Development and Biological Evaluation of Potent and Specific Inhibitors of Mitotic Kinesin Eg5

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Anticancer drugs that perturb mitosis, for example, vinca alkaloids, taxol and epothilone, play a major role in the therapy of malignant diseases. One of their major drawbacks is that they are all directed against the same protein, tubulin—the microtubule subunit which forms the mitotic spindle.[1,2] However, microtubules are also involved in many other cellular processes such as maintenance of organelles and cell shape, cell motility, synaptic vesicles and intracellular transport phenomena.[3,4,5] Interference with their formation or depolymerisation often leads to dose-limiting side effects like, for example, neurotoxicity.

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The discovery of a new class of proteins, the mitotic kinesins, presents a novel approach to cancer treatment.[6] These proteins are exclusively involved in the formation and function of the mitotic spindle and some of them are only expressed in proliferating cells.[7,8] Their inhibition leads to cell cycle arrest and ultimately to apoptosis without interfering with other microtubule-dependent processes.[6]

The mitotic kinesin, Eg5 (also called kinesin-5 or kinesin spindle protein, KSP) plays an important role in the early stages of mitosis. It mediates centrosome separation and formation of the bipolar mitotic spindle.[9] Inhibition of Eg5 leads to cell-cycle arrest during mitosis and cells with a monopolar spindle, so-called monoasters.[10] In 1999 the screening of a large library of synthetic compounds identified racemic monastrol, a 4-aryl-3,4-dihydropyrimidin-2(1*H*)-thione derivative, as the first small-molecule inhibitor of Eg5.[11] Monastrol is a moderate allosteric inhibitor ($IC_{50} = 30\ \mu M$) of Eg5[12] which binds some 12 Å away from the nucleotide binding site of the protein. In doing so, it triggers both local and distal structural changes throughout the motor domain.[13] In the meantime, four other inhibitors of Eg5 have been published: terpendole E ($IC_{50} = 14.6\ \mu M$), *S*-trityl-L-cysteine ($IC_{50} = 1.0\ \mu M$), HR22C16 ($IC_{50} = 0.8\ \mu M$) and CK0106023 ($IC_{50} = 12\ nM$)[14–17]. According to recent investigations monastrol does not display any neurotoxicity in fact, short-term treatment with monastrol has been reported to enhance axonal growth—in contrast to anticancer drugs such as the taxanes which are highly deleterious to axonal formation and growth. There is no indication of any kind of toxicity caused by monastrol to cultures of sympathetic neurons over longer exposure times.[18] For the other Eg5 inhibitors mentioned above such studies have not been reported. For these reasons we focused on the development of potent, specific and cell permeable monastrol analogues. Here we describe the discovery of such derivatives and their inhibitory activity against Eg5 as well as their ability to inhibit cell division.

The monastrol analogues **1**–**4** (Scheme 1 A, B) were synthesized as racemic mixtures by using the Biginelli reaction. Either the appropriate aldehyde, urea and ethyl acetoacetate,[19] were heated or the appropriate aldehyde, urea or thiourea and the 1,3-dicarbonyl compound were irradiation together with polyphosphate ester (PPE) in a domestic microwave oven[20–23] (Scheme 1 A, B and Table 1). Alcohol **5** was synthesized from **4a** by selective Luche reduction of the 5-carbonyl function and used as a 3:1 diastereomeric mixture—every diastereomer as enantiomeric mixture (Scheme 1 C).[24]

Subsequently we screened 40 compounds for their ability to inhibit Eg5 by using an in vitro steady-state ATPase assay. We found that most of the synthesized compounds were less potent Eg5 inhibitors as compared to 36% inhibition by monastrol (**2c**). Nine of the compounds showed less than 5% inhibition of Eg5 activity under the assay conditions (Table 2). Three compounds, however, were significantly more potent than monastrol (Figure 1 A, Table 2). The assay showed that enhancement of inhibition cannot be achieved by variation of the aromatic substitution pattern of the 4-aryl moiety in 4-aryl-3,4-dihydropyrimidin-2(1*H*)-ones or -thiones, compared to monastrol (**2c**). Furthermore, the use of sterically demanding

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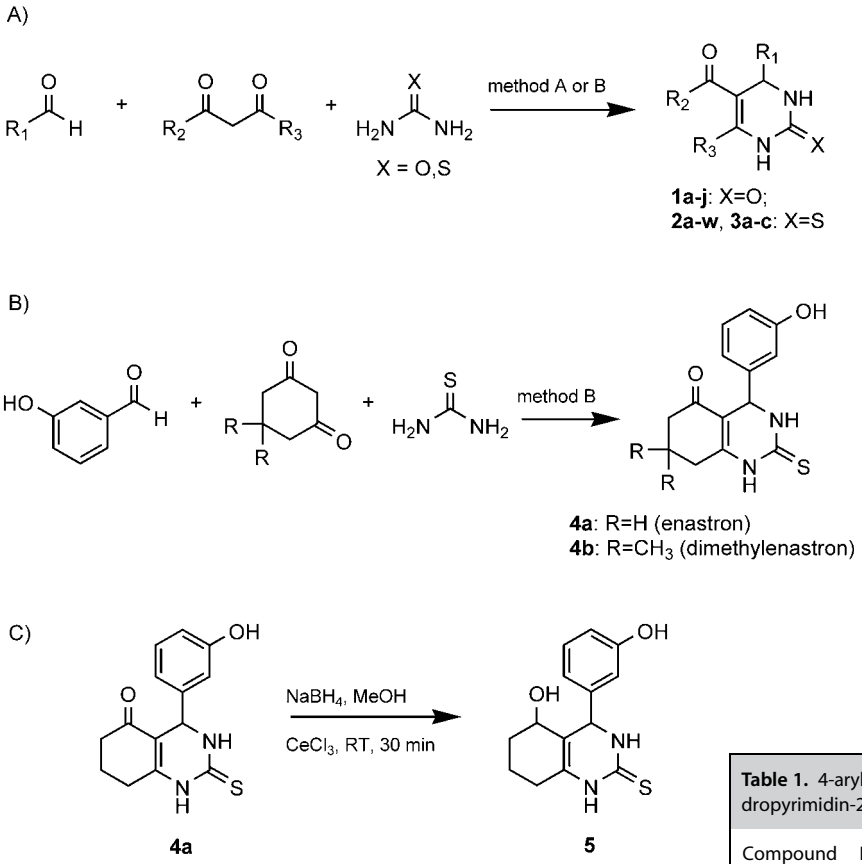

Scheme 1. Synthesis of monastrol analogues. A) Synthesis of **1a–j**, **2a–w** and **3a–c** derivatives. B) Synthesis of enastron (**4a**) and dimethylenastron (**4b**); yields are 13% and 22%, respectively. C) Synthesis of enastrol (**5**). Method A: 105 °C, 2 h; method B: polyphosphate ester, microwave irradiation.

residues at the 4-position, like naphthyl- or styryl-residues, led to a decreased Eg5 inhibition (e.g., compounds **2o** and **2p**). Also, substitution of the methyl group by a phenyl group at 6-position of the 3,4-dihydropyrimidin-2(1*H*)-thione scaffold resulted in a decrease of Eg5 inhibition as demonstrated by the monastrol analogue **3a**. Nevertheless, compounds with a thiocarbonyl group in 2-position, in general showed higher inhibition levels than the same compounds with a carbonyl group at this position (for example compound **1e** vs. **2c**). However, a distinct increase in Eg5 inhibition compared to monastrol was achieved with the bicyclic compounds **4a**, **5** and particularly **4b**. Taken together, these results show that the thiocarbonyl group at 2-position as well as the 3-hydroxyphenyl group at 4-position of the 4-aryl-3,4-dihydropyrimidin derivatives seem to be necessary for high inhibition levels of the mitotic kinesin, Eg5. However, a 3-carboxyphenyl group at 4-position of the heterocyclic part of these derivatives is also tolerated. In fact, the corresponding derivative **2u** has a similar potency to monastrol ($IC_{50} = 32\ \mu M$). Furthermore, conformational rigidization through cyclisation of the side chains, which results in a cyclic ketone, leads to a significantly better inhibition compared to monastrol. Reduction of the carbonyl group (**4a**) to the corresponding alcohol does not affect inhibitory properties. However, the two additional methyl groups at 7-position in **4b** lead to a distinct increase of Eg5 inhibition compared to **4a**. These results are supported by data from the molecular modelling program MOLOC[25,26] based on the published X-ray structure of monastrol with Eg5.[13] Especially the methyl group with *syn*-orientation relative to the phenol ring, established strong, attractive interactions with the protein. The aromatic ring of Tyr211 and the side chains and backbones of Glu215 and Ala218 are involved in this interaction.

We propose the names enastron (**4a**), dimethylenastron (**4b**) and enastrol (**5**) for these compounds (Greek en = one, astron = aster, star). We measured the IC_{50} values for these three significantly improved inhibitors and found that enastron and enastrol had an IC_{50} of 2 μM, that is, they were more than ten-times more potent inhibitors of Eg5 as compared to monastrol, which has an IC_{50} of 30 μM (Figure 1B, D). Dimethylenastron with an IC_{50} of 200 nM, was more than 100-times more potent

Table 1. 4-aryl-3,4-dihydropyrimidin-2(1*H*)-ones (**1a–j**) and 4-aryl-3,4-dihydropyrimidin-2(1*H*)-thiones (**2a–w**, **3a–c**).

Compound	R_1	R_2	R_3	X	yield [%]	method
1a	4-(OMe)-C_6H_4	OEt	Me	O	63	A
1b	4-($N(Me)_2$)-C_6H_4	OEt	Me	O	34	A
1c	3-(F)-4-(OMe)-C_6H_3	OEt	Me	O	29	A
1d	2-(NO_2)-C_6H_4	OEt	Me	O	31	B
1e	3-(OH)-C_6H_4	OEt	Me	O	26	B
1f	2-(NO_2)-5-(OH)-C_6H_3	OEt	Me	O	67	B
1g	3,5-$(F)_2$-C_6H_3	OEt	Me	O	60	B
1h	3-(Br)-C_6H_4	OEt	Me	O	54	B
1i	2-(Br)-C_6H_4	OEt	Me	O	47	B
1j	3-(F)-C_6H_4	OEt	Me	O	77	B
2a	C_6H_5	OEt	Me	S	22	B
2b	2-(NO_2)-C_6H_4	OEt	Me	S	38	B
2c	3-(OH)-C_6H_4	OEt	Me	S	39	B
2d	2-(NO_2)-5-(OH)-C_6H_3	OEt	Me	S	39	B
2e	3,5-$(F)_2$-C_6H_3	OEt	Me	S	61	B
2f	3-(F)-4-(OMe)-C_6H_3	OEt	Me	S	70	B
2g	3-(Br)-C_6H_4	OEt	Me	S	59	B
2h	2-(Br)-C_6H_4	OEt	Me	S	58	B
2i	3-(F)-C_6H_4	OEt	Me	S	8	B
2j	2-Furfuryl	OEt	Me	S	9	B
2k	1-Naphthyl	OEt	Me	S	33	B
2l	2,5-$(OMe)_2$-C_6H_3	OEt	Me	S	18	B
2m	3-(NO_2)-C_6H_4	OEt	Me	S	43	B
2n	4-(NO_2)-C_6H_4	OEt	Me	S	33	B
2o	2-Naphthyl	OEt	Me	S	50	B
2p	Ph-CH = CH	OEt	Me	S	11	B
2q	2-(NO_2)-4-($N(CH_3)_2$)-C_6H_3	OEt	Me	S	9	B
2r	2-(OCF_3)-C_6H_4	OEt	Me	S	57	B
2s	2-(NO_2)-Ph-CH = CH	OEt	Me	S	12	B
2t	2-(CF_3)-C_6H_4	OEt	Me	S	36	B
2u	3-(COOH)-C_6H_4	OEt	Me	S	28	B
2v	4-(COOH)-C_6H_4	OEt	Me	S	33	B
2w	4-(NO_2)-Ph-CH = CH	OEt	Me	S	12	B
3a	3-(OH)-C_6H_4	OEt	Ph	S	63	B
3b	3-(COOH)-C_6H_4	OEt	Ph	S	37	B
3c	4-(COOH)-C_6H_4	OEt	Ph	S	31	B

Table 2. Screening of the synthesized compounds for Eg5 inhibition. The inhibition values were determined by ATPase assay and are indicated as a percentage of the solvent only control. Results are the means of two independent measurements. The final concentration of the compounds in the screening assay was 46 μM.

compound	inhibition [%]	compound	inhibition [%]
1a	6	**2k**	12
1b	0	**2l**	3
1c	12	**2m**	0
1d	3	**2n**	0
1e	27	**2o**	0
1f	15	**2p**	6
1g	18	**2q**	22
1h	23	**2r**	12
1i	14	**2s**	23
1j	19	**2t**	12
2a	4	**2u**	33
2b	23	**2v**	20
2c (monastrol)	36	**2w**	14
2d	19	**3a**	26
2e	23	**3b**	16
2f	21	**3c**	11
2g	14	**4a** (enastron)	59
2h	23	**4b** (dimethylenastron)	66
2i	17	**5**	64
2j	3		

than monastrol (Figure 1C). All three compounds inhibited Eg5 activity completely at concentrations above the IC_{50}. In control experiments inhibition of kinesins belonging to five other kinesin subfamilies (kinesin-1, kinesin-4, kinesin-7, kinesin-10 and one ungrouped—originating from 4 different organisms) was not observed with these three compounds (see Supporting Information). This indicates that the inhibition of Eg5 is specific.

We then tested the effect of enastron, dimethylenastron and enastrol on exponentially growing cultured human cells. HeLa cells were incubated for 18 h in medium supplemented with various concentrations of one of the three compounds, monastrol or DMSO. Cells were then fixed and analyzed by fluorescence-activated cell sorter (FACS) analysis and immunofluorescence (Figure 2). FACS analysis showed that monastrol blocked cells efficiently in the G2/M phase of the cell cycle (74%) at 100 μM. A lower concentration (10 μM) promoted only a very small increase in the number of G2/M cells (31% versus 23% in the control). This is in agreement with previous reports.[11] The three compounds tested also blocked cells in G2/M but did so at considerably lower concentrations than monastrol. Indeed, 10 μM enastron or enastrol had the same effect as 100 μM monastrol, promoting the accumulation of 77% of

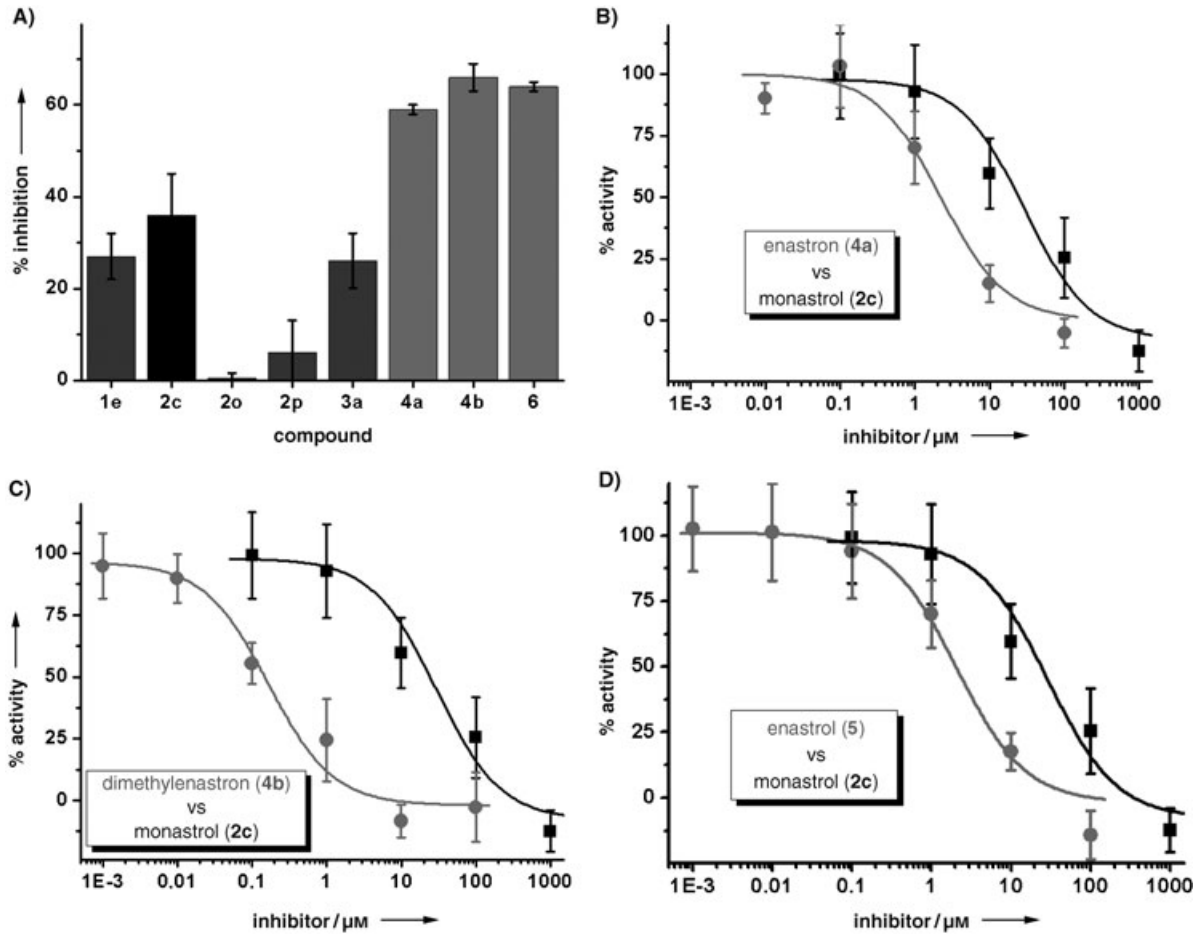

Figure 1. Inhibition of the microtubule-stimulated ATPase activity of Eg5. A) Relative inhibition of Eg5 by selected compounds in the initial screen. Eg5 inhibition is dependent on the concentrations of the three best inhibitors identified in the screen. B) Enastron (**4a**), C) dimethylenastron (**4b**) and, D) enastrol (**5**) as compared to monastrol (**2c**).

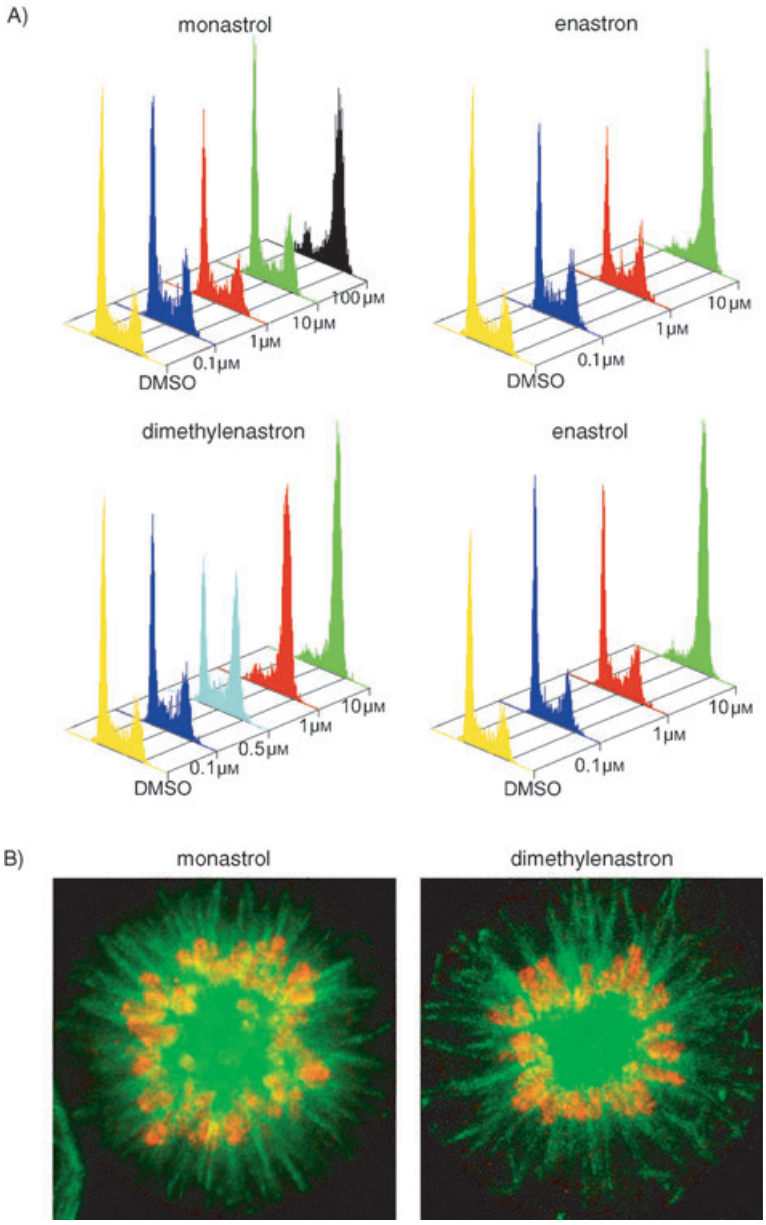

Figure 2. Effect of enastrol (**6**), enastron (**4a**) and dimethylenastron (**4b**) on exponentially growing HeLa cells. A) FACS analysis of exponentially growing HeLa cells after 18 h incubation with various concentrations of monastrol, enastron, dimethylenastron and enastrol. The amplitude of the curves corresponds to the cell number. A peak on the left side represents cells in G1 phase of the cell cycle, while a peak on the right side represents cells in G2/M phase. B) Representative confocal images of cells treated with monastrol and dimethylenastron (right). Microtubules are shown in green and DNA in red.

cells in G2/M. Strikingly, only 1 μM dimethylenastron was sufficient for the accumulation of 75% of the cells in G2/M cells. No cell cycle distribution abnormalities were observed in cells treated with 0.1 μM dimethylenastron. We therefore looked at the effect of intermediate concentrations of this compound and found that 0.5 μM dimethylenastron resulted in the accumulation of 47% of cells in G2/M. The concentrations required for mitotic arrest were similar to those required for the in vitro inhibition of Eg5 activity. This suggests a mechanism for cell-cycle arrest mediated specifically through Eg5 inhibition.

One useful characteristic of monastrol is that it is nontoxic to cells when applied transiently—once washed out, cells resume mitosis. We therefore tested whether the three new compounds also shared this property. HeLa cells were incubated with various concentrations of the different compounds. After 18 h the compounds were washed out and the cells incubated in fresh medium for 24 h before analysis. Cells were found to have reentered cell cycle under the conditions tested. This indicated that like monastrol, the three new compounds can be washed out from the cells and are nontoxic (data not shown).

We then examined the morphology of cells treated with the different compounds with immonufluorescence by using an anti-tubulin antibody and Hoechst to stain DNA. The same phenotype that is typical for Eg5 inhibition was observed for monastrol and the three test compounds: cells were arrested in mitosis with a radial arrangement of microtubules and chromosomes (Figure 2).

Finally, we tested whether the compounds would generate the same effect in *Xenopus* egg extracts as with HeLa cells. 100 μM monastrol, 10 μM enastron or enastrol or 1 μM dimethylenastron were added to a *Xenopus* egg extract. Spindle assembly was monitored and we found that all compounds inhibited bipolar spindle formation and promoted the formation of circular figures formed by radial arrangement of microtubules and chromosomes (data not shown) as was previously described for monastrol[11] and as we observed with cultured cells.

In summary we have identified novel, specific and very potent cell-permeable inhibitors of the mitotic motor, Eg5. These inhibitors of the quinazoline-2(1*H*)-thione class of compounds were obtained by modifying the 4-aryl-3,4-dihydropyrimidin-2(1*H*)-thione scaffold of monastrol in a systematic fashion. The most potent compound, dimethylenastron, is up to more than 100-times more potent than monastrol, both in vitro and with arresting mitosis of cultured cells. Comparisons between more and less potent compounds of a synthesized library of monastrol derivatives allowed us to establish the structure–activity relationship of this class of inhibitors. These novel inhibitors have the potential to be very potent anticancer drug candidates.[27]

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