at 5.5% yield with 34.9% of unreacted **5a** remaining after 24 h at RT. Performing the reaction at 40 C led to the formation of **6a** or **7a** in 29.5 and 25.5% yield, respectively, while the amounts of ureido derivatives decreased to 25.2 and 34.2%, respectively. Further extension of the reaction time up to 72 h increased the yield of the desired products. In comparison, a similar study performed for the reaction of **2a** with urea or thiourea and ethyl acetoacetate confirmed that the desired products **4a** and **3a** were formed as the major products in yields of 73.8 and 57.7%, respectively within 24h, together with ethyl 2-ferrocenylmethylideneacetoacetate **13** (12.3–17.3%) and trace amounts of ureido derivatives **12a** and **11b**. (1.7 and 2.4%) (Supporting Scheme S1).

Based on the above results, we decided to perform the reactions of **5a–d** with 3-hydroxybenzaldehyde, thiourea or urea and SbCl3 at 40 C for 72 h, which allowed the isolation of **6a**, **6c**, **6d** and **7a** in yields of 10–54% (Scheme 2, condition A). Any further effort to prepare **6b** or **7b–d** under the same conditions failed. Further studies revealed that **7c** and **7d** were formed in 6.2 and 1.4% yield when the reactions were carried out in a microwave reactor in the presence of 10 mol% of Yb(OTf)3 in 2,2,2-trifluoroethanol at 110 C for 30 min. We also observed the formation of trace amounts of ureido derivatives **8c** and **8d** (1.4–2.5%). Interestingly, under microwave condition, **6a** was formed in only 1.6% yield, while ortho-substituted **6b** or **7b** were not found (Scheme 2, condition B).



Scheme 3. Synthesis of the type II ferrocenyl monastrol analogs. Conditions: A) 40 C, 72 h; B) CF3CH2OH, Yb(OTf)3 (10%mol), MW, 110 C, 30 min; C) SbCl3 (1 eq), RT, 72 h.

The structures and purities of all compounds were confirmed by the NMR spectroscopy and HPLC-ESI-MS analysis (Supporting Figures S7-S60). In the 1H NMR spectra in DMSO-d6 of **3a–d** and **6b–d**, we observed two signals assigned to the NH-1 and NH-3 protons at ca. 10.5 and 9.7 ppm, respectively, while in the oxo-analogs **4a–d** these protons were observed at ca. 9.1 and 7.5 ppm. However, in the case of **6a**, the order of the NH-1 and NH-3 proton peaks was inversed, and the corresponding signals were present at ca. 8.7 and 9.9 ppm. In comparison to monastrol derivatives, the 2-acyl-3-(ureido)propanoates **8a–d** presented much more complicated spectra as they can exist in both keto and enol forms, as confirmed by NMR spectroscopy. In the 1H NMR spectra of **8a–d** in DMSO-d6, the signals of the NH2 group were observed at ca 5.6 ppm while the NH protons resonated at ca. 6.6 ppm as broad singlets or doublets. In the 13C{1H} NMR spectra of **8a-d**, a set of three carbonyl atom signals was present at ca. 195, 168 and 158 ppm, which were assigned to keto CO, ester and 158 ppm urea moieties, correspondingly. The HPLC-ESI-MS analysis of the monastrol derivatives shown the major peaks (with area in a range of 84.1 – 95.8%) preceded by minor peaks (with area in a range of 3.1 - 10%) both assigned to cation [M]+ formed by oxidation of studied compound, and only in a case of **6a** we observed molecular ion assigned to [M+H]+ (Supporting Figures S43-S60). The presence of the major peak together with trace amounts of minor peak observed in the HPLC-ESI-MS spectra of studied compounds can be assigned to the keto-enol forms.

The molecular structures of the organometallic analogs of monastrol, i.e., **3a** and **6a**, were determined by single-crystal X-ray diffraction analysis (Supporting Figures S1-S3, Supporting Tables S1-S3). Similarly, to **1**, both **3a** and **6a** crystallized in the centrosymmetric space groups C2/c (**3a**) and P21/c (**6a**) as racemic mixtures. In both instances, a single molecule of the compound was found in the asymmetric unit. Compound **6a** co-crystallized with one molecule of CH2Cl2 which was found to be severely disordered.

The antiproliferative activity of the synthesized compounds was studied in a set of human cancer cell lines, namely A549 (alveolar basal epithelial cell adenocarcinoma), Colo 205 (colorectal adenocarcinoma), HCT 116 (colorectal carcinoma), Hep G2 (hepatocellular carcinoma), MCF7 (breast adenocarcinoma), and SW620 (colorectal adenocarcinoma), as well as in a panel of five multidrug-resistant (MDR) cell lines derived from SW620 and characterized by overexpression of various ABC proteins, namely ABCG2 (SW620C), ABCC1 (SW620M and SW620E) and ABCB1 (SW620D, SW620E, and SW620V).64 We intentionally did not incorporate any of so called “normal” (i.e., non-cancerous) cells in our study as the potential target of our compounds is crucial for cell division, so it is obvious that all dividing cells would be affected (as it happens during chemotherapy in the patient’s body when bone marrow, intestinal lining and hair follicles are involved). Initially, we evaluated the impact of **3a–4d**, **6a, 6c, 6d**, **7a**, **7c**, **7d** and **8a–d** on the cell viability at the IC50 of **1** of the respective cell line (Figure 2). Such screening allowed us to choose 10 of the most active compounds and study them further in a more detailed way. In the next step, we determined the IC50 values for selected compounds in the same set of cancer cell lines (Table 1) and in the MDR cell line panel (Table 2).

Analysis of the antiproliferative potential of the synthesized compounds in the A549, Colo 205, Hep G2, HCT 116, SW620 and MCF7 cell lines revealed that ferrocenyl analogs of monastrol **3** and **6** and of its oxo-analogs **4** and **7** were more active than **1**. Notably, their activity strongly depended on the ferrocenyl substituents and the type of heteroatom at C-2. For example, a simple replacement of the 3-hydroxyphenyl moiety in **1** with a ferrocenyl group (**3a**) resulted only in slightly increased antiproliferative potency towards Colo 205, HepG2 and SW620 cells. The biological activity of type I analogs could be further increased by substituting the 3-hydroxyphenyl group in **1** with a ferrocenylphenyl moiety, with the most active representative being the 4-ferrocenylphenyl comObraz zawierający stół

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**Figure 2**. Antiproliferative activity of the synthesized compounds in comparison to **1**. All compounds were administered at concentrations equal to the IC50 values of **1** in the respective cell lines (51.0 M (A549), 112 M (Colo 205), 78.1 M (Hep G2), 41.9 M (HCT 116), 29.4 M (MCF7), and 84.7 M (SW620)). The results are presented as relative viability compared to non-treated controls (mean value SD from three independent experiments).

Exchanging the sulfur atom for oxygen usually diminished the biological potency of the type I compounds (**3a–d** vs. **4a–d**; Figure 2 and Table 1) with one significant exception being **4b** bearing a 2-ferrocenylphenyl moiety instead of the 3-hydroxyphenyl group. Also, replacing the 6-methyl group in **1** with ferrocenyl or ferrocenylphenyl moieties in compounds **6a, 6c, 6d** and **7d** (Table 1) significantly affected the biological activity of type II compounds. The thiones exhibited substantially higher activity than **1**, while only one oxo-analog **7a** exerted moderate activity. It should be noted that the oxo-analog **6c**, bearing a 3-ferrocenylphenyl moiety instead of the 6-methyl group, exhibited the highest antiproliferative potency towards all studied cell lines with IC50 values in the low micromolar range. Overall, MCF7 and Hep G2 cells were more sensitive to the investigated compounds while the colorectal and lung cancer cell lines were usually slightly more resistant.

Activity of the multidrug resistance pumps can be an obstacle in delivering a drug into the target cell. Therefore, we studied the biological potency of the compounds in cells overexpressing various ABC transporters responsible for MDR. The synthesized compounds were more active than **1** with the exception of **3a** and **4b** in SW620M and SW620V cells (Table 2). However, no clear effect of MDR pump overexpression on cell sensitivity was observed, neither in case of **1** nor its analogs. The overall pattern of activity in MDR cells was identical to that observed in non-MDR cell lines. Based on these results, we have selected compounds **3a**, **4b** and **6c** for more detailed studies on their biological activity.

Table 1. Cytotoxicity of the ferrocenyl analogs of monastrol (3a-d, 6a, 6c and 6d) and of oxo-monastrol (4b, 4d and 7a) in cancer cell lines. IC50 values are presented with the respective 95% confidence intervals given in brackets. Data presented are derived from three independent experiments

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Compound | A549 | Colo 205 | Hep G2 | HCT 116 | MCF7 | SW620 |
| **1** | 51.0  [42.0-63.4] | >100 | 78.1  [52.8-182.3] | 41.9  [33. 7-53.6] | 29.4  [20.5-44.9] | 84.7  [64.4-120] |
| **3a** | **75.8**  **[44.1-218.2]** | **37.3**  **[28.9-47.4]** | **6.20**  **[3.94-9.88]** | **80.8**  **[64.0-107.8]** | **26.1**  **[21.8-31.0]** | **50.6**  **[44.0-58.8]** |
| **3b** | 11.4  [8.16-16.0] | 24.9  [17.5-37.2] | 5.38  [4.22-6.84] | 17.7  [15.1-20.9] | 5.38  [4.35-6.61] | 21.3  [16.2-28.7] |
| **3c** | 16.5  [12.1-23.1] | 34.5  [26.3-46.9] | 31.5  [24.6-41.8] | 32.5  [24.7-44.0] | 6.57  [5.62-7.72] | 41.5  [36.5-47.4] |
| **3d** | 6.71  [5.45-8.29] | 14.6  [11.5-19.0] | 10.2  [9.14-11.5] | 23.3  [17.2-32.8] | 1.13  [0.81-1.57] | 65.1  [50.4-87.2] |
| **4b** | **6.40**  **[5.15-7.94]** | **6.53**  **[5.29-8.05]** | **5.95**  **[4.73-7.47]** | **6.95**  **[5.73-8.43]** | **6.41**  **[5.16-7.96]** | **8.97**  **[7.09-11.3]** |
| **4d** | >100 | 72.3  [52.6-187] | 52.9  [42.9-67.2] | 50.5  [39.2-67.7] | 14.8  [11.1-20.0] | 54.1  [45.1-66.1] |
| **6a** | 37.1  [25.9-56.7] | 34.6  [20.3-39.9] | 12.8  [9.23-17.6] | 51.6  [46.1-57.9] | 11.2  [9.04-13.9] | 38.7  [31.1-18.9] |
| **6c** | **3.58**  **[2.91-4.40]** | **3.64**  **[2.88-4.59]** | **2.39**  **[2.10-2.73]** | **3.54**  **[3.09-4.06]** | **4.02**  **[3.34-4.83]** | **7.19**  **[4.59-11.1]** |
| **6d** | 14.5  [13.2-15.9] | 14.9  [ 11.0 -18.8] | 11.5  [8.23-15.10] | 6.68  [5.69-7.84] | NA | 14.2  [12.1-16.6] |
| **7a** | 24.8[a] | 21.9[a] | 21.3[a] | 23.3[a] | 21.7  [18.7-25.5] | 25.9  [20.4-34.3] |

Table 2. Cytotoxicity of the ferrocenyl analogues of monastrol (3a-d, 6a, 6c and 6d) and of oxo-monastrol (4b, 4d and 7a) in MDR cancer cell lines. IC50 values are presented with the respective 95% confidence intervals given in brackets. Data presented are derived from three independent experiments

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Compound | SW620 | SW620C | SW620D | SW620E | SW620M | SW620V |
| **1** | 84.7  [64.4-120] | 85.8  [79.9-134.1] | >100 | 86.8  [61.2-139] | 33.5  [29.8-37.8] | 45.9  [34.9-62.9] |
| **3a** | **50.6**  **[44.0-58.8]** | **37.2**  **[30.0-46.8]** | **20.9**  **[13.7-31.6]** | **28.0**  **[23.1-33.7]** | **45.0**  **[36.6-56.0]** | **63.7**  **[41.5-113]** |
| **3b** | 21.3  [16.2-28.7] | 24.0  [18.2-32.7] | 11.1  [8.53-14.42] | 17.1  [11.4-26.1] | 19.1  [13.6-27.7] | 38.9  [22.8-81.0] |
| **3c** | 41.5  [36.5-47.4] | 68.3  [55.5-87.0] | 35.3  [30.3-41.7] | 26.5  [22.2-32.0] | 32.4  [26.1-40.9] | 21.1  [17.3-26.1] |
| **3d** | 65.1  [50.4-87.2] | 34.1  [25.7-46.8] | 17.8  [15.1-21.0] | 20.4  [15.6-27.3] | 12.4  [10.4-14.9] | 10.2  [8.28-12.7] |
| **4b** | **8.97**  **[7.09-11.3]** | **10.9**  **[9.07-13.3]** | **13.0**  **[10.94-15.5]** | **12.0**  **[9.75-14.8]** | **8.72**  **[6.59-11.6]** | **17.4**  **[15.0-20.3]** |
| **4d** | 54.1  [45.1-66.1] | 46.9  [34.3-68.0] | 56.2  [39.3-86.6] | 52.9  [41.0-71.0] | 48.4  [40.0-59.8] | 65.2  [50.2-88.9] |
| **6a** | 38.7  [31.1-18.9] | 29.3  [23.2-36.6] | 22.9  [17.7-29.2] | 19.3  [14.4-25.4] | 18.9  [11.7-31.3] | 12.7  [9.95-16.1] |
| **6c** | **7.19**  **[4.59-11.1]** | **3.19**  **[2.37-4.26]** | **5.02**  **[3.69-6.81]** | **4.68**  **[3.41-6.41]** | **9.42**  **[7.59-11.7]** | **4.99**  **[4.18-5.96]** |
| **6d** | 14.2  [12.1-16.6] | 8.40  [7.01-10.1] | 16.6  [12.2-22.5] | 23.0  [18.6-27.9] | 14.3  [10.2-19.9] | 15.5  [1.98-19.9] |
| **7a** | 25.9  [20.4-34.3] | 19.7  [16.9-23.0] | 32.2  [26.5-39.6] | 25.4[a] | 25.5  [21.1-31.4] | 27.3  [23.7-32.0] |

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**Figure 3**. Relative ATPase activity in presence of 10 µM of **1**, **3a**, **4b** and **6c**. Solvent control activity was referred to as 100%. Data are presented as mean ± SEM, n = 3 or 4 (in case of KSP). Data analyzed by one-way ANOVA and post hoc Tukey's test. \*\*\* P < 0.001, \*\*\*\* P < 0.0001

Kinesins utilize ATP to slide along microtubules and therefore their activity can be assessed by measuring the ATP hydrolysis rate. Since **1** is known to be a specific inhibitor of KSP activity, we tested compounds **3a**, **4b** and **6c** in comparison to **1** for their inhibitory activity on KSP at a concentration of 10 µM. Kinesin inhibition was determined with the Kinesin ATPase Endpoint Biochem Kit (see Supporting Information for details). The residual KSP ATPase activity was two times lower in case of ferrocenyl analogs (**3a** – 7.2%, **4b** – 8.8% and **6c** – 9.8%) than in case of **1** (18.7%) (Figure 3). To assess whether such effects were selective towards KSP, we used also other motor proteins, kinesin 4 family motor (KIF4A), kinesin-like protein KIF23 and mitotic centromere-associated kinesin (MCAK) but none of the compounds inhibited these proteins. It must be stressed here that the nucleotide binding sequence of all four proteins is highly conserved as revealed by the UniProt alignment tool. It is therefore unlikely that the compounds interact with the ATP-binding domain of KSP in which case they would inhibit all the other proteins too. Thus, it must be implied that the investigated modifications of the core structure do not alter the specificity of the monastrol analogs.

Obraz zawierający grafika wektorowa

Opis wygenerowany automatycznieThe antiproliferative activity of ferrocenyl compounds is believed to be associated with their increased ability to generate reactive oxygen species (ROS) in target cells.36 To verify the hypothesis that introducing a ferrocenyl moiety to **1** impacts the mechanism of action, we investigated the ability of **3a**, **4b** and **6c** to induce intracellular ROS formation (Figure 4). The rate of dihydrorhodamine 123 oxidation was considered the indicator for ROS production in SW620 cells. Compounds **4b** and **6c** were approximately 25% more active in terms of ROS generation than **1**, which supports the hypothesis of ROS involvement in the anticancer activity of such ferrocenyl compounds.

**Figure 4**. ROS induction by **3a**, **4b** and **6c** in SW620 cells as compared to verapamil, an ABCB1 inhibitor used to increase rhodamine 123 retention in vulnerable cells, and **1**. Data presented as means ± SEM, n = 3. Data analysed by one-way ANOVA and post hoc Tukey's test. \* P < 0.05, \*\*\* P < 0.001

As a KSP inhibitor, **1** leads to the formation of monopolar mitotic spindles and chromosome segregation blockade in cancer cells. Flow cytometry was used to assess the cell cycle distribution and in particular to monitor an increase in G2/M phase cells (Table 3). SW620 and SW620V cells, the latter being twice as sensitive to **1** than the parental SW620 cells were exposed to **4b** and **6c** for 24 h to detect the potential detrimental effects of ROS formation. We did not observe any signs of mitotic arrest in the cells exposed either to **1** or its ferrocenyl analogs. This is not really surprising considering the typical length of human cancer cell cycle ranging between 20 and 24 hours, and the incubation period being relatively short. Indeed, pronounced effects of monastrol are observed after 48-hour exposure – twice the length of the cell cycle duration.65 However, an increased number of apoptotic cells (sub-G1 fraction) and a reduced percentage of cells in the G0/G1 phase could be clearly seen, especially in SW620 cells exposed to **4b** and **6c**. Such short-term effects support the potential role of ROS formation in the biological activity of the compounds studied.

Table 3. Cell cycle distribution in SW620 and SW620V cells after treatment with monastrol 1 and its ferrocenyl derivatives 3a, 4b and 6c at a concentration equal to the IC90 value of 1 for SW620. Compounds were used at concentrations equal to the respective IC90 values. Data presented as means ± SEM, n = 3.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Cell line |  | sub-G1 | G0/G1 | S | G2/M |
| SW620 | ctrl | 0.9 ± 0.2 | 73.7 ± 4.0 | 13.9 ± 2.6 | 12.1 ± 1.5 |
|  | **1** | 12.4 ± 4.1 | 63.4 ± 3.4 | 10.9 ± 3.2 | 11.1 ± 1.7 |
|  | **3a** | 15.7 ± 4.6 | 65.2 ± 5.7 | 10.0 ± 3.4 | 9.8 ± 2.5 |
|  | **4b** | 28.2 ± 2.6 | 50.6 ± 2.8 | 13.2 ± 4.4 | 8.0 ± 2.3 |
|  | **6c** | 32.1 ± 3.8 | 45.4 ± 6.7 | 13.5 ± 1.8 | 9.3 ± 2.3 |
| SW620V | ctrl | 1.1 ± 0.2 | 75.2 ± 5.0 | 13.8 ± 2.8 | 9.0 ± 3.0 |
|  | **1** | 5.5 ± 2.2 | 69.7 ± 7.9 | 12.3 ± 4,5 | 10.0 ± 2.1 |
|  | **3a** | 6.6 ± 3.2 | 68.0 ± 6.0 | 10.9 ± 5.4 | 11.4 ± 2.8 |
|  | **4b** | 11.7 ± 1.9 | 61.0 ± 5.7 | 10.4 ± 4.0 | 11.2 ± 2.3 |
|  | **6c** | 12.6 ± 2.2 | 62.8 ± 8.9 | 10.3 ± 2.2 | 13.9 ± 2.6 |

Molecular modeling was used to investigate the possible binding mode(s) for the ferrocenyl monastrol and oxo-monastrol derivatives. Docking of the (*R*)- and (*S*)-enantiomers of the ferrocenyl derivatives **3a–7d** revealed that the docking is largely independent whether the compound derived of dihydropyrimidin-2(1H)-one or a dihydropyrimidin-2(1H)-thione (Supporting Information) and similar predicted binding modes and intermolecular interactions were found.66 In general, the (*S*)-enantiomers showed higher predicted binding affinities than the (*R*)-isomers (Supporting Table S5). The latter have relatively weak pose prediction consistencies between docking runs, i.e., the algorithm predicts many different poses. Several of these configurations were regarded to be implausible, e.g., the 3-hydroxyphenyl ring being predicted to be in the aqueous phase. These results are suggestive of weak binding affinities and biological effects for the (*R*)-isomers. Evidence of this is apparent as Maliga et al.67 demonstrated (*S*)-1 has ~15 times greater potency than its enantiomeric counterpart, (*R*)-1.

Molecular docking revealed that most of the (*S*)-isomers with 6-(ferrocenylphenyl) substituents (**6c**, **6d** and **7d**) retained similar binding poses and intermolecular interactions comparable to (*S*)-monastrol, e.g., the docked configuration of (*S*)-**6c** (Figure 3A); the 3-hydroxyphenyl moiety remained in the hydrophobic cavity, the thione facing Ile136, and most of the main hydrogen bonding interactions were retained, i.e., the 3-NH tetrahydropyrimidine formed a hydrogen bond with Glu116 and the phenol with Glu118. The introduced 6-(ferrocenylphenyl) moiety is predicted to be outside of the allosteric pocket but is situated close to the hydrophobic regions partly formed by Ala218 and the alkyl side chain of Arg221 where they are expected to form favorable hydrophobic contacts. In contrast to the 4-ferrocenyl or 4-(ferrocenylphenyl) derivatives (**3a–4d**), there is a lack of key predicted hydrogen bonding patterns resulting from the occupation of the ferrocenyl moiety of the hydrophobic cavity and the resulting change in the tetrahydropyrimidine ring orientation; indeed it is pointing out of the binding pocket (Figure 3B). Additionally, the thione functional group faces away from Ile136 and the ethanoate is directed towards the allosteric cavity. These poses have reversed the position of the ligands as compared to (*S*)-**1**, depriving them of good binding within the allosteric pocket. They lack a favorable mix of hydrogen bonding for specificity and lipophilic contacts for affinity.68

Obraz zawierający wewnątrz, różny, kilka

Opis wygenerowany automatycznie

**Figure 3**. Docked configurations of (*S*)-**6c** (A) and (*S*)-**3a** (B). The predicted hydrogen bonds are depicted as green dotted lines between the derivative and the residues Glu116 (blue) and Glu118 (red). The protein surface is rendered. Red, blue and grey regions represents partially negative, positive and hydrophobic regions. Hydrogen atoms are hidden for clarity.

The 6-(ferrocenylphenyl) substituted compounds **6c** and **6d** were the most cytotoxic derivatives (Table 1). The molecular modeling results indicate that the substitution of the 3-hydroxyphenyl group with the 4-(ferrocenylphenyl) moiety leads to different orientations of the derivatives thus impairing hydrogen bonding networks within the allosteric cavity. Derivative **4b** contradicts this interpretation as it has good cytotoxic potency (Table 1); it can be speculated that **4b** does not occupy the allosteric pocket but exerts its biological effect by other means.

In conclusion, we report a series of systematically-modified ferrocenyl derivatives of monastrol and oxo-monastrol which were evaluated for their antiproliferative activity and we aimed to rationalize the results by a series of complementary biological studies inspired the biological activity of monastrol. We found that introducing a ferrocenyl moiety leads to a broad spectrum of activity towards all the investigated cells including the MDR panel cell lines. The derivatives **3a**, **4b** and **6c** inhibited KSP with the highest potency. The higher cytotoxic activity of **4b** and **6c** is in agreement with their ability to induce ROS generation, and their pro-apoptotic effects. These data suggest that the cytotoxic potency of the compounds is related also to the level of ROS generation, not only to KSP inhibition, which suggests an additional mechanism of action. Based on docking studies, we cannot explain the higher activity of oxo-monastrol **4b** in relation to monastrol **3b**. Interestingly, we found that the replacement of the 3-hydroxyphenyl group by a 4-ferrocenylphenyl group induces the high antiproliferative activity towards the MCF7 breast cancer cell line, which is currently being further investigated.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Synthesis of all reagents and target compounds, copies of the NMR spectra and HPLC-ESI-MS analysis, X-ray analysis, condition for biological assays, antiproliferative/cytotoxic activity assay, cell cycle analysis, reactive oxygen species formation, kinesin ATPase inhibition assay, docking studies protocol (PDF).

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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REFERENCES

1. Dumontet, C.; Jordan, M. A., Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat. Rev. Drug Discov.* **2010,** *9* (10), 790-803.

2. Tischer, J.; Gergely, F., Anti-mitotic therapies in cancer. *J. Cell Biol.* **2018,** *218* (1), 10-11.

3. Desai, A.; Mitchison, T. J., Microtubule polymerization dynamics. *Annu. Rev. Cell Dev. Biol.* **1997,** *13* (1), 83-117.

4. Joshi, H. C., Microtubule dynamics in living cells. *Curr. Opin. Cell Biol.* **1998,** *10* (1), 35-44.

5. Lane, J., Microtubule-based membrane movement. *Biochim. Biophys. Acta* **1998,** *1376*, 27-55.

6. Canta, A.; Chiorazzi, A.; Cavaletti, G., Tubulin: a target for antineoplastic drugs into the cancer cells but also in the peripheral nervous system. *Curr. Med. Chem.* **2009,** *16* (11), 1315-1324.

7. Crown, J.; O'Leary, M.; Ooi, W. S., Docetaxel and paclitaxel in the treatment of breast cancer: a review of clinical experience. *Oncologist* **2004,** *9*, 24-32.

8. Rowinsky, M., Eric K, The development and clinical utility of the taxane class of antimicrotubule chemotherapy agents. *Annu. Rev. Med.* **1997,** *48* (1), 353-374.

9. Marzo, I.; Naval, J., Antimitotic drugs in cancer chemotherapy: promises and pitfalls. *Biochem. Pharmacol.* **2013,** *86* (6), 703-710.

10. and, L. S. B. G.; Philp, A. V., The Road Less Traveled: Emerging Principles of Kinesin Motor Utilization. *Annu. Rev. Cell Dev. Biol.* **1999,** *15* (1), 141-183.

11. Sauer, G.; Körner, R.; Hanisch, A.; Ries, A.; Nigg, E. A.; Silljé, H. H. W., Proteome Analysis of the Human Mitotic Spindle\*. *Mol. Cell Proteomics* **2005,** *4* (1), 35-43.

12. Cochran, J. C.; Gatial, J. E.; Kapoor, T. M.; Gilbert, S. P., Monastrol inhibition of the mitotic kinesin Eg5. *J. Biol. Chem.* **2005,** *280* (13), 12658-12667.

13. Myers, K. A.; Baas, P. W., Kinesin-5 regulates the growth of the axon by acting as a brake on its microtubule array. *J. Cell Biol.* **2007,** *178* (6), 1081-1091.

14. Sharp, D. J.; Rogers, G. C.; Scholey, J. M., Microtubule motors in mitosis. *Nature* **2000,** *407* (6800), 41-47.

15. Wittmann, T.; Hyman, A.; Desai, A., The spindle: A dynamic assembly of microtubules and motors. *Nat. Cell Biol.* **2001,** *3* (1), E28-E34.

16. Sarli, V.; Giannis, A., Targeting the Kinesin Spindle Protein: Basic Principles and Clinical Implications. *Clin. Cancer Res.* **2008,** *14* (23), 7583.

17. Ding, S.; Xing, N.; Lu, J.; Zhang, H.; Nishizawa, K.; Liu, S.; Yuan, X.; Qin, Y.; Liu, Y.; Ogawa, O., Overexpression of Eg5 predicts unfavorable prognosis in non‐muscle invasive bladder urothelial carcinoma. *Int. J. Urol.* **2011,** *18* (6), 432-438.

18. Liu, C.; Zhou, N.; Li, J.; Kong, J.; Guan, X.; Wang, X., Eg5 Overexpression Is Predictive of Poor Prognosis in Hepatocellular Carcinoma Patients. *Dis. Markers* **2017,** *2017*, 2176460-2176460.

19. Liu, M.; Wang, X.; Yang, Y.; Li, D.; Ren, H.; Zhu, Q.; Chen, Q.; Han, S.; Hao, J.; Zhou, J., Ectopic expression of the microtubule‐dependent motor protein Eg5 promotes pancreatic tumourigenesis. *J. Pathol.* **2010,** *221* (2), 221-228.

20. Sun, D.; Lu, J.; Ding, K.; Bi, D.; Niu, Z.; Cao, Q.; Zhang, J.; Ding, S., The expression of Eg5 predicts a poor outcome for patients with renal cell carcinoma. *Med. Oncol.* **2013,** *30* (1), 476.

21. DeBonis, S.; Skoufias, D. A.; Lebeau, L.; Lopez, R.; Robin, G.; Margolis, R. L.; Wade, R. H.; Kozielski, F., In vitro screening for inhibitors of the human mitotic kinesin Eg5 with antimitotic and antitumor activities. *Mol. Cancer Ther.* **2004,** *3* (9), 1079-1090.

22. Duan, L.; Wang, T. Q.; Bian, W.; Liu, W.; Sun, Y.; Yang, B. S., Centrin: Another target of monastrol, an inhibitor of mitotic spindle. *Spectroc. Acta Pt. A-Molec. Biomolec. Spectr.* **2015,** *137*, 1086-1091.

23. Good, J. A.; Berretta, G.; Anthony, N. G.; Mackay, S. P., The discovery and development of Eg5 inhibitors for the clinic. In *Kinesins and Cancer*, Springer: 2015; pp 27-52.

24. Myers, S. M.; Collins, I., Recent findings and future directions for interpolar mitotic kinesin inhibitors in cancer therapy. *Future Med. Chem.* **2016,** *8* (4), 463-489.

25. Wood, K. W.; Cornwell, W. D.; Jackson, J. R., Past and future of the mitotic spindle as an oncology target. *Curr. Opin. Pharmacol.* **2001,** *1* (4), 370-377.

26. Mayer, T. U.; Kapoor, T. M.; Haggarty, S. J.; King, R. W.; Schreiber, S. L.; Mitchison, T. J., Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* **1999,** *286* (5441), 971-974.

27. Kaan, H. Y. K.; Ulaganathan, V.; Rath, O.; Prokopcova, H.; Dallinger, D.; Kappe, C. O.; Kozielski, F., Structural basis for inhibition of Eg5 by dihydropyrimidines: stereoselectivity of antimitotic inhibitors enastron, dimethylenastron and fluorastrol. *J. Med. Chem.* **2010,** *53* (15), 5676-5683.

28. Gartner, M.; Sunder-Plassmann, N.; Seiler, J.; Utz, M.; Vernos, I.; Surrey, T.; Giannis, A., Development and Biological Evaluation of Potent and Specific Inhibitors of Mitotic Kinesin Eg5. *ChemBioChem* **2005,** *6* (7), 1173-1177.

29. Garcia-Saez, I.; DeBonis, S.; Lopez, R.; Trucco, F.; Rousseau, B.; Thuéry, P.; Kozielski, F., Structure of human Eg5 in complex with a new monastrol-based inhibitor bound in the R configuration. *J. Biol. Chem.* **2007,** *282* (13), 9740-9747.

30. Yang, L.; Jiang, C.; Liu, F.; You, Q.-D.; Wu, W.-T., Cloning, Enzyme Characterization of Recombinant Human Eg5 and the Development of a New Inhibitor. *Biol. Pharm. Bull.* **2008,** *31* (7), 1397-1402.

31. Garcia-Saez, I.; Skoufias, D. A., Eg5 targeting agents: From new anti-mitotic based inhibitor discovery to cancer therapy and resistance. *Biochem. Pharmacol.* **2021,** *184*, 114364.

32. El-Nassan, H. B., Advances in the discovery of kinesin spindle protein (Eg5) inhibitors as antitumor agents. *Eur. J. Med. Chem.* **2013,** *62*, 614-631.

33. Ghosh, S., Cisplatin: The first metal based anticancer drug. *Bioorg. Chem.* **2019,** *88*, 102925.

34. Wheate, N. J.; Walker, S.; Craig, G. E.; Oun, R., The status of platinum anticancer drugs in the clinic and in clinical trials. *Dalton. Trans.* **2010,** *39* (35), 8113-8127.

35. Steel, T. R.; Walsh, F.; Wieczorek-Błauż, A.; Hanif, M.; Hartinger, C. G., Monodentately-coordinated bioactive moieties in multimodal half-sandwich organoruthenium anticancer agents. *Coord. Chem. Rev.* **2021,** *439*, 213890.

36. Patra, M.; Gasser, G., The medicinal chemistry of ferrocene and its derivatives. *Nat. Rev. Chem.* **2017,** *1* (9), 0066.

37. Chellan, P.; Sadler, P. J., Enhancing the Activity of Drugs by Conjugation to Organometallic Fragments. *Chem. Eur. J.* **2020,** *26* (40), 8676-8688.

38. Jaouen, G.; Vessières, A.; Top, S., Ferrocifen type anti cancer drugs. *Chem. Soc. Rev.* **2015,** *44* (24), 8802-8817.

39. Costa, N. C.; Piccoli, J. P.; Santos-Filho, N. A.; Clementino, L. C.; Fusco-Almeida, A. M.; De Annunzio, S. R.; Fontana, C. R.; Verga, J. B.; Eto, S. F.; Pizauro-Junior, J. M., Antimicrobial activity of RP-1 peptide conjugate with ferrocene group. *PLoS One* **2020,** *15* (3), e0228740.

40. Daniluk, M.; Buchowicz, W.; Koszytkowska-Stawińska, M.; Jarząbek, K.; Jarzembska, K. N.; Kamiński, R.; Piszcz, M.; Laudy, A. E.; Tyski, S., Ferrocene Amino Acid Ester Uracil Conjugates: Synthesis, Structure, Electrochemistry and Antimicrobial Evaluation. *ChemistrySelect* **2019,** *4* (37), 11130-11135.

41. Patra, M.; Gasser, G.; Metzler-Nolte, N., Small organometallic compounds as antibacterial agents. *Dalton. Trans.* **2012,** *41* (21), 6350-6358.

42. Wenzel, M.; Patra, M.; Senges, C. H. R.; Ott, I.; Stepanek, J. J.; Pinto, A.; Prochnow, P.; Vuong, C.; Langklotz, S.; Metzler-Nolte, N.; Bandow, J. E., Analysis of the mechanism of action of potent antibacterial hetero-tri-organometallic compounds: A structurally new class of antibiotics. *ACS Chem. Biol.* **2013,** *8* (7), 1442-1450.

43. Ludwig, B. S.; Correia, J. D. G.; Kühn, F. E., Ferrocene derivatives as anti-infective agents. *Coord. Chem. Rev.* **2019,** *396*, 22-48.

44. Dive, D.; Biot, C., Ferroquine as an oxidative shock antimalarial. *Curr. Top. Med. Chem.* **2014,** *14* (14), 1684-1692.

45. Dubar, F.; Slomianny, C.; Khalife, J.; Dive, D.; Kalamou, H.; Guérardel, Y.; Grellier, P.; Biot, C., The ferroquine antimalarial conundrum: redox activation and reinvasion inhibition. *Angew. Chem. Int. Ed.* **2013,** *52* (30), 7690-7693.

46. Wells, T.; van Huijsduijnen Hooft, R., Ferroquine: welcome to the next generation of antimalarials. *Lancet Infect. Dis.* **2015,** *15* (12), 1365-1366.

47. Al-Masoudi, W. A.; Al-Masoudi, N. A., A ruthenium complexes of monastrol and its pyrimidine analogues: Synthesis and biological properties. *Phosphorus Sulfur Silicon Relat. Elem.* **2019,** *194* (11), 1020-1027.

48. Al-Masoudi, W. A.; Al-Masoudi, N. A.; Weibert, B.; Winter, R., Synthesis, X-ray structure, in vitro HIV and kinesin Eg5 inhibition activities of new arene ruthenium complexes of pyrimidine analogs. *J. Coord. Chem.* **2017,** *70* (12), 2061-2073.

49. Łomzik, M.; Hanif, M.; Budniok, A.; Błauż, A.; Makal, A.; Tchoń, D. M.; Leśniewska, B.; Tong, K. K. H.; Movassaghi, S.; Söhnel, T.; Jamieson, S. M. F.; Zafar, A.; Reynisson, J.; Rychlik, B.; Hartinger, C. G.; Plażuk, D., Metal-Dependent Cytotoxic and Kinesin Spindle Protein Inhibitory Activity of Ru, Os, Rh, and Ir Half-Sandwich Complexes of Ispinesib-Derived Ligands. *Inorg. Chem.* **2020,** *59* (20), 14879-14890.

50. Csámpai, A.; Györfi, A.; Turos, G. I.; Sohar, P., Application of Biginelli reaction to the synthesis of ferrocenylpyrimidones and [3]-ferrocenophane-containing pyrimido [4, 5-d] pyrimidinediones. *J. Organomet. Chem.* **2009,** *694* (22), 3667-3673.

51. Fu, N.-Y.; Yuan, Y.-F.; Pang, M.-L.; Wang, J.-T.; Peppe, C., Indium(III) halides-catalyzed preparation of ferrocene-dihydropyrimidinones. *J. Organomet. Chem.* **2003,** *672* (1), 52-57.

52. Kiss, K.; Csámpai, A.; Sohár, P., New ferrocenyl-substituted heterocycles. Formation under Biginelli conditions, DFT modelling, and structure determination. *J. Organomet. Chem.* **2010,** *695* (15-16), 1852-1857.

53. Wang, R.; Liu, Z.-Q., Ferrocene as a functional group enhances the inhibitive effect of dihydropyrimidine on radical-induced oxidation of DNA. *Org. Chem. Front.* **2014,** *1* (7), 792-797.

54. Chrabaszcz, K.; Blauz, A.; Gruchala, M.; Wachulec, M.; Rychlik, B.; Plazuk, D., Synthesis and Biological Activity of Ferrocenyl and Ruthenocenyl Analogues of Etoposide: Discovery of a Novel Dual Inhibitor of Topoisomerase II Activity and Tubulin Polymerization. *Chem. Eur. J.* **2021,** *n/a* (n/a).

55. Kowalczyk, K.; Blauz, A.; Ciszewski, W. M.; Wieczorek, A.; Rychlik, B.; Plazuk, D., Colchicine metallocenyl bioconjugates showing high antiproliferative activities against cancer cell lines. *Dalton. Trans.* **2017,** *46* (48), 17041-17052.

56. Plażuk, D.; Wieczorek, A.; Błauż, A.; Rychlik, B., Synthesis and biological activities of ferrocenyl derivatives of paclitaxel. *MedChemComm* **2012,** *3* (4), 498-501.

57. Plażuk, D.; Wieczorek, A.; Ciszewski, W. M.; Kowalczyk, K.; Błauż, A.; Pawlędzio, S.; Makal, A.; Eurtivong, C.; Arabshahi, H. J.; Reynisson, J.; Hartinger, C. G.; Rychlik, B., Synthesis and in vitro Biological Evaluation of Ferrocenyl Side-Chain-Functionalized Paclitaxel Derivatives. *ChemMedChem* **2017,** *12* (22), 1882-1892.

58. Wieczorek, A.; Blauz, A.; Makal, A.; Rychlik, B.; Plazuk, D., Synthesis and evaluation of biological properties of ferrocenyl-podophyllotoxin conjugates. *Dalton. Trans.* **2017,** *46* (33), 10847-10858.

59. Wieczorek, A.; Blauz, A.; Zal, A.; Arabshahi, H. J.; Reynisson, J.; Hartinger, C. G.; Rychlik, B.; Plazuk, D., Ferrocenyl Paclitaxel and Docetaxel Derivatives: Impact of an Organometallic Moiety on the Mode of Action of Taxanes. *Chem. Eur. J.* **2016,** *22* (32), 11413-21.

60. Cepanec, I.; Litvić, M.; Filipan-Litvić, M.; Grüngold, I., Antimony(III) chloride-catalysed Biginelli reaction: a versatile method for the synthesis of dihydropyrimidinones through a different reaction mechanism. *Tetrahedron* **2007,** *63* (48), 11822-11827.

61. Russowsky, D.; Canto, R. F. S.; Sanches, S. A. A.; D'Oca, M. G. M.; de Fatima, A.; Pilli, R. A.; Kohn, L. K.; Antonio, M. A.; de Carvalho, J. E., Synthesis and differential antiproliferative activity of Biginelli compounds against cancer cell lines: Monastrol, oxo-monastrol and oxygenated analogues. *Bioorg. Chem.* **2006,** *34* (4), 173-182.

62. Plazuk, D.; Vessieres, A.; Hillard, E. A.; Buriez, O.; Labbe, E.; Pigeon, P.; Plamont, M. A.; Amatore, C.; Zakrzewski, J.; Jaouen, G., A [3]ferrocenophane polyphenol showing a remarkable antiproliferative activity on breast and prostate cancer cell lines. *J. Med. Chem.* **2009,** *52* (15), 4964-7.

63. Pidathala, C.; Amewu, R.; Pacorel, B.; Nixon, G. L.; Gibbons, P.; Hong, W. D.; Leung, S. C.; Berry, N. G.; Sharma, R.; Stocks, P. A.; Srivastava, A.; Shone, A. E.; Charoensutthivarakul, S.; Taylor, L.; Berger, O.; Mbekeani, A.; Hill, A.; Fisher, N. E.; Warman, A. J.; Biagini, G. A.; Ward, S. A.; O’Neill, P. M., Identification, Design and Biological Evaluation of Bisaryl Quinolones Targeting Plasmodium falciparum Type II NADH:Quinone Oxidoreductase (PfNDH2). *J. Med. Chem.* **2012,** *55* (5), 1831-1843.

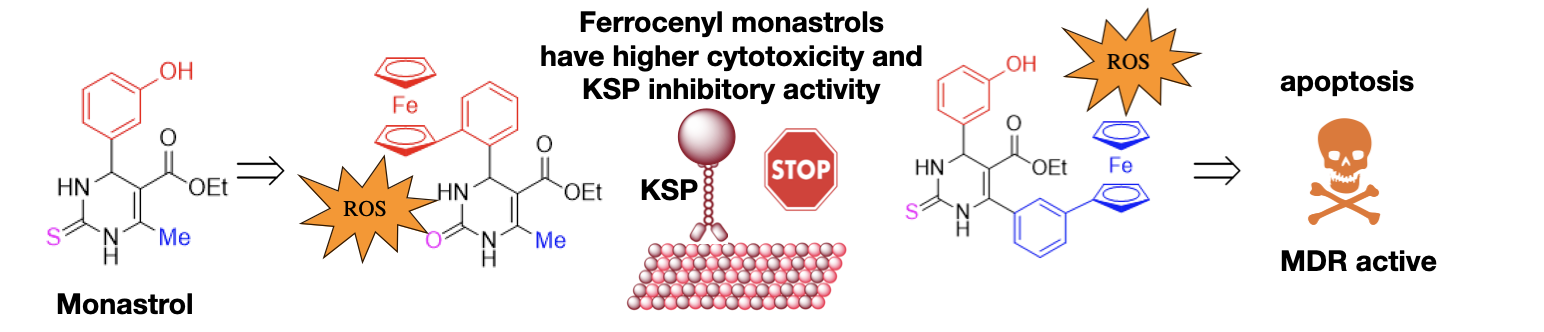
64. Blauz, A.; Rychlik, B., Drug-selected cell line panels for evaluation of the pharmacokinetic consequences of multidrug resistance proteins. *J. Pharmacol. Toxicol. Methods* **2017,** *84*, 57-65.

65. Asraf, H.; Avunie-Masala, R.; Hershfinkel, M.; Gheber, L., Mitotic Slippage and Expression of Survivin Are Linked to Differential Sensitivity of Human Cancer Cell-Lines to the Kinesin-5 Inhibitor Monastrol. *PLoS One* **2015,** *10* (6), e0129255.

66. Soumyanarayanan, U.; Bhat, V. G.; Kar, S. S.; Mathew, J. A., Monastrol mimic Biginelli dihydropyrimidinone derivatives: synthesis, cytotoxicity screening against HepG2 and HeLa cell lines and molecular modeling study. *Org. Med. Chem. Lett.* **2012,** *2* (1), 23.

67. Maliga, Z.; Kapoor, T. M.; Mitchison, T. J., Evidence that Monastrol Is an Allosteric Inhibitor of the Mitotic Kinesin Eg5. *Chem. Biol.* **2002,** *9* (9), 989-996.

68. Fersht, A., *Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding*. Macmillan: 1999.



Introduction of the ferrocenyl group into the monastrol scaffold improved the cytotoxic activity, increased the kinesin-spindle-protein (KSP) inhibitory activity as well as its ability to generate reactive oxygen species (ROS) which led to increased levels of apoptotic cells.