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New chemical tools for investigating human mitotic kinesin Eg5

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Abstract—We have designed and synthesized a series of monastrol derivatives, an allosteric inhibitor of Eg5, a motor protein responsible for the formation and maintenance of the bipolar spindle in mitotic cells. Sterically demanding structural modifications have been introduced on the skeleton of the parent drug either via a multicomponent Biginelli reaction or a stepwise modification of monastrol. The ability of these compounds to inhibit Eg5 activity has been investigated using two in vitro steady-state ATPase assays (basal and microtubule-stimulated) as well as a cell-based assay. One compound in the series appeared more potent than monastrol by a fivefold factor. Three other compounds that were unable to inhibit Eg5 ATPase activity in vitro proved potent Eg5 inhibitors in the cell-based assay. The results obtained led to the identification of structure–activity relationships further used to design an affinity matrix that can be used for fast and efficient purification of Eg5 from crude lysate of eukaryotic cells.

1. Introduction

Kinesin motors are specialized enzymes that use ATP hydrolysis to generate force and movement along their cellular tracks, the microtubules (MTs).[1] They are implicated in cell division and therefore have generated interest as potential protein targets in the treatment of cancer.[2–4] Indeed one of the major drawbacks associated with anticancer drugs that perturb mitosis (e.g., vinca alkaloids, taxanes, epothilones, . . .) is that they are all directed against the same protein, tubulin, the microtubule subunit which forms the mitotic spindle.[5] As 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, interference with their formation or depolymerization often leads to dose-limiting side effects like, for example, peripheral neuropathy.[6]

One of these targets in the kinesin superfamily, Eg5, a member of the kinesin-5 family, is responsible for the formation and maintenance of the bipolar spindle.[7,8] When inhibited by specific inhibitors, cells arrest in mitosis with a monoastral spindle phenotype. Human Eg5 has been recently identified as one of the potential targets of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester **1**, further named monastrol[9,10] (Scheme 1). That racemic dihydropyrimidone (DHPM) is an allosteric inhibitor of Eg5[11] and recent in vitro studies with cultured neurons showed that it does not display any toxicity, short term treatment even enhancing axonal growth[12,13] in contrast to anticancer drugs such as the taxanes which are highly deleterious to axonal formation and growth. Whether these results will translate into a lack of neurotoxicity in animals or even in humans is a key question which has not been answered so far.

DHPM is obtained via probably what is one of the first described multicomponent reactions. Indeed the venerable Biginelli dihydropyrimidine synthesis was discovered by Pietro Biginelli who reported in 1893 on the acid-catalyzed cyclocondensation reaction of ethyl acetoacetate, benzaldehyde, and urea[14] (Scheme 1). During more than a century, only few chemists investigated the synthetic utility and scope of the Biginelli reaction and less than 40 reports have appeared in the literature before 1999. The discovery of the potential anticancer activity of monastrol probably is not irrelevant to, or at least

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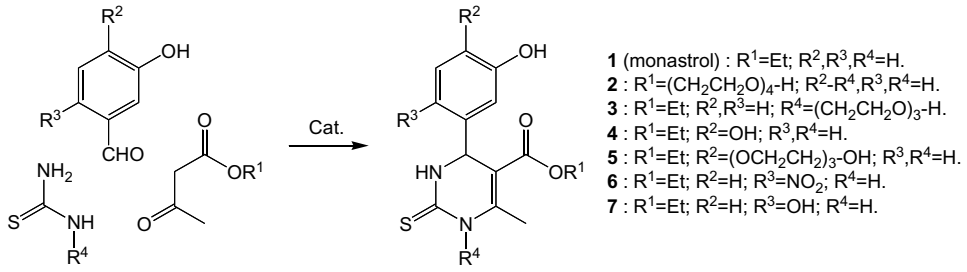


Scheme 1. The Biginelli condensation reaction and structure of monastrol **1** and analogs.

coincides with, an outbreak in the interest for the old transformation as more than 350 articles have been published on the Biginelli condensation since 1999.

In the course of a research program focused on the structural analysis of kinesins, especially Eg5, we were confronted with the necessity to probe and purify kinesins from different sources as well as mutants. Thus we synthesized a series of monastrol derivatives for a rapid determination of the structure–activity relationship, only scarce data being available in the literature.[9,15,16] The results obtained from in vitro and cell-based assays allowed us to design an affinity matrix for purification of Eg5 recombinantly expressed in *Escherichia coli* and from HeLa cells. Herein we describe the synthesis of such monastrol derivatives, their potency to inhibit Eg5 ATPase activity, and their mitotic arrest phenotype. We report as well on the immobilization of a monastrol derivative onto a polymer matrix. Finally a protocol for the quick and efficient purification of full-length Eg5 from eukaryotic cells is discussed.

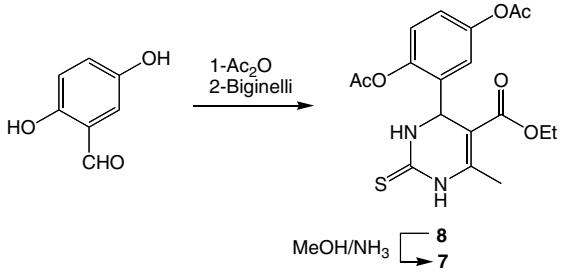
2. Results and discussion

2.1. Synthesis of monastrol derivatives

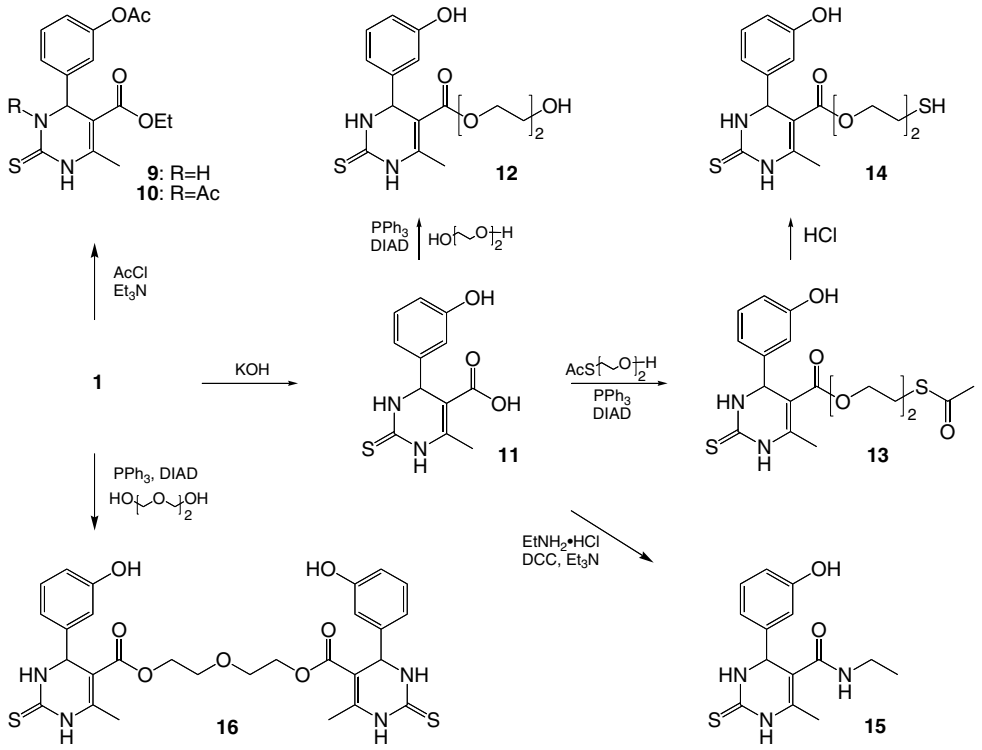
We coarsely identified three possible anchoring sites on the monastrol backbone. The first one is the ester moiety (C^5), the second one is the allylic nitrogen atom (N^1), and the last one is the phenol ring ($C^{4'}$ and $C^{6'}$). A fourth possibility for structural modification of the monastrol backbone has been investigated consisting in alkylation or replacement of sulfur at C^2 on the pyrimidine ring.

The different monastrol derivatives were synthesized either via a one-pot classical Biginelli procedure or by a linear reaction sequence starting from monastrol. Compound **2** was prepared applying the classical Biginelli condensation procedure[14] with 3-hydroxybenzaldehyde, thiourea, and 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl acetoacetate (Scheme 1). Compound **3** was synthesized using a similar protocol starting from the corresponding *N*-substituted thiourea, 3-hydroxybenzaldehyde, and ethyl acetoacetate. The low yield (7%) obtained for that reaction accounts for the poor tolerance of the Biginelli reaction with regard to urea *N*-substitution.[17,18] Compounds **4** and **5** resulted from the condensation of thiourea and ethyl acetoacetate with 3,4-dihydroxybenzaldehyde and the corresponding 4-alkoxy-3-hydroxybenzaldehyde, respectively. Similarly, compounds **6** and **7** were obtained starting from 5-hydroxy-2-nitro-benzaldehyde and 2,5-dihydroxy-benzaldehyde, respectively. However direct condensation of 2,5-dihydroxy-benzaldehyde with thiourea and ethyl acetoacetate in the Biginelli reaction failed to yield **7** whatever the experimental conditions used. The target compound was finally obtained via a two-step procedure involving 2,5-diacetoxy-benzaldehyde in the Biginelli condensation reaction to form 4-(2,5-diacetoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester **8** that was subsequently submitted to careful hydrolysis of acetates (Scheme 2). Both compounds **7** and **8** were poorly stable explaining the low yields obtained in these two transformations (see Section 4). Compounds **9**–**16** were obtained via stepwise transformations of monastrol (Scheme 3). Monoacetylation of monastrol **1** gave compound **9**, and diacetylated compound **10** was obtained as a side product. Hydrolysis of ethyl ester in **1** afforded acid **11** that was further condensed with different alcohols and amines to yield **12**, **13**, **15**, and **16**. Saponification of thioacetate **13** led to thiol **14**. A first modification at C^2 on the pyrimidine ring was introduced by substituting thiourea for selenourea in the Biginelli reaction to get compound **17** (Scheme 4). Adapting the alternate strategy for the synthesis of dihydropyrimidones developed by Atwal,[19] we prepared as well *S*-alkylated compounds **18** and **19**.

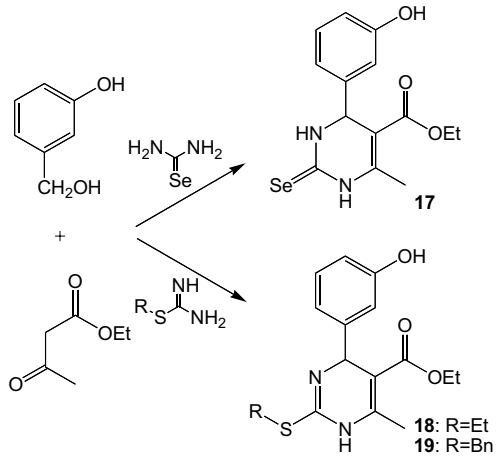
All the compounds prepared were analyzed for their ability to inhibit Eg5 by using in vitro steady state ATPase assays (basal and microtubule-stimulated activities) as well as a cell-based assay leading to the induction of monastrol spindles in HeLa cells (see Section 4). The results obtained are reported in Table 1.



Scheme 2. Synthesis of compounds **7** and **8**.



Scheme 3. Synthesis of compounds **9**–**16**.



Scheme 4. Synthesis of compounds **17**–**19**.

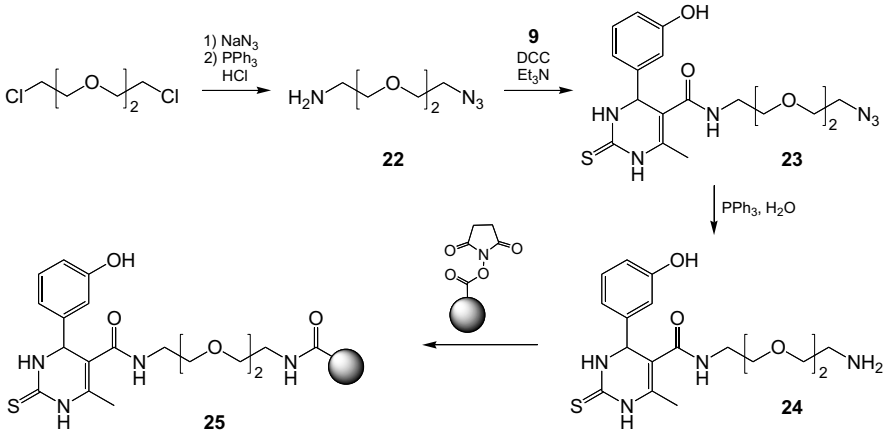
2.2. Inhibition of basal Eg5 ATPase activity

We found that the compounds modified at the aromatic ring in the $C^{4'}$ (compounds **4** and **5**), $C^{6'}$ (compounds **6**, **7**, and **8**), or $O^{3'}$ position (compounds **9** and **10**) do not inhibit Eg5 basal ATPase activity. Substitution at N^1 (compound **3**) was as well definitely detrimental to activity. More interesting are the compounds modified at the carboxyl group in the C^5 position. All of them are Eg5 inhibitors though a little less potent than monastrol in general. Carboxylic acid **11** resulting from ethyl ester hydrolysis in monastrol exhibits an IC_{50} value of 32 μM. Replacement of the ethyl ester in monastrol with the corresponding ethyl amide leads as well to a decrease in the basal ATPase activity, amide **15** being less active than monastrol by a fourfold factor. Increasing the length of the amide chain confirms that effect (compound **23**, see Scheme 5). However, introduction of a hydrophilic end group (NH_2, compound **24**) at the opposite of the chain has a reverse effect, presumably because of improved aqueous solubility. The same is observed when the ethyl ester is replaced with a long chain ester (compound **2**). For esters with intermediate chain length (compounds **12**–**14**), the initial basal ATPase activity of parent monastrol is preserved. It is likely that the difference in potency between **2** (long chain ester) and **12** (short chain ester) does not strictly result from steric hindrance within the binding site of the protein. If so, compound **16** would exhibit a poor inhibition score which is not the case (IC_{50} 10 μM) even considering the dimeric structure of the compound. In the same line of thought, the substitution of the free hydroxyl group at the extremity of the diethylene glycol arm in **12** for the bulkier acetylsulfanyl moiety in **13** does not result in a decrease of the activity, on the contrary. To explore one step further the influence of substitution at C^5, we introduced a furyl residue as a planar sterically demanding substituent at that position. The corresponding compound **20** (Fig. 1) proved more potent than monastrol by a fourfold factor. Finally, modifications performed at C^2 on the pyrimidine moiety gave contrasted results. Sulfur replacement for selenium (compound **17**, Scheme 4) provoked a twofold factor decrease in the inhibition efficacy. A recent report by Gartner et al. indicates that the same substitution for oxygen is detrimental to activity as well.[15] Concerning *S*-alkylated compounds **18** and **19**, no inhibition was observed. That may be due either to steric or electronic (or both) effects as both the size of the molecule and the

Table 1. Inhibition of basal and MT-stimulated Eg5 ATPase activity by different monastrol analogs (see Section 4 for details)

Inhibitor	Inhibition of basal ATPase activity IC_{50} (μM)	Inhibition of MT-stimulated ATPase activity IC_{50} (μM)	Monoastral spindles in cell-based assays		
			100 μM	50 μM	IC_{50} (μM)
Monastrol **1**	6.1 ± 1.5	12.3 ± 1.2	94.4 ± 3.4	45.5 ± 4.5	51.3
2	23.0 ± 9.0	62.5 ± 15.0	16.8 ± 7.0	n.s.	n.d.
3	n.i.	n.i.	13.6 ± 2.1	n.s.	n.d.
4	n.i.	n.i.	n.d.	n.s.	n.d.
5	n.i.	n.i.	10.3 ± 2.1	n.s.	n.d.
6	n.i.	n.i.	n.d.	n.s.	n.d.
7	n.i.	n.i.	n.d.	MTs affected	n.d.
8	n.i.	n.i.	n.d.	56.2 ± 10.0	n.d.
9	n.i.	n.i.	89.6 ± 3.7	49.7 ± 11.7	50.0
10	n.i.	n.i.	83.5 ± 4.4	39.3 ± 3.2	62.5
11	32.0 ± 4.5	72.2 ± 17.0	15.5 ± 2.7	n.s.	n.d.
12	9.5 ± 2.0	21.0 ± 10.0	50.5 ± 4.8	n.s.	n.d.
13	4.5 ± 0.3	11.5 ± 2.0	32.5 ± 8.5	n.s.	n.d.
14	4.0 ± 1.8	5.5 ± 2.8	15.2 ± 1.3	n.s.	n.d.
15	23.0 ± 9.0	82.3 ± 20.2	17.8 ± 4.1	n.s.	n.d.
16	10.0 ± 5.0	24.0 ± 0.4	11.4 ± 3.8	n.s.	n.d.
17	11.5 ± 0.7	23.0 ± 7.0	33.2 ± 5.7	n.d.	n.d.
18	n.i.	n.i.	n.s.	n.s.	n.d.
19	n.i.	n.i.	n.s.	n.s.	n.d.
20	1.5 ± 0.1	3.0 ± 0.1	n.d.	96.8 ± 3.6	9.2
21	n.i.	n.i.	n.d.	36.9 ± 7.8	n.d.
23	39.0 ± 15.0	69.1 ± 2.0	14.9 ± 3.1	n.s.	n.d.
24	22.0 ± 3.0	18.3 ± 2.0	15.8 ± 1.9	n.s.	n.d.

The percentage of mitotic cells showing monoastral spindles at drug concentrations 100 μM and 50 mM, and the calculated IC_{50} are indicated on the right. n.i.: no inhibition at the higher concentration tested (500 μM). n.d.: not determined. n.s.: no significant induction of monoastral spindles in treated cells (<25%).



Scheme 5. Synthesis of the monastrol-based affinity matrix.

electron density at the heterocycle are modified when compared to parent monastrol.

That first set of results indicates there is no or little restriction in the accessibility of the monastrol binding site of Eg5 for dihydropyrimidinethiones with bulky substituents in the C^5 position. That conclusion is supported as well by recent results reported by Gartner et al. who described interesting inhibition of Eg5 ATPase activity by enastron and dimethylenastron (Fig. 1).[15]

2.3. Inhibition of microtubule-stimulated ATPase activity

Results are roughly parallel to those obtained in the basal ATPase activity inhibition assay. The previously active compounds do inhibit Eg5 microtubule-stimulated ATPase activity though less efficiently than basal ATPase activity. One exception is met with compound **14** presenting equivalent inhibition scores in both basal and MT-stimulated assays and being twice as potent as monastrol in the MT-stimulated assay (IC_{50} 5.5 μM). The unexpectedly high inhibition of MT-stimulated ATPase activity for compound **14** could result from a covalent reaction of the free thiol group with some cystein residue or disulfide bridge in the proximity of the protein region interacting with microtubules, further hindering or preventing stimulation of ATPase activity by microtubules. Incubation under reductive conditions (TCEP, 1 mM) however has no influence on the activity of the compound and more sophisticated

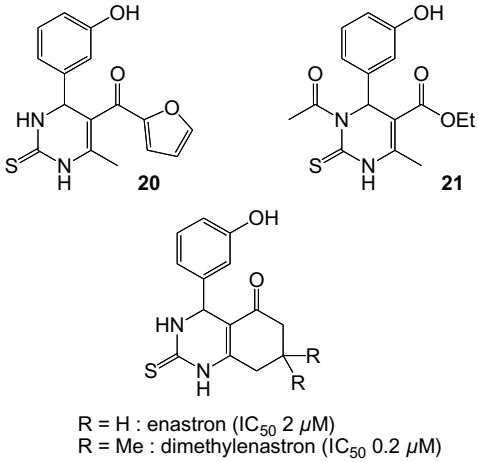


Figure 1. Additional monastrol analogs referred to in the body text.

experiments are required to confirm or rule out that hypothesis.

2.4. Cell-based assays

We looked at the ability of the different compounds to induce monoastral spindle in asynchronous HeLa cells.[20] The compounds were evaluated at 50 and 100 μM, and the proportion of cells with the characteristic monoastral spindle phenotype was quantified. The higher the inhibition of ATPase activity, the more frequent monoastral phenotype.

However, compound **13** which is more potent than monastrol to inhibit both basal and microtubule-stimulated Eg5 ATPase activities appears three times less active in the cell-based assay. The same and even more striking behavior is observed with compound **16** that does not induce any monoastral spindle phenotype (11.4% monoastral spindles to be compared to 9.6% observed in the absence of inhibitor) though it is a potent inhibitor of basal as well as MT-stimulated Eg5 ATPase activities. On the other hand, compounds **9** and **10** that do not inhibit at all Eg5 ATPase activity in the in vitro assays proved to be among the most active compounds in the cell-based assay and can be compared within that respect to parent monastrol (Fig. 2). The IC_{50} of compounds **9** and **10** were calculated to be 50.0 and 62.5 μM, respectively, and are close to that of monastrol (51.3 μM). That result could be explained by a difference in terms of drug delivery efficiency inside the cells during the test. Indeed monastrol monoacetate **9** and diacetate **10** are more lipophilic than the other compounds in the series and so are capable to better cross the cell membrane. Once in the cytoplasm they are transformed into parent monastrol by cellular esterases or chemical hydrolysis. In other words, compounds **9** and **10** act as prodrugs of monastrol. The fact that the phenol acetate moiety is more sensitive to hydrolysis than *N*-acyl ureas is consistent with the higher activity observed for monoacetate **9** when compared to diacetate **10**, that one being not fully hydrolyzed at the end of the 7 h incubation period with HeLa cells. To further support that hypothesis, we preincubated compounds **9**, **10**, and N^3-acetyl monastrol **21** (Fig. 1) in the presence and absence of medium used in the cell-based assay (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum) and subsequently determined the inhibition of basal Eg5 ATPase activity (Fig. 3). Compounds **9** and **10** do not inhibit Eg5 ATPase activity when preincubated in the absence of medium, whereas low inhibition is observed with compound **21**. After incubation in cell-based assay medium for 12 h, compounds **9**, **10**, and **21** do inhibit basal Eg5 activity with IC_{50} values of 3.9, 5.0, and 3.8 μM, respectively. These results are in very good agreement with the value obtained for monastrol (6.1 μM) and are consistent with our hypothesis on a preliminary enzymatic transformation of the compounds into monastrol. Finally, ethyl acetate extraction of cell-based assay medium incubated with diacylated compound **10** (2 mM) for different periods of time and thin layer chromatography analysis of the extracts do confirm the quantitative transformation of the compound into monastrol (by comparison with an authentic sample). Half-life of diacetyl monastrol **10** in

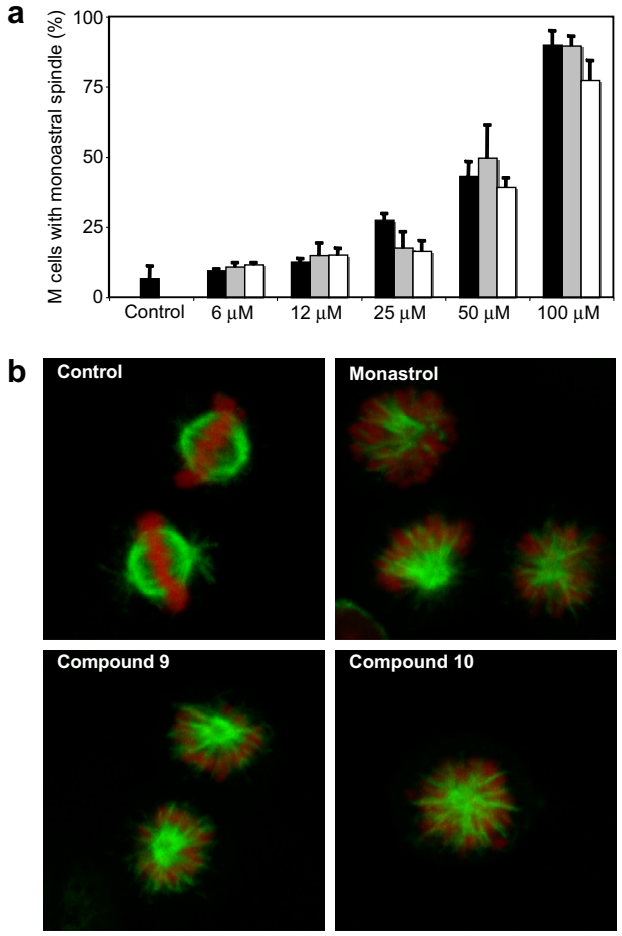


Figure 2. Induction of monoastral spindles by increasing concentrations of monastrol and acetylated derivatives **9** and **10**. HeLa cells were exposed for **7** h to increasing concentrations of monastrol **1** (black bars), compound **9** (gray bars), and compound 10 (open bars). (a) The percentage of monoastral spindles was determined from the total number of cells in M phase by staining fixed cells for β-tubulin (green) and chromatin (red). (b) Representative spindles at 100 μM drug concentration for compounds **1**, **9**, and **10**. Control refers to absence of drug.

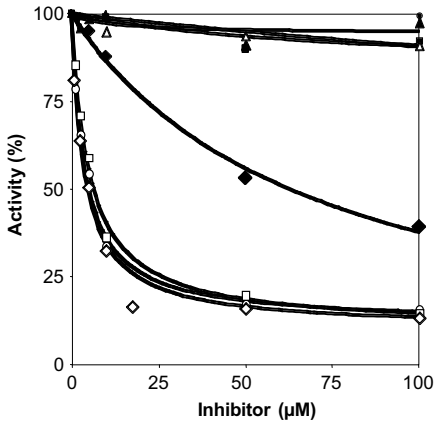


Figure 3. Inhibition of basal Eg5 ATPase activity by acetylated monastrol derivatives in the absence and presence of medium used in cell-based assays. Compounds **8** (▲), **9** (●), **10** (■), and **21** (♦) in the absence of medium, and the same compounds in the presence of medium (△, ○, □, and ◊, respectively).

these conditions was estimated to be less than 100 min. Formation of the intermediate monoacetyl monastrol **9** was observed before final transformation into monastrol.

More puzzling are the results obtained for compounds **7** and **8**. These two compounds do not inhibit Eg5 ATPase activities. However, in the cell-based assay microtubules appeared to be affected by 50 μM of dihydroxy compound **7**, chromosomes being misaligned, and **8** proves more potent than monastrol. The interpretation of that is not clear as compound **8** is expected to be enzymatically converted into **7** by cellular esterases (vide supra). Moreover preincubation of **8** in the medium used in the cell-based assay did not transform the compound into an inhibitor of basal Eg5 ATPase activity (Fig. 3) what is consistent with the results obtained with **7** in that assay. A possible explanation for these results would be a chemical degradation of diacetyl compound **8** into an active compound to be identified. That hypothesis could be partly supported by the poor chemical stability of the compound observed during its synthesis.

Last but not least, compound **20** appeared to be far more potent than parent monastrol in inducing monoastral spindles (Fig. 4a). Its IC_{50} value was determined to be 9.2 μM versus 51.3 μM for monastrol (Table 1 and Fig. 4b). We exposed HeLa cells for 24 h with compound **20** and compared its ability to block cells in mitosis with that of nocodazole, a well-known mitotic inhibitor targeting microtubules, as well as monastrol (Fig. 4c). Cell populations were examined by two-dimensional flow cytometry (cells were labeled with the MPM2 monoclonal antibody recognizing phosphospecific mitotic antigens, and propidium iodide for DNA labeling). Compound **20** blocked cells with 4 N DNA content at 25 μM. In addition, cells were arrested in mitosis since 72% of the 4 N arrested cells were MPM2 positive, whereas nocodazole treated cells were 82% positive. At 100 μM of compound **20**, 77% of the cells were mitotic whereas in the presence of 100 μM of monastrol only 48% were mitotic. Thus compound

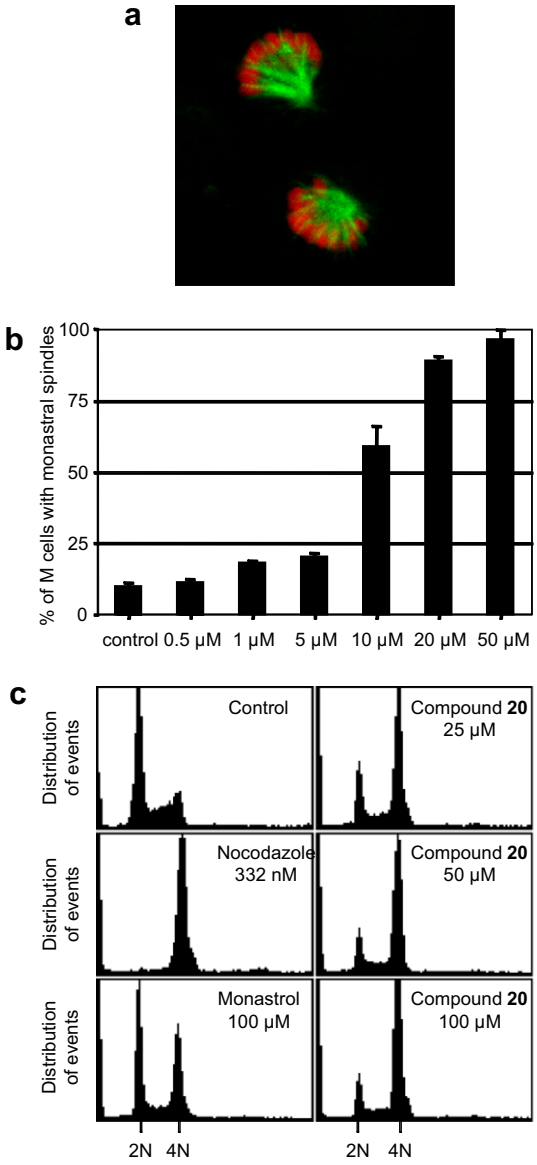


Figure 4. Induction of monoastral spindles and mitotic arrest by compound **20**. (a) Representative spindles at 10 μM drug concentration for compound **20**. Cells were fixed and stained for immunofluorescence microscopy as in Figure 2. (b) Percentage of monoastral spindles determined from the total number of mitotic cells after exposure to compound **20**. (c) Histograms obtained by flow cytometric analysis of untreated cells and cells treated with nocodazole, monastrol, and three different concentrations of compound **20**.

20 is far more potent in arresting cells in mitosis than parent monastrol.

2.5. Synthesis of a monastrol affinity matrix and Eg5 purification procedure

Considering the results described above, an affinity matrix was designed in order to improve our protocols for purification of human full-length Eg5 from eukaryotic cells (Scheme 5). Azidoamine **22** (prepared in 2 steps from commercially available 1,2-bis(2-chloroethoxy)-ethane) was condensed with carboxylic acid **11** to yield monastrol derivative **23**. A subsequent Staudinger reaction afforded the reduced primary amino compound **24**

that was then reacted with NHS-activated Sepharose. The affinity matrix thus immobilizes monastrol through two amide bonds which guarantees hydrolytic stability for safe and efficient recycling of the functionalized support.

The spacer arm between monastrol and the polymer resin—three ethylene oxide units, approximately 1.1 nm long—is expected to provide the immobilized dihydropyrimidinethione with enough degree of freedom so as to allow binding and immobilization of kinesin from eluting cells homogenate.

To test whether Eg5 can bind to and elute off the monastrol affinity matrix, we first applied purified $Eg5_{2–386}$, a monomeric recombinant Eg5 protein expressed in *E. coli*,[20] to the column. After extensive washing, we eluted $Eg5_{2–386}$ off the column using excess amount of (*S*)-trityl-L-cysteine (STLC), another potent Eg5 inhibitor that shares the same binding pocket with monastrol (data not shown).[21] STLC was preferred to monastrol in the elution step because of higher availability and lower cost. As a second step, we purified recombinantly expressed Eg5 from an *E. coli* crude extract using the same elution conditions. Mass spectrometry of the STLC released material revealed a single peak corresponding to a peptide with molecular weight fitting to that predicted for the expressed human Eg5 motor domain based on amino acid sequence (Fig. 5a). A control experiment with *E. coli* cells not expressing Eg5 and subsequent analysis by Western blot indicated that no significant material was retained on the column (Fig. 5b).

Finally, we have extended the use of the monastrol affinity matrix to the purification of the endogenous Eg5 protein from mammalian cells (HeLa) (Fig. 6). Following high speed centrifugation, the supernatant obtained from a lysate of mitotically arrested HeLa cells using nocodazole was applied on the column. The latter was washed extensively and elution of native Eg5 was performed with STLC (see Section 4 for details). The eluted fractions with Eg5 contained 15–35 μg of protein. Immunoblot analysis using human Eg5 specific polyclonal antibody revealed that Eg5 was retained almost quantitatively by the column and released by STLC (Fig. 6b). An experiment performed under similar conditions but using the control matrix (i.e., without immobilized monastrol derivative) did not reveal any binding of wild-type Eg5 (Fig. 6a). That gives confirmation of the specificity of the interaction between Eg5 and the affinity matrix which is strictly mediated by the immobilized monastrol. The eluted samples were applied to a SDS–PAGE for protein analysis. Several faint Coomassie G-250 stained bands were detected and analyzed by peptide mass fingerprinting and MALDI-TOF/TOF mass spectrometry. Following that procedure, five different proteins were identified (Table 2) of which human mitotic Eg5 was probably the full-length protein because the 21 peptides of the peptide mass fingerprint covered the amino acid sequence range from 16 to 1033 (total 1057). Eg5 was identified by MS/MS of peptide $_{932}$SYLYPSTLVR$_{941}$(Fig. 7). We also identified β-tubulin of the αβ-tubulin heterodimer, the building block of MTs, to

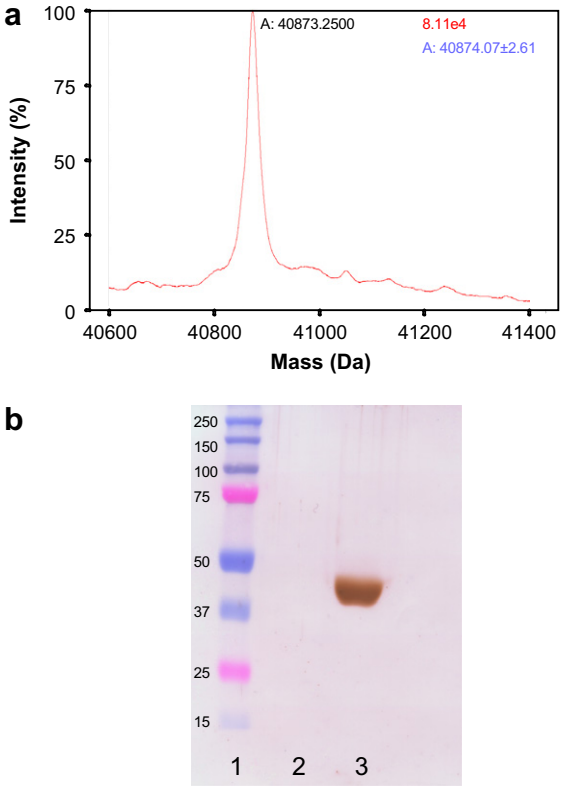


Figure 5. Purification of human Eg5 by chromatography on the monastrol-based affinity matrix. (a) Analysis of the released peptides following STLC elution by mass spectrometry shows a major peptide with a molecular weight of 40,873 Da corresponding to the bacterially expressed human Eg5 motor domain. (b) Western blot analysis of *E. coli* cells in the absence and presence of the expression plasmid coding for recombinant Eg51-386. Lane 1: protein standard; lane 2: fraction eluted with STLC using *E. coli* cells (control); lane 3: fraction eluted with STLC using cells expressing recombinant $Eg5_{1–386}$.

which Eg5 binds during mitosis to crosslink and slide antiparallel MTs apart.[8] It has previously been shown that human Eg5 is in the 'ATP-like' conformation (high-affinity for MTs), when either monastrol or an analog is bound in the inhibitor-binding pocket, even when MgADP is bound in the nucleotide-binding pocket.[22,23] It is therefore likely that tubulin copurifies with Eg5. Two additional cytoskeleton proteins identified were actin, that—like tubulin—forms filaments and is a major constituent of eukaryotic cells, and α-actinin, that crosslinks and bundles actin filaments. Finally we identified the heat shock cognate 71 kDa protein (Hsc70), which is a constitutively expressed member of the 70 kDa heat-shock protein family and functions as a molecular chaperon. However, by using the control matrix (i.e., without immobilized monastrol derivative) we could show that actin and Hsc70 are simply abundant housekeeping proteins that bind unspecifically to the matrix.

3. Conclusion

A series of monastrol derivatives have been prepared by introducing sterically demanding structural modifications on the skeleton of the parent drug. The ability of

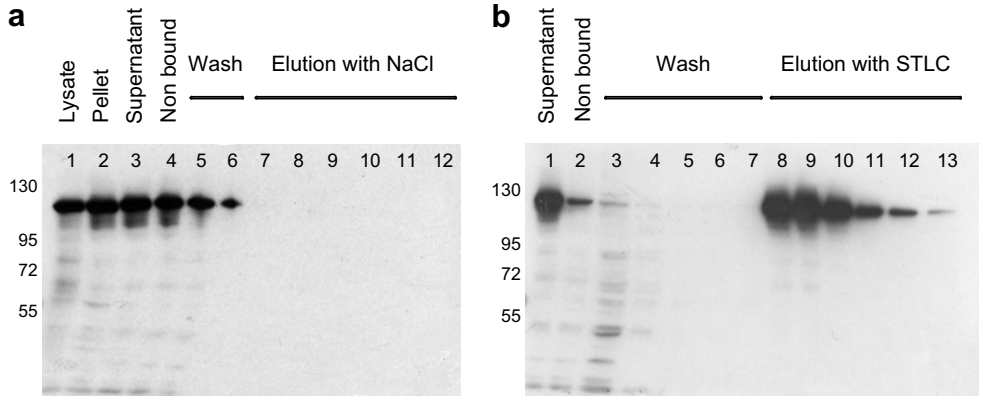


Figure 6. Western blot analysis with Eg5 specific polyclonal antibody of the different fractions obtained during the affinity chromatography purification process (fraction size: 500 μL). The supernatant from HeLa cells arrested in mitotis with nocodazole was loaded onto the non-active column (a) to eliminate proteins that bind non-specifically to the polymer matrix and the flow through was then loaded onto the monastrol-based affinity matrix 25 (b). The columns were washed with lysis buffer, and proteins bound to the columns were eluted either with 2 M NaCl (a) or with 1 mM STLC (b). The eluted fractions from the monastrol affinity column were subsequently analyzed by mass spectrometry. (a) Lane 1: total lysate from HeLa crude extract; lane 2: pellet after centrifugation; lane 3: supernatant after centrifugation; lane 4: flow-through; lanes 5–6: washes; lanes 7–12: fractions eluted with 2 M NaCl. (b) Lane 1: supernatant after centrifugation; lane 2: flow-through; lanes 3–7: washes; lanes 8–13: fractions eluted with 1 mM STLC.

Table 2. Proteins identified from eluted fractions of the monastrol-based affinity column by mass spectrometry

Protein name	Accession No.	MW (kDa)	Mascot protein score	SC (%)	No. pep	MS/MS sequences [*m*/*z*] (Mascot ions score)
Actin, cytoplasmic	P60709	42	102	19	8	$_{29}$AVFPSIVGRPR$_{39}$ [1198.71 Da] (52)
α-Actinin-4	O43707	105	136	17	17	$_{194}$DGLAFNALIHR$_{204}$ [1226.67 Da] (40)
Heat shock cognate 71 kDa protein	P11142	71	85	14	8	$_{160}$DAGTIAGLNVLR$_{171}$ [1199.68 Da] (27) $_{37}$TTPSYVAFTDTER$_{49}$ [1487.71 Da] (26)
Kinesin Eg5	P52732	119	168	16	21	$_{932}$32SYLYPSTLVR$_{941}$ [1198.69 Da] (50)
β-Tubulin	P07437	50	117	28	14	$_{253}$LAVNMVPFPR$_{262}$ [1143.65 Da] (21)

Protein name, Accession No., and theoretical molecular weight (MW) were derived from the Swiss-Prot entries. Sequence coverage (SC) and number of peptides (No. pep) of the identified proteins are indicated. The Mascot protein score of the merged peptide mass fingerprints and MS/MS spectra, and Mascot ion scores of individual fragmented peptides are shown.

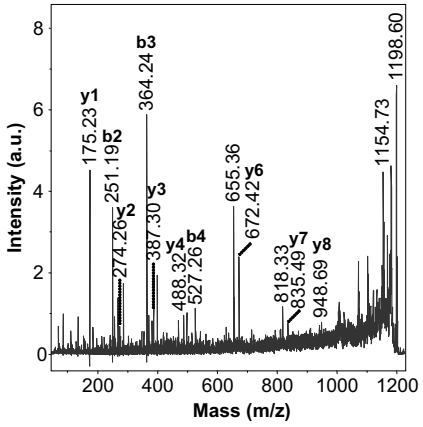


Figure 7. Identification of human mitotic Eg5 by MALDI-TOF/TOF. The MALDI-TOF/TOF spectrum of the peptide with *m*/*z* 1198.60 Da is presented. Kinesin Eg5 was unequivocally identified with the b-ions b2–b4, and the y-ions y1–y4, and y6–y8 of the tryptic peptide SYLYPSTLVR.

these compounds to inhibit Eg5 activity in in vitro ATPase assays and in cell-based assay was investigated. Three compounds that were unable to inhibit Eg5 ATPase activity in vitro proved potent Eg5 inhibitors in the cell-based assay, acting as prodrugs that are transformed into monastrol inside the cellular compartment before migrating to the nucleus. Eg5 tolerance for structural modifications at the C^5 position on monastrol backbone allowed optimization and furyl derivative **20** appears four to five times more active than monastrol. The identification of structure–activity relationships resulted in the design of an affinity matrix that has been used for fast and efficient purification of Eg5 from crude lysate of eukaryotic cells and mammalian cells. Mass spectrometry analysis of the purified samples revealed purification of Eg5 with β-tubulin. Two additional proteins have been shown to bind non-specifically to the column material.

So the monastrol affinity matrix described in that work can be used for the purification not only of the bacterially expressed Eg5 but also for the purification of the native human Eg5 protein from cellular extracts. Furthermore the purification of the native molecule should allow a number of investigations that are not relevant with truncated Eg5 motor domain, that is, identification of possible Eg5 interacting proteins through the cell cycle and studies on Eg5 post-translational modifications or oligomerization process. The problem of monastrol specificity and monastrol-targets other than

Eg5 can be tentatively addressed as well with such an affinity matrix.

4. Experimental

4.1. Chemistry

All reactions were carried out with reagent grade solvents. Commercially available reagents were used without further purification. NHS-activated Sepharose 4 FastFlow was purchased from Amersham Biosciences. Chemicals for Eg5 ATPase assays were from sources indicated by Hackney and Jiang.[24] ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 200 and 300 MHz instruments, and chemical shifts are reported in ppm downfield from TMS. IR spectra were recorded on a Perkin-Elmer-1600-FTIR spectrometer and absorption values v are in cm^{-1}. Mass Spectra (MS) were recorded on a Finnigan-4600 quadrupole instrument at chemical ionization. Mass data are reported in mass units (m/z).

4.1.1. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester (2). 3-Oxo-butyric acid 2-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-ethyl ester (prepared by trans-esterification of methyl acetoacetate with tetraethylene glycol over K10 montmorillonite[25]) (1.20 g, 4.3 mmol), thiourea (0.22 g, 2.9 mmol), and 3-hydroxybenzaldehyde (0.35 g, 2.9 mmol) were refluxed in i-PrOH (10 mL) with concentrated hydrochloric acid (0.5 mL) for 16 h. Isopropyl alcohol was removed in vacuo, methylene chloride (20 mL) was added, and the solution was washed twice with HCl 5% (10 mL), dried over $MgSO_4$, and reduced under vacuum. The crude residue was purified by silica gel chromatography (AcOEt/EtOH 100:0 to 90:10) to yield compound **2** (0.79 g, 62%) as a hygroscopic glassy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.53 (s, 1H); 8.42 (s, 1H); 7.18 (t, J = 7.3 Hz, 1H); 6.91 (m, 1H); 6.80 (d, J = 7.9 Hz, 1H); 6.72 (dd, J = 1.5, 7.9 Hz, 1H); 5.26 (d, J = 2.6 Hz, 1H); 4.38–4.31 (m, 1H); 4.04–3.97 (m, 1H); 3.78–3.50 (m, 14H); 2.33 (s, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 173.5; 164.9; 156.0; 144.3; 143.8; 130.1; 118.1; 115.0; 114.9; 102.1; 72.3; 70.6; 70.5; 69.9; 69.8; 69.2; 62.7; 61.4; 55.9; 17.8. IR (neat) v 3253 (b); 2949; 2440 (b). MS (m/z) 441.26 [M+H]$^+$.

4.1.2. Synthesis of 3-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethyl}-4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (3). 2-[2-(2-Amino-ethoxy)-ethoxy]ethanol[26] (1.05 g, 7.0 mmol) in anhydrous acetonitrile (5 mL) was added dropwise to benzoyl isothiocyanate (0.95 mL, 7.0 mmol) in anhydrous acetonitrile (5 mL) at room temperature. The reaction mixture was stirred for 30 min before the solvent was removed in vacuo. The crude residue was purified by silica gel chromatography to yield 1-benzoyl-3-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethyl-thiourea (2.2 g, 48%) as a hygroscopic glassy solid. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 10.94 (br s, 1H); 9.15 (br s, 1H); 7.84 (dd, J = 1.7, 6.8 Hz, 2H); 7.60–7.44 (m, 3H); 3.92 (td, J = 4.9, 5.1 Hz, 2H); 3.76–3.67 (m, 8H); 3.61 (t, J = 4.9 Hz, 2H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 179.8; 166.9; 133.4; 131.7; 129.0; 127.4; 72.6; 70.4; 70.2; 68.1; 61.7; 45.5. The N-alkyl-N'-benzoyl thiourea (0.57 g, 1.8 mmol) and sodium hydroxide (0.13 g, 3.2 mmol) were stirred in water/acetonitrile (5 mL, 1:1) for 30 min at room temperature. The reaction mixture was neutralized by adding diluted HCl and evaporated under vacuum. The residue was suspended in CH_2Cl_2, washed with water, dried over $MgSO_4$, and evaporated to yield 1-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethyl-thiourea (0.31 g, 82%) as a hygroscopic glassy solid. ^{1}H NMR (CD_3OD/D_2O 1:1, 200 MHz) δ 3.67–3.57 (m, 12 H). ^{13}C NMR (CD_3OD/D_2O 1:1, 50 MHz) δ 159.6; 72.8; 70.7; 70.6; 69.8; 61.5; 40.9. The latter compound (0.23 g, 1.1 mmol) was refluxed in ethanol (10 mL) with ethyl acetoacetate (0.2 mL, 1.6 mmol), 3-hydroxybenzaldehyde (0.13 g, 1.1 mmol), and concentrated hydrochloric acid (0.4 mL) for 24 h. Ethyl acetate was added and the resulting mixture was washed with brine, dried over $MgSO_4$, and reduced under vacuum. The residue was purified by silica gel chromatography (EtOAc/EtOH 92:8) to yield compound **3** (33 mg, 7%) as a glassy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.08 (t, J = 7.8 Hz, 1H); 6.76–6.67 (m, 3H); 5.27 (s, 1H); 4.16 (q, J = 7.2 Hz, 2H); 3.74–3.47 (m, 12H); 2.47 (s, 3H); 1.25 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 167.2; 157.5; 156.4; 143.6; 143.5; 129.6; 118.7; 114.8; 113.5; 113.4; 72.6; 70.3; 70.1; 69.6; 61.4; 60.2; 42.2; 29.7; 24.0; 14.3. MS (m/z) 425.24 [M+H]$^+$.

4.1.3. Synthesis of 4-(3,4-dihydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (4). Compound **4** (63 mg, 39%) was prepared from 3,4-dihydroxybenzaldehyde following the same procedure as described for **2**. ^{1}H NMR (CD_3OD, 300 MHz) δ 6.73 (d, J = 2.0 Hz, 1H); 6.70 (d, J = 8.7 Hz, 1H); 6.61 (dd, J= 2.0, 8.7 Hz, 1H); 5.17 (s, 1H); 4.08 (q, J = 7.0 Hz, 1H); 2.33 (s, 3H); 1.18 (t, J = 7.0 Hz, 3H). ^{13}C NMR (CD_3OD, 50 MHz) δ 175.6; 167.5; 146.4; 146.2; 145.1; 136.5; 119.4; 116.2; 114.9; 103.6; 61.2; 56.2; 17.6; 14.5. IR (neat) v 3312; 3182; 2991; 2923; 1687. MS (m/z) 308.98 [M+H]$^+$.

4.1.4. Synthesis of 4-(3-hydroxy-4-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (5). 2-[2-(2-Chloro-ethoxy)-ethoxy]-ethanol (2.9 mL, 20.0 mmol), 3,4-dihydroxybenzaldehyde (2.76 g, 20.0 mmol), potassium carbonate (2.76 g, 20.0 mmol), and sodium iodide (0.05 g, 0.3 mmol) were stirred in anhydrous DMF (20 mL) at 80 °C for 18 h. Methylene chloride (75 mL) was added and the solution was washed twice with NaOH 3 N (50 mL). The aqueous phase was acidified with HCl 12 N, reduced in vacuo, and the residue was triturated twice with EtOAc/MeOH (20 mL, 1:1). The organic phase was reduced under vacuum and the residue was purified by silica gel chromatography (CH_2Cl_2/EtOAc/EtOH 50:50:5) to yield the expected 3-hydroxy-4-{2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy}-benzaldehyde (2.78 g, 52%) as a glassy solid. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 9.80 (s, 1 H); 7.39 (d, J = 2.0 Hz, 1H); 7.34 (dd, J = 2.0, 8.3 Hz, 1H); 6.91 (d, J = 8.3 Hz,

1H); 4.21–4.16 (m, 1H); 3.90–3.85 (m, 1H); 3.75–3.66 (m, 10H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 191.1; 151.6; 147.2; 131.1; 123.5; 115.9; 112.1; 72.3; 70.6; 70.0; 69.0; 67.9. IR (neat) ν 3498 (b); 2890 (b). MS (*m*/*z*) 271.04 $[M+H]^+$. The latter compound (0.49 g, 1.8 mmol), ethyl acetoacetate (0.35 mL, 2.7 mmol), thiourea (0.15 g, 2.0 mmol), and HCl 12 N (0.4 mL) were refluxed for 4 d in EtOH (10 mL). Alcohol was removed under reduced pressure, methylene chloride (50 mL) was added, and the solution was washed twice with HCl 2 N. The organic layer was dried over $MgSO_4$, reduced under vacuum, and the residue was purified by silica gel chromatography (CH_2Cl_2/EtOH 100:0 to 95:5). Compound **5** (0.73 g, 91%) was obtained as a glassy solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.85 (d, J = 2.2 Hz, 1H); 6.81 (d, J = 5.0 Hz, 1H); 6.70 (dd, J= 2.2, 8.1 Hz, 1H); 5.27 (d, J = 3.4 Hz, 1H); 4.14–4.05 (m, 4H); 3.86–3.62 (m, 12H); 2.34 (s, 3H); 1.17 (t, J = 7.2 Hz, 3H). ^{13}C NMR (CD_3OD, 75 MHz) δ 175.8; 167.3; 148.1; 147.7; 145.4; 138.4; 119.1; 115.2; 114.6; 103.3; 73.6; 71.5; 71.3; 70.7; 69.5; 62.1; 61.2; 56.1; 17.7; 14.5. IR (neat) ν 3194 (b); 2930 (b). MS (*m*/*z*) 441.25 $[M+H]^+$.

4.1.5. Synthesis of 4-(3-hydroxy-6-nitro-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (6). A mixture of 3-hydroxy-6-nitrobenzaldehyde (127 mg, 0.76 mmol), thiourea (116 mg, 1.52 mmol), ethyl acetoacetate (97 μL, 0.76 mmol), and ytterbium(III) trifluoromethanesulfonate hydrate (48 mg, 0.08 mmol) was refluxed in acetonitrile (5 mL) for 4 h before the solvent was removed under vacuum. The crude residue was purified by silica gel chromatography (Et_2O/hexane 50:50 to 70:30) to yield **6** (166 mg, 64%) as a yellow powder. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.94 (d, J= 9.0 Hz, 1H); 6.87 (d, J = 2.7 Hz, 1H); 6.82 (dd, J = 2.7, 9.0 Hz, 1H); 6.04 (br s, 1H); 3.96 (q, J = 7.0 Hz, 2H); 2.43 (s, 3H); 1.00 (t, J= 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 176.3; 166.5; 164.5; 147.3; 141.5; 141.3; 128.8; 116.5; 116.2; 101.7; 68.8; 61.3; 17.5; 14.2. IR (neat) ν 3195 (b); 2981. MS (*m*/*z*) 338.20 $[M+H]^+$.

4.1.6. Synthesis of 4-(2,5-dihydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (7). Compound **8** (76 mg, 0.2 mmol) was stirred for 2 h in methanolic ammonia (2 mL) at 0 °C. The solvent was removed under vacuum and the residue was purified by silica gel chromatography (AcOEt) to yield **7** (9 mg, 15%) as a slightly yellow solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 6.59 (d, J = 8.4 Hz, 1H); 6.47 (dd, J = 3.0, 8.4 Hz, 1H); 6.32 (d, J = 3.0 Hz, 1H); 5.55 (s, 1H); 4.15–4.04 (m; 2H); 2.47 (s, 3H); 1.19 (t, J = 7.2 Hz, 3H). IR (neat) ν 3324 (b); 3201.

4.1.7. Synthesis of 4-(2,5-diacetoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (8). Acetic anhydride (1.2 mL, 12.7 mmol) was added dropwise to a suspension of 2,5-dihydroxybenzaldehyde (361 mg, 2.6 mmol) and potassium carbonate (722 mg, 5.2 mmol) in anhydrous diethyl ether at 0 °C. The mixture was stirred for 2 h at room temperature, filtered, and the solid was washed with ethyl acetate. The combined filtrate was washed with aqueous $NaHCO_3$, water, and brine, and dried over $MgSO_4$. The solvent was removed in vacuo to yield pure 2,5-diacetoxy-benzaldehyde (580 mg, 100%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 10.06 (s, 1H); 7.60 (d, J = 3.0 Hz, 1H); 7.35 (dd, J = 3.0, 8.7 Hz, 1H); 7.19 (d, J = 8.7 Hz, 1H); 2.37 (s, 3H); 2.30 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 187.5; 169.0; 168.8; 148.9; 148.3; 128.6; 128.4; 124.5; 123.2; 20.9; 20.7. IR (neat) ν 1759; 1692. A mixture of the latter compound (448 mg, 2.0 mmol), thiourea (153 mg, 2.0 mmol), ethyl acetoacetate (230 μL, 3.0 mmol), and ytterbium(III) trifluoromethanesulfonate hydrate (63 mg, 0.1 mmol) in anhydrous acetonitrile (10 mL) was stirred at 55–60 °C for 2 h. The solvent was removed under vacuum and the crude residue was purified by silica gel chromatography (Et_2O/CH_2Cl_2 40:60) to yield **8** (132 mg, 17%) as a slightly yellow solid. ^{1}H NMR (CD_3OD/$CDCl_3$ 1:1, 200 MHz) δ 7.09 (d, J= 8.8 Hz, 1H); 6.99 (dd, J = 2.6, 8.8 Hz, 1H); 6.69 (d, J = 2.6 Hz, 1H); 5.42 (s, 1H); 4.08 (qd, J = 2.0, 7.0 Hz, 2H); 2.48 (s, 3H); 2.35 (s, 3H); 2.22 (s, 3H); 1.17 (t, J= 7.0 Hz, 3H). ^{13}C NMR (CD_3OD/$CDCl_3$ 1:1, 75 MHz) δ 170.2; 167.6; 162.8; 160.6; 157.9; 148.8; 145.3; 134.9; 124.4; 122.2; 120.9: 101.4; 61.1; 36.7; 23.8; 21.2; 21.1; 14.4. IR (neat) ν 1762; 1207; 1170. MS (*m*/*z*) 393.27 $[M+H]^+$.

4.1.8. Synthesis of 4-(3-acetoxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (9). Acetic anhydride (0.12 mL, 1.3 mmol) was added to a mixture of monastrol **1** (0.32 g, 1.1 mmol), triethylamine (0.20 mL, 1.4 mmol), and 4-DMAP (6 mg, 0.05 mmol) in anhydrous THF (5 mL). The resulting solution was stirred at room temperature overnight. THF was removed under vacuum, ethyl acetate (10 mL) was added, and the solution was washed with diluted hydrochloric acid. The organic layer was dried over $MgSO_4$, reduced in vacuo, and the residue was purified by silica gel chromatography (Et_2O/hexane 50:50 to 70:30) to yield **9** (0.31 g, 84%) as a slightly yellow powder. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.82 (s, 1H); 8.29 (s, 1H); 7.27 (t, J = 8.2 Hz, 1H); 7.12 (d, J = 7.8 Hz, 1H); 7.00–6.96 (m, 2H); 5.33 (d, J = 2.9Hz, 1H); 4.06 (q, J = 7.1 Hz, 2H); 2.31 (s, 3H); 2.24 (s, 3H); 1.13 (t, J= 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 173.8; 169.2; 165.0; 150.6; 144.0; 143.5; 129.6; 124.0; 121.2; 119.9; 102.2; 60.3; 55.2; 21.0; 17.9; 13.9. IR (neat) ν 3190 (b); 2986. MS (*m*/*z*) 335.26 $[M+H]^+$. A fraction eluting before compound **9** was identified as diacetylated compound **10** (0.05 g, 12%). ^{1}H NMR ($CDCl_3$, 200 MHz) δ 8.61 (s, 1H); 7.29 (t, J = 7.1 Hz, 1H); 7.14 (d, J = 5.9 Hz, 1H); 7.03–7.00 (m, 2H); 6.66 (s, 1H); 4.23 (q, J = 7.1 Hz, 2H); 2.78 (s, 3H); 2.36 (s, 3H); 2.27 (s, 3H); 1.28 (t, J = 7.1 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 178.0; 173.2; 169.2; 164.9; 150.7; 143.6; 140.4; 129.4; 123.7; 121.3; 119.7; 107.9; 60.9; 53.4; 27.6; 21.1; 17.3; 14.1. IR (neat) ν 3270 (b); 2987; 1699. MS (*m*/*z*) 377.20 $[M+H]^+$.

4.1.9. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid (11). Monastrol **1** (0.18 g, 0.6 mmol) and potassium hydroxide (0.10 g, 1.8 mmol) were stirred in water (2 mL) for 3 d. The solution was acidified with HCl 2 N and

extracted with EtOAc. The organic layer was dried over $MgSO_4$, reduced under vacuum, and the residue was purified over silica gel (EtOAc/CH_2Cl_2 50:50 to 100:0) to yield compound **11** (0.13 g, 79%) as a white powder. ^1H NMR (CD_3OD, 300 MHz) δ 7.12 (t, J = 7.5 Hz, 1H); 6.79–6.69 (m, 2H); 6.67 (dd, J = 5.6, 7.1 Hz, 1H); 5.27 (s, 1H); 2.34 (s, 3H). ^{13}C NMR (CD_3OD, 75 MHz) δ 176.2; 169.4; 158.7; 146.0; 145.3; 130.6; 118.9; 115.8; 114.4; 103.7; 56.3; 17.7. IR (neat) ν 3226 (b). MS (*m*/*z*) 265.10 $[M+H]^+$.

4.1.10. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid 2-(2-hydroxy-ethoxy)-ethyl ester (12). To a mixture of carboxylic acid **11** (57 mg, 0.22 mmol), diethylene glycol (0.21 mL, 2.2 mmol), and triphenyl phosphine (117 mg, 0.44 mmol) in anhydrous THF (3 mL) was added DIAD (64 μL, 0.33 mmol) at room temperature. The solution was stirred for 2 h before the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (EtOAc/CH_2Cl_2 50:50 to 100:0) to yield compound **12** (39 mg, 51%) as a white powder. ^1H NMR (CD_3OD, 300 MHz) δ 7.13 (t, J= 7.9 Hz, 1H); 6.79 (d, J = 8.5 Hz, 1H); 6.77 (d, J = 2.6 Hz, 1H); 6.68 (dd, J = 2.6, 8.5 Hz, 1H); 5.28 (s, 1H); 4.26–4.12 (m, 2H); 3.64–3.61 (m, 4H); 3.52–3.45 (m, 2H); 2.34 (s, 3H). ^{13}C NMR (CD_3OD, 50 MHz) δ 176.1; 167.2; 158.7; 146.1; 146.0; 130.7; 118.9; 115.8; 114.6; 102.9; 73.5; 70.0; 64.5; 62.2; 56.2; 17.8. IR (neat) ν 3256 (b); 2952; 2447 (b). MS (*m*/*z*) 353.19 $[M+H]^+$.

4.1.11. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid 2-(2-acetylsulfanyl-ethoxy)-ethyl ester (13). Potassium thioacetate (0.39 g, 3.4 mmol) and 2-[2-hydroxy-ethoxy)-ethyl methanesulfonate][26] (0.33 g, 1.8 mmol) in anhydrous DMF (5 mL) were stirred at 80 °C for 4 h. Diethyl ether (25 mL) was added and the resulting solution was washed with water. The organic layer was dried over $MgSO_4$, reduced under vacuum, and the residue was purified by silica gel chromatography (Et_2O/hexane 75:25) to yield expected *S*-2-(2-hydroxy-ethoxy)-ethyl ethanethioate (0.26 g, 89%). ^1H NMR ($CDCl_3$, 300 MHz) δ 3.66 (t, J = 5.0 Hz, 2H); 3.58 (t, J = 6.3 Hz, 2H); 3.53 (t, J = 5.0 Hz, 2H); 3.06 (t, J = 6.3 Hz, 2H); 2.31 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 195.4; 71.9; 69.5; 61.5; 30.4; 28.7. MS (*m*/*z*) 164.99 $[M+H]^+$. DIAD (77 μL, 0.37 mmol) was added dropwise at room temperature to a mixture of the latter compound (38 mg, 0.23 mmol), acid **11** (50 mg, 0.19 mmol), and triphenyl phosphine (106 mg, 0.40 mmol) in anhydrous THF (2 mL). The solution was stirred for 1 h, reduced in vacuo, and the residue was purified by silica gel chromatography (Et_2O/hexane 70:30) to yield compound **13** (32 mg, 41%) as a white solid. ^1H NMR ($CDCl_3$, 200 MHz) δ 8.40 (s, 1H); 8.07 (s, 1H); 7.13 (t, J = 8.1 Hz, 1H); 6.82–6.72 (m, 3H); 5.32 (t, J = 2.6 Hz, 1H); 4.22–4.15 (m, 2H); 3.64–3.60 (m, 2H); 3.53 (t, J = 6.5 Hz, 2H); 3.05 (t, J = 6.5 Hz, 2H); 2.34 (s, 3H); 2.32 (s, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 196.6; 174.0; 165.3; 156.4; 143.9; 143.8; 130.1; 118.6; 115.5; 113.8; 102.3; 69.5; 68.7; 63.3; 55.6; 30.6; 28.7; 18.3. MS (*m*/*z*) 411.27 $[M+H]^+$.

4.1.12. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid 2-(2-mercapto-ethoxy)-ethyl ester (14). Compound **13** (21 mg, 0.05 mmol) was stirred at room temperature for 24 h in *i*-PrOH (2 mL) with 3 drops of concentrated hydrochloric acid. The solvent was removed in vacuo and the residue was filtered over silica gel (Et_2O) to yield **14** (13 mg, 69%) as a glassy solid. ^1H NMR (CD_3OD, 200 MHz) δ 7.13 (t, J = 7.7 Hz, 1H); 6.81–6.66 (m, 3H); 5.28 (s, 1H); 4.27–4.15 (m, 2H); 3.60 (t, J = 4.8 Hz, 2H); 3.49 (t, J = 6.0 Hz, 2H); 2.58 (t, J = 6.5 Hz, 2H); 2.36 (s, 3H). ^{13}C NMR (CD_3OD, 50 MHz) δ 176.3; 167.2; 158.8; 146.2; 146.1; 130.7; 118.9; 115.9; 114.6; 102.9; 74.0; 69.8; 64.4; 56.3; 24.7; 17.7. IR (neat) ν 3242 (b); 1185. MS (*m*/*z*) 335.14 $[M-HS]^+$.

4.1.13. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethylamide (15). DCC (55 mg, 0.26 mmol) was added to a mixture of acid **11** (35 mg, 0.13 mmol), ethylamine hydrochloride (26 mg, 0.32 mmol), and triethylamine (45 μL, 0.32 mmol) in anhydrous THF (2 mL). The solution was stirred at room temperature overnight. The precipitate was removed by filtration, the filtrate was reduced in vacuo, and the residue was purified by silica gel chromatography (EtOAc/CH_2Cl_2 70:30 to 100:0) to yield amide **15** (38 mg, 96%) as a white solid. ^1H NMR ($CDCl_3$/CD_3OD 1:1, 200 MHz) δ 7.13 (t, J = 8.8 Hz, 1H); 6.75–6.69 (m, 3H); 5.20 (s, 1H); 3.17–3.06 (m, 2H); 2.02 (s, 3H); 0.95 (t, J= 7.3 Hz, 3H). ^{13}C NMR ($CDCl_3$/CD_3OD 1:1, 50 MHz) δ 174.4; 167.7; 157.9; 143.7; 133.8; 130.4; 118.2; 115.9; 113.8; 108.2; 57.0; 34.8; 16.6; 14.4. MS (*m*/*z*) 292.15 $[M+H]^+$.

4.1.14. Synthesis of 1,5-di-[4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxy]-3-oxapentane (16). DIAD (60 μL, 0.30 mmol) was added dropwise at room temperature to a mixture of diethylene glycol (10 μL, 0.10 mmol), acid **11** (49 mg, 0.18 mmol), and triphenyl phosphine (112 mg, 0.43 mmol) in anhydrous THF (2 mL). The solution was stirred for 1 h, reduced in vacuo, and the residue was purified by silica gel chromatography (EtOAc) to yield compound **16** (49 mg, 87%) as a white solid. ^1H NMR (CD_3OD, 200 MHz) δ 7.10 (t, J = 7.8 Hz, 2H); 6.79–6.64 (m, 6H); 5.26 (s, 2H); 4.17–4.07 (m, 4H); 3.51 (t, J = 4.5 Hz, 4H); 2.33 (s, 6H). ^{13}C NMR (CD_3OD, 50 MHz) δ 176.1; 167.2; 158.8; 146.1; 146.0; 130.7; 119.0; 115.9; 114.6; 102.9; 70.0; 64.6; 56.3; 17.8. IR (neat) ν 3239 (b); 2443 (b). MS (*m*/*z*) 599.45 $[M+H]^+$.

4.1.15. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-selenoxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (17). A mixture of finely ground selenourea (246 mg, 2.00 mmol), 3-hydroxybenzaldehyde (105 mg, 0.85 mmol), and magnesium chloride hexahydrate (75 mg, 0.37 mmol) was stirred with ethyl acetoacetate (120 μL, 0.94 mmol) at 80 °C for 3 h. The reaction mixture was purified by silica gel chromatography (Et_2O/hexane 67:33) to yield **17** (221 mg, 76%) as a white solid. ^1H NMR ($CDCl_3$, 200 MHz) δ 8.84 (sb, 2H); 7.09

(t, J = 7.8 Hz, 1H); 6.85–6.70 (m, 3H); 5.32 (s, 1H); 4.07 (q, J = 6.6 Hz, 2H); 2.29 (s, 3H); 1.14 (t, J = 6.6 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 169.8 (t, J= 189.6 Hz); 165.8; 156.2; 143.2; 142.5; 130.0; 118.8; 115.7; 113.9; 103.2; 60.8; 55.7; 56.3; 18.1; 14.0. IR (neat) ν 3287; 3159; 3106; 2981; 1663. MS (m/z) 340.98 $[M+H]^+$.

4.1.16. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-(ethylthio)-1,4-dihydro-pyrimidine-5-carboxylic acid ethyl ester (18). Sodium hydrogenocarbonate (328 mg, 3.91 mmol) and 2-ethylisothiourea[27] (105 mg, 1.01 mmol) were added to ethyl 2-(3-hydroxybenzylidene)-3-oxobutanoate[28] (182 mg, 0.78 mmol) in anhydrous DMF (2 mL). The resulting mixture was stirred for 3 h at 80 °C, and volatile was removed under reduced pressure. The crude residue was purified by silica gel chromatography (Et_2O/hexane 70:30) to yield **18** (103 mg, 41%) as a white solid. ^{1}H NMR (CD_3OD, 300 MHz) δ 7.09 (t, J = 7.8 Hz, 1H); 6.77 (d, J = 8.1 Hz, 1H); 6.74 (d, J = 1.8 Hz, 1H); 6.64 (dd, J = 2.1, 8.1 Hz, 1H); 5.46 (s, 1H); 4.09 (q, J = 6.9 Hz, 2H); 3.10–3.04 (m, 1H); 2.86–2.79 (m, 1H); 2.30 (s, 3H); 1.21 (t, J = 6.9 Hz, 3H); 1.20 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CD_3OD/CDCl_3$ 1:1, 50 MHz) δ 168.3; 157.7; 153.2; 146.9; 140.0; 130.0; 118.9; 114.9; 114.4; 101.5; 60.7; 58.7; 25.7; 19.1; 14.8; 14.6. IR (neat) ν 3253; 2974; 1692. MS (m/z) 321.36 $[M+H]^+$.

4.1.17. Synthesis of 4-(3-hydroxy-phenyl)-6-methyl-2-(benzylthio)-1,4-dihydro-pyrimidine-5-carboxylic acid ethyl ester (19). Compound **19** (188 mg, 69%) was prepared from 2-benzylisothiourea[29] following the same procedure as for **18**. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.36–7.22 (m, 5H); 7.20 (t, J = 7.8 Hz, 1H); 6.89 (dd, J = 0.6, 7.2 Hz, 1H); 6.78 (m, 1H); 6.77 (d, J = 7.8 Hz, 1H); 5.65 (sb, 1H); 4.43 (d, J = 13.8 Hz, 2H); 4.14 (q, J = 6.6 Hz, 2H); 2.36 (s, 3H); 1.22 (t, J = 7.2 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 167.1; 156.7; 154.0; 145.9; 137.4; 133.3; 130.0; 129.4; 129.0; 127.7; 119.1; 115.2; 114.4; 102.3; 60.5; 58.0; 30.1; 20.1; 14.6. IR (neat) ν 3237; 2926; 1684. MS (m/z) 383.41 $[M+H]^+$.

4.1.18. Synthesis of 5-furanoyl-4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine (20). Ethyl furoate (5.16 g, 40.0 mmol) in anhydrous acetone (2.9 mL, 40.0 mmol) was added to a suspension of t-BuOK (9.00 g, 80.0 mmol) in anhydrous toluene (75 mL) at 0 °C. The mixture was stirred for 6 h at room temperature before acetic acid (4.60 mL, 80.0 mmol) and water (30 mL) were added. The organic layer was washed with water, brine, dried over $MgSO_4$, and reduced in vacuo. Purification by silica gel chromatography (Et_2O/hexane 10:90) afforded 1-(furan-2-yl)butane-1,3-dione (3.85 g, 63%) as a viscous oil that slowly crystallized at room temperature. Both conjugated enol and diketone forms are observed by NMR in a 80/20 ratio. ^{1}H NMR ($CDCl_3$, 300 MHz) *enol form* δ 7.55 (dd, J = 0.9, 1.8 Hz, 1H); 7.13 (dd, J = 0.9, 3.6 Hz, 1H); 6.52 (dd, J = 1.8, 3.6 Hz, 1H); 6.05 (s, 1H); 2.12 (s, 3H); *diketone form* δ 7.59 (dd, J = 0.9, 1.8 Hz, 1H); 7.25 (dd, J = 0.6, 3.6 Hz, 1H); 6.55 (dd, J = 1.8, 3.6 Hz, 1H); 3.94 (s, 1H); 2.28 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) *enol form* δ 189.4; 176.0; 145.9; 145.8; 115.5; 112.4; 96.0; 24.4; *diketone form* δ 201.3; 82.1; 150.4; 147.1; 118.5; 112.7; 54.2; 30.5. IR (neat) ν 3130; 1586. MS (m/z) 153.07 $[M+H]^+$. The latter compound (643 mg, 4.2 mmol) was stirred with thiourea (355 mg, 4.7 mmol), 3-hydroxybenzaldehyde (516 mg, 4.2 mmol), and ytterbium(III) trifluoromethanesulfonate hydrate (288 mg, 0.5 mmol) in refluxing anhydrous THF (10 mL) for 10 h. The solvent was removed under vacuum and the residue was purified by silica gel chromatography (Et_2O/hexane 50:50 to 100:0) to yield **20** (1.22 g, 93%) as a yellow powder. ^{1}H NMR (DMSO-d_6, 200 MHz) δ 10.27 (br s, 1H); 9.57 (d, J = 3.2 Hz, 1H); 9.43 (s, 1H); 7.92 (br s, 1H); 7.14 (d, J = 3.6 Hz, 1H); 7.06 (d, J = 7.8 Hz, 1H); 6.67–6.56 (m, 4H); 5.33 (d, J = 3.2 Hz, 1H); 1.90 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.6; 174.0; 157.4; 152.6; 146.9; 144.2; 140.4; 129.5; 118.1; 116.8; 114.7; 113.2; 112.5; 109.5; 55.0; 17.1. IR (neat) ν 3181 (b); 1614; 1590; 1563; 1212. MS (m/z) 315.15 $[M+H]^+$.

4.1.19. Synthesis of 2-acetyl-4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxylic acid ethyl ester (21). Diacetylated compound **10** (52 mg, 0.14 mmol) in methyl alcohol/water (5 mL, 4:1) was stirred for 1 h with aqueous $NaHCO_3$ saturated solution (1 mL) at room temperature. The pH of the solution was brought to 7 with diluted HCl before solvent was removed under vacuum. The residue was suspended in ethyl acetate and washed with water, dried over $MgSO_4$, reduced in vacuo, and purified by silica gel chromatography (Et_2O/hexane 50:50) to yield **21** (36 mg, 78%) as a slightly yellow powder. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.62 (s, 1H); 7.15 (t, J = 7.8 Hz, 1H); 6.84 (d, J = 7.8 Hz, 1H); 6.79 (s, 1H); 6.74 (d, J = 7.8 Hz, 1H); 6.64 (s, 1H); 4.22 (q, J = 7.0 Hz, 2H); 2.78 (s, 3H); 2.36 (s, 3H); 1.28 (t, J= 7.0 Hz, 3H). ^{13}C NMR ($CDCl_3$, 50 MHz) δ 178.3; 173.6; 165.3; 156.1; 143.5; 140.4; 130.1; 118.9; 115.3; 113.6; 108.4; 61.3; 53.8; 27.9; 17.6; 14.3. IR (neat) ν 3270 (b); 2982. MS (m/z) 335.36 $[M+H]^+$.

4.1.20. Synthesis of 2-[2-(2-azido-ethoxy)-ethoxy]-ethylamine (22). 1,2-Bis(2-chloroethoxy)-ethane (10.6 g, 56.7 mmol), sodium azide (12.0 g, 184.6 mmol), and sodium iodide (1.2 g, 8.0 mmol) were stirred in refluxing acetonitrile (60 mL) for 7 d. The solvent was removed under vacuum and diethyl ether (100 mL) was added. The resulting suspension was successively washed with water, aqueous $Na_2S_2O_5$, water, and brine. The organic layer was dried over $MgSO_4$ and reduced in vacuo to yield analytically pure 1,2-bis(2-azidoethoxy)-ethane (11.2 g, 99%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.71–3.68 (m, 8H); 3.39 (t, J = 5.1 Hz, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 71.2; 70.6; 51.1. MS (m/z) 200.78 $[M+H]^+$. Triphenyl phosphine (9.09 g, 34.6 mmol) in diethyl ether (50 mL) was added dropwise over a 3-h period to 1,2-bis(2-azidoethoxy)-ethane (6.94 g, 34.6 mmol) in Et_2O/THF/HCl 1 N (100 mL, 5:1:5) at room temperature. The reaction mixture was vigorously stirred for 12 h before the organic layer was washed with HCl 4 N. The aqueous layer was then washed twice with

diethyl ether and brought to pH 14 by adding sodium hydroxide pellets. The resulting aqueous solution was washed twice with CH_2Cl_2, the organic layers were pulled, dried over $MgSO_4$, and reduced under vacuum to yield analytically pure compound **22** (6.01 g, 99%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 3.62–3.57 (m, 6H); 3.49 (t, J = 5.1 Hz, 2H); 3.35 (t, J = 5.1 Hz, 2H); 2.84 (t, J = 5.4 Hz, 2H); 2.32 (s, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 73.6; 70.9; 70.6; 70.4; 51.0; 42.0. MS (*m*/*z*) 174.96 $[M+H]^+$.

4.1.21. Synthesis of 1-azido-5-[4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxamido]-3-oxa-pentane (23). DCC (252 mg, 1.22 mmol) was added to a mixture of acid **11** (265 mg, 1.00 mmol), azidoamine **22** (188 mg, 1.08 mmol), and triethylamine (200 μL, 1.43 mmol) in anhydrous THF (10 mL). The solution was stirred at room temperature overnight. The precipitate was removed by filtration, the filtrate was reduced under vacuum, and the residue was purified by silica gel chromatography (EtOAc/CH_2Cl_2 80:20 to 100:0) to yield compound **23** (260 mg, 62%) as a white solid. ^{1}H NMR ($CDCl_3$/CD_3OD 1:1, 300 MHz) δ 7.13 (t, J = 8.4 Hz, 1H); 6.73–6.71 (m, 3H); 5.24 (s, 1H); 3.61 (t, J = 4.8 Hz, 4H); 3.55–3.34 (m, 6H); 3.35 (t, J = 5.1 Hz, 2H); 2.07 (s, 3H). ^{13}C NMR ($CDCl_3$/CD_3OD 1:1, 50 MHz) δ 174.0; 167.6; 157.7; 143.5; 134.7; 130.4; 118.2; 115.8; 113.7; 107.6; 70.6; 70.2; 70.1; 69.7; 56.7; 50.8; 39.6; 16.7. IR (neat) ν 3262 (b); 2924; 2360 (b); 2108. MS (*m*/*z*) 422.24 $[M+H]^+$.

4.1.22. Synthesis of 1-amino-5-[4-(3-hydroxy-phenyl)-6-methyl-2-thioxo-1,2,3,4-tetrahydro-pyrimidine-5-carboxamido]-3-oxa-pentane (24). Azide **23** (210 mg, 0.50 mmol) and triphenyl phosphine (198 mg, 0.75 mmol) were stirred with water (250 μL) in THF (5 mL) at room temperature overnight. THF was removed under reduced pressure, water (10 mL) was added, and the solution was washed twice with CH_2Cl_2. The aqueous layer was lyophilized to yield analytically pure amine **24** (159 mg, 81%). ^{1}H NMR (CD_3OD, 300 MHz) δ 6.91 (t, J = 8.1 Hz, 1H); 6.52–6.45 (m, 3H); 5.05 (s, 1H); 3.30–3.02 (m, 10H); 2.53 (t, J = 5.4 Hz, 2H); 1.83 (s, 3H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 175.6; 169.2; 159.3; 145.2; 135.0; 130.9; 118.5; 116.3; 114.7; 108.9; 73.1; 71.2; 71.1; 70.3; 57.6; 41.9; 40.4; 16.8. IR (neat) ν 3257 (b); 2926; 2360. MS (*m*/*z*) 396.21 $[M+H]^+$.

4.1.23. Synthesis of monastrol affinity matrix (25). NHS-activated Sepharose 4 Fast Flow (16–23 μmol/mL, 6 mL) was washed with HCl 1 mM (75 mL) at 4 °C and suspended in water (10 mL). Amine **24** (56 mg, 0.14 mmol) was added, the suspension was brought to pH 7 with Et_3N and gently shaken overnight at room temperature. The resin was filtered, washed successively with water (10 mL), 1 mM ethanolamine (20 mL, pH 8), 50 mM Tris buffer (10 mL, pH 8), and 70 mM AcOH (10 mL, pH 4). Tris buffer and AcOH washings were repeated twice and, finally, the resin was extensively washed with ultra pure water, and stored in water/ethyl alcohol (8:2) until use.

The control (non-derivatized) chromatography matrix was prepared differently. NHS-activated Sepharose 4 Fast Flow (16–23 μmol/mL, 1 mL) was washed with HCl 1 mM (15 mL) at 4 °C and suspended in 100 mM ethanolamine (10 mL). The suspension was gently shaken for 1 h, extensively washed with ultra pure water and stored in water/ethyl alcohol (8:2) until use.

4.2. ATPase assays

Measurements of ATPase rates in the absence and presence of MTs were performed in duplicate at room temperature. Steady-state basal and MT-activated ATPase rates were measured with $Eg5_{2–386}$ using the pyruvate kinase/lactate dehydrogenase-linked assay.[24] To test the inhibition of MT-stimulated Eg5 ATPase activity, Eg5 was used at 40 nM in the presence of 1 μM MTs. The assays were performed in the presence of DMSO (2.5%). The maximal inhibitor concentrations tested were 500 μM. The data were treated using Kaleidagraph 3.0 (Synergy Software). The inhibition of the basal Eg5 ATPase activity (in the absence of MTs) was measured in the presence of 1.0 μM Eg5. Paralleled experiments were carried out with acetylated monastrol derivatives **8**, **9**, **10**, and **21** with prior incubation of the drugs (2 mM) for 12 h at 37 °C in Dulbecco's modified Eagle's medium (GIBCO, BRL) supplemented with 10% fetal bovine serum (Hyclone) and subsequent measurement of the basal Eg5 ATPase activity.

4.3. Cell-based assays

HeLa cells were grown on Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and maintained in a humid incubator at 37 °C in 5% CO_2. Cells were left to adhere for at least 24 h on poly-D-lysine-coated glass coverslips before the addition of the drugs. Following 7-h incubation with drugs, cells were fixed with 1% paraformaldehyde-PBS at 37 °C for 3 min followed by 5-min incubation in 100% methanol at −20 °C. Coverslips were then washed with PBS and cells were stained with anti-β-tubulin monoclonal antibodies for 1 h and then with an FITC-conjugated goat anti-mouse secondary antibody (Jakson ImmunoResearch Laboratories, West Grove, PA USA) for 30 min and counterstained with propidium iodide. Images were collected with a MRC-600 Laser Scanning Confocal apparatus (BioRAD Laboratories) coupled to a Nikon Optiphot microscope.

Cells were analyzed by two-dimensional flow cytometry using MPM-2 monoclonal antibody recognizing mitosis specific phosphoepitopes,[30] and propidium iodide, a marker of DNA content. Cells were fixed, incubated with MPM-2 antibodies, and labeled with FITC-conjugated goat anti-mouse IgG secondary antibodies and propidium iodide as described previously.[31] Data were collected using a FACScan flow cytometer (Becton Dickinson, San Jose, CA), with propidium iodide in the first, and MPM-2 in the second dimension, using Cellquest software, and for each sample 10,000 events were collected and aggregated cells were gated out.

4.4. Protocol for Eg5 purification by affinity chromatography

HeLa cells were plated in 10 × 15-cm plates and blocked in mitosis with 100 ng/mL nocodazole (SIGMA). All subsequent steps were performed at 4 °C. Mitotic cells were collected by shake off, washed with PBS, resuspended in lysis buffer (20 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, 0.2 M NaCl, 1 mM DTT), and then lysed by homogenization. Following centrifugation at 100 kg for 1 h, the supernatant was collected (3 mL; protein content: 2.0 mg/mL) and loaded onto a column conditioned with the control (non-derivatized) chromatography matrix (1 mL). The flow-through was directly loaded onto a second column packed with the affinity matrix **25** (Sepharose-monastrol beads; 1 mL; flow-through: 3 mL; protein content: 1.2 mg/mL). Both columns were washed with 5 mL of lysis buffer. Material from the non-active first column was eluted with 2 M NaCl, whereas bound Eg5 was released from the second column with 5 mL of lysis buffer supplemented with 1 mM STLC. The eluates were collected as 500 μL fractions. The eluted fractions with Eg5 contained 30–70 μg/mL of protein. Equal volumes from each fraction were taken and SDS gel samples were prepared in Lammli buffer and loaded in 8% SDS-gel electrophoresis followed by transfer to nitrocellulose sheets using a semi-dry transfer apparatus. Blots were incubated with rabbit anti-Eg5 antibodies (directed against the specific C-terminal part of the human protein) used at 1/1000 dilution and then exposed to horseradish peroxidase-conjugated goat anti-rabbit IgG, diluted 1/5000, and developed by echochemiluminescence (ECL, Amersham). A similar procedure was applied using the control resin.

4.5. Protein identification by mass spectrometry

Proteins from the eluted fractions were further separated by 10% SDS–PAGE with a MiniProtean 3 cell (Bio-Rad, Munich, Germany), stained with Coomassie G-250, and identified by peptide mass fingerprinting and tandem mass spectrometry (MS/MS). The gel bands were excised with a scalpel for in-gel digestion with 0.1 μg of trypsin in 20 μL of 50 mM ammonium hydrogenocarbonate, pH 7.8. An ULTRAFLEX II (Bruker Daltonics, Bremen, Germany) MALDI-TOF/TOF mass spectrometer was used with a mass accuracy of 50 ppm after external calibration. The samples were analyzed in the MS mode (for generation of peptide mass fingerprints) as well as in the TOF/TOF mode (for fragmentation analysis of the 1–2 most intense peaks). The matrix used was α-cyano-4-hydroxycinnamic acid (20 mg/mL) in 0.3% aqueous trifluoroacetic acid/acetonitrile (2:1). The samples were applied to a stainless steel sample holder and introduced into the mass spectrometer after drying. Mass spectra were transformed into peak lists by using FlexAnalysis v.2.4 (Bruker Daltonics, Bremen, Germany). The peak lists of the MS and MS/MS spectra were merged by BioTools v.3.0 (Bruker Daltonics, Bremen, Germany). Proteins were identified using an in-house copy of the protein identification software program Mascot.[32] Mass spectra were searched against the Swiss-Prot database, setting mass accuracy to 50 ppm (MALDI-TOF/TOF) for peptide mass fingerprinting and 0.4 Da for MS/MS, allowing for up to one missed cleavage site, pyro-Glu formation at N-terminal Gln, oxidation of methionine, N-terminal acetylation of the protein, and modification of cysteines by acrylamide. The MS/MS spectra were further manually confirmed considering the criteria for MALDI-TOF/TOF described elsewhere.[33]

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