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Synthesis and biological evaluation of L-cysteine derivatives as mitotic kinesin Eg5 inhibitors

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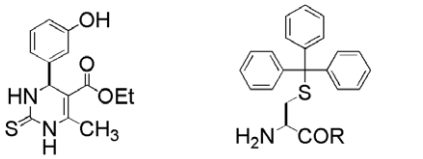
Abstract—Inhibition of Eg5 represents a novel approach for the treatment of cancer. Here, we report the synthesis and structure-activity relationship of *S*-trityl-L-cysteine (STLC) derivatives as Eg5 inhibitors. Some of these derivatives such as **4f** demonstrated enhanced inhibitory activity against Eg5 and induced mitotic arrest with characteristic monoastral spindles in HeLa cells.

Antimitotic agents such as taxol, epothilone, and vinca alkaloids have found clinical utility as cancer chemotherapeutic agents.[1] These agents bind to the β-tubulin protein a component of the mitotic spindle microtubules. Although microtubules play important roles throughout the cell cycle of cancer cells, disruption of microtubule dynamics also produces undesirable side effects, such as toxicity in non-dividing cells like peripheral neurons, leading to peripheral neuropathy in patients.[2]

The microtubule associated kinesin Eg5 also known as kinesin spindle protein (KSP), a member of the kinesin-5 family, plays an important role in the early stages of mitosis. It is responsible for the formation and maintenance of the bipolar spindle.[3] Inhibition of Eg5 leads to cell cycle arrest during mitosis and causes cells with a monopolar spindle, so called monoasters.[4] Because Eg5 is not expressed in postmitotic neurons and is likely to act only in dividing cells, its inhibitors might provide better specificity than microtubule inhibitors in the treatment of human malignancies.[5] In 1999, Monastrol, the first small-molecule inhibitor of Eg5, was discovered in a phenotype-based screening.[6] Since then, a number of Eg5 inhibitors have been found and some of them appeared as the therapeutic potential for anticancer drugs.[7]

In order to find a new structural class of kinesin Eg5 inhibitors, we screened our chemical library using in vitro ATPase assay measuring the test compound's ability to prevent the ATP hydrolysis by Eg5.[8] As the results of screening efforts, we identified L-cysteine derivatives **1** and **2** (Fig. 1) as kinesin Eg5 inhibitors. Although compound **1** (*S*-trityl-L-cysteine, STLC) was known as an Eg5 inhibitor,[9,10] there were no reports on structure-activity relationships (SARs) for the related analogues. Therefore, we decided to make synthetic efforts on its derivatizations to find potent and selective inhibitors of Eg5. In this paper, we describe the synthesis of L-cysteine derivatives and their inhibitory activity against Eg5 as well as their ability to inhibit cell division.



Monastrol

1: R=OH IC_{50}= 1.8 μM
2: R=NH_2 IC_{50}= 3.1 μM

Figure 1. Eg5 inhibitors: Monastrol, cysteine derivatives (**1**), and (**2**).

Keywords: *S*-Trityl-L-cysteine; Mitotic kinesin; Eg5; Inhibitor; Anticancer.
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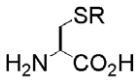
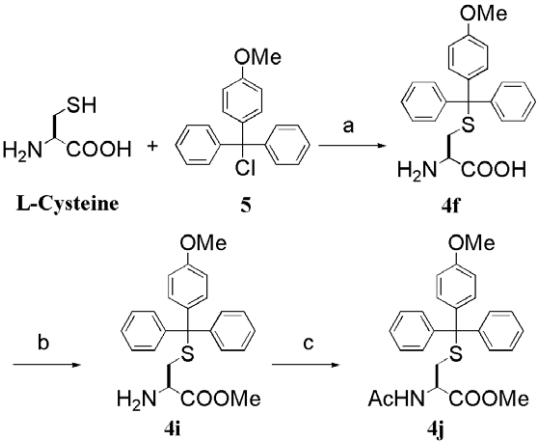
Table 1. SAR of thiol substituent of L-cysteine



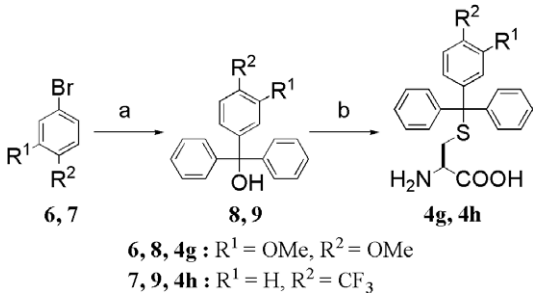
3

Compound	R	ATPase IC_{50}[a] (μM)	Cytotoxicity IC_{50}[a] (μM)
3a (L-cysteine)	H	>63	>50
3b	CH_3	>63	>50
3c	*t*-Bu	>63	>50
3d	CH_2Ph	>63	>50
3e	CH_2-4′-anisole	>63	>50
3f	CH_2COOH	>63	>50
3g	$CH_2CH_2NH_2$	>S3	>50
3h	CH-9*H*-fluorene	>63	>50
3i	$CHPh_2$	>63	>50
1 (STLC)	CPh_3	1.80	3.29

[a] IC_{50} values were derived from dose–response curves generated from triplicate data points.



Scheme 1. Reagents and conditions: (a) DMF, rt; (b) $SOCl_2$, MeOH, 0 °C then reflux; (c) AcCl, pyridine, CH_2Cl_2, rt.



Scheme 2. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C; then benzophenone, THF; (b) L-cysteine, TFA, rt.

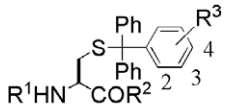
During our first attempts to develop a SAR and to identify the key features that would drive the inhibition, we focused on modifying the thiol substituents in the L-cysteine. At first, we confirmed the lack of potency of commercially available triphenylmethane and triphenylmethanethiol against Eg5 (ATPase assay $IC_{50} > 63$ μM). Then, we purchased compounds (**3a–i**) from several suppliers and evaluated for their activity against Eg5 and an anti-proliferation activity in HeLa cells (Table 1).[11] Surprisingly, all examination of substituents on the thiol group in the L-cysteine resulted in loss of potency in both assays (**3a–i**). Based on these initial results, we expected that cysteine derivatives containing triarylmethyl group would have a greater potential for high inhibition levels of the Eg5. Next we focused on modifications for the triarylmethyl, amino, and carboxyl moieties in STLC.

The synthetic procedures for the target compounds are summarized in Schemes 1 and 2. Compound **4f** was easily prepared by treating L-cysteine with commercially available triarylmethyl chloride **5**.[12] Compound **4f** was converted to the methyl ester **4i** with $SOCl_2$ in MeOH, and subsequently acylation by AcCl led to compound **4j**. Compounds **4g** and **4h** were synthesized as follows; triphenylmethanols **8** and **9** were prepared by treating corresponding aryl bromide **6** or **7** with *n*-BuLi at −78 °C in THF to form aryl lithium which upon treatment with benzophenone, and condensation with L-cysteine in the presence of TFA provided target compounds **4g** and **4h**, respectively.

As expected, triarylmethyl group in L-cysteine derivatives was essential for high inhibition level of both in Eg5 ATPase and cytotoxic activities and close correlation for inhibitory activities between Eg5 ATPase and cytotoxicity was observed across almost all compounds (Tables 1 and 2). In general, protection of the amino terminal in **1** resulted in loss of both activities (**4a–d, 4j**). Modification of the carboxylic acid terminal in **1** to the primary amide as in **2** or the methyl ester as in **4e** yielded little loss of Eg5 ATPase activity. On the other hand, cytotoxicity of **2** and **4e** was slightly improved compared to **1**, presumably reflecting different cell permeability properties. There is a possibility that the major cellular uptake pathway of STLC is amino acid transporter such as System L. System L and other amino acid transporters require free amino and carboxyl moiety as substrate.[13] However, we showed that the cytotoxicity of **2**, **4e**, and **4i** was improved compared to **1**. These results suggest that hydrophobicity of STLC derivatives is more important for incorporation.

Drastic changes both in Eg5 ATPase and cytotoxic activities were observed by incorporation of substituents onto the phenyl ring (**1** versus **4f–h**, **4e** versus **4i**). Remarkably, compound **4f** showed >10-fold potent inhibition both in Eg5 ATPase and cytotoxic activities as compared to **1**. However, incorporation of 3,4-dimethoxy substituents onto the phenyl ring resulted in loss of potency (**4g**). Among 4-MeO analogues (**4f**, **4i**, and **4j**), the same SAR for L-cysteine moiety above mentioned was obtained. That is, modification of carboxylic acid terminal to methyl ester yielded little loss of Eg5 ATPase activity (**4f** versus **4i**), and acetyl protection of amino terminal resulted in activity losses of the both (**4i** versus **4j**). Introduction of trifluoromethyl group provided compound **4h** with sevenfold increase in Eg5 ATPase and sixfold increase in cytotoxic activity over **1**. We then chose three compounds (**4f**, **4h**, and **4i**) and evaluated for inhibition of four other structurally and

Table 2. SAR of side chain (R^1, R^2) and phenyl substituent (R^3)



4

Compound	R^1	R^2	R^3	ATPase IC_{50}[a] (μM)	Cytotoxicity IC_{50}[a] (μM)	Mitotic arrest MI_{50}[b] (μM)
1 (STLC)	H	OH	H	1.8	3.29	1.4
2	H	NH_2	H	3.1	1.78	NT
4a	Ac	OH	H	>63	>50	NT
4b	Fmoc[c]	OH	H	55.0	11.9	NT
4c	Boc	OH	H	>63	>50	NT
4d	Trityl	OH	H	>63	16.5	NT
4e	H	OMe	H	5.8	1.72	NT
4f	H	OH	4-OMe	0.15	0.21	0.15
4g	H	OH	3,4-Dimethoxy	14.8	49.7	NT
4h	H	OH	4-CF_3	0.22	0.53	0.34
4i	H	OMe	4-OMe	0.68	0.32	NT
4j	Ac	OMe	4-OMe	>63	>50	NT

[a] IC_{50} values were derived from dose–response curves generated from triplicate data points.
[b] MI_{50} values were derived from dose–response curves generated from triplicate data points; NT, not tested.
[c] Fmoc; 9-fluorenylmethoxycarbonyl.

Table 3. ATPase inhibitory activity of selected compounds[a]

Compound	Eg5	CENP-E	Kid	MKLP-1	KIF-4
1 (STLC)	89	0	0	2	4
4f	98	0	0	10	5
4h	79	0	0	7	3
4i	100	0	0	5	4

[a] The inhibition values were determined by ATPase assay and are indicated as a percentage of the solvent only control. Values are the means of three independent measurements. The final concentration of the compounds in the assay was 20 μM.

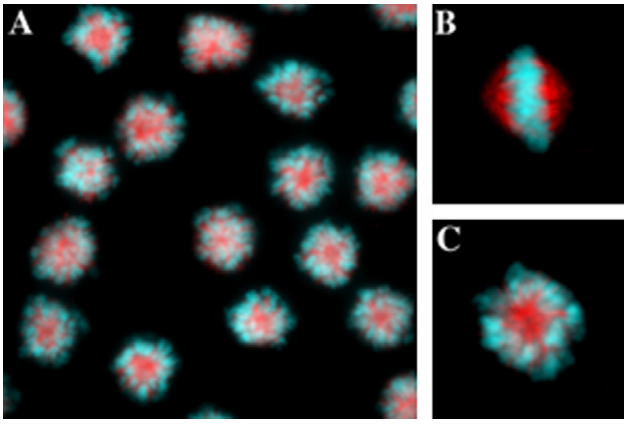


Figure 2. HeLa cells with normal spindle (B) and with monoasters after treatment with compound **4f** (1 μM) (A and C).

functionally related mitotic kinesins (CENP-E, kid, MKLP1, KIF4).[14] All these kinesins were not inhibited by the tested compounds including **1** at 20 μM, respectively (Table 3). These results indicate that the selected derivatives are specific Eg5 inhibitors as STLC is.

In order to confirm that the cytotoxicity displayed by the STLC derivatives was a consequence of Eg5 inhibition, we further characterized the cellular activity of compounds **4f** and **4h**. Cells with suppressed Eg5 function, for example by RNAi or small molecule inhibitors, display a characteristic monoastral phenotype and arrest in mitosis. HeLa cells treated with compounds **4f** or **4h** for 18 h, corresponding to approximately one cell cycle, showed an increase in the mitotic index relative to untreated cells. The MI_{50} values (concentration of 50% induction in M phase in HeLa cells at 20 h) of both of compounds (**4f** and **4h**) were lower than that of STLC (Table 2).[15] We next examined the mitotic phenotype of the cells treated with compound **4f**.[16] As shown in the fluorescent microscopy images of Figure 2, compound **4f** induced the formation of monopolar mitotic spindles in HeLa cells after 20-h treatment. Multiple monopolar mitotic spindles but no bipolar mitotic spindles were observed (Fig. 2). Several DAPI-stained chromosomes (blue) are detected in the focal plane with microtubules of the mitotic spindle (red). As expected, the monoastral phenotype was observed in cells treated with **4f**.

In conclusion, we have identified specific and potent cell-permeable inhibitors of the mitotic kinesin Eg5. Structural optimization of the screening hits **1** and **2** led to the sub-micromolar inhibitors **4f**, **4h**, and **4i**. The most potent compound **4f** was >10-fold more potent than initial compound **1**, both in Eg5 ATPase and cytotoxic activities. Future efforts will focus on further improvement in potency for this compound series.

Acknowledgments

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14. Other kinesins' inhibitory activity was assessed by measuring the amount of ATP resulting from in vitro reaction using Kinase-Glo Plus luminescent kinase assay (Promega). The in vitro ATPase assay was performed as described as Eg5.
15. Mitotic arrest was measured by assessing the mitosis-specific phosphorylation of Histone H3 using anti-phospho-Histone H3 (Ser10) antibody (Upstate). The positive cells to the antibody were counted under the microscope while total cell number was measured by assessing the DAPI-stained cells.
16. HeLa cells treated with STLC derivatives were fixed by the conventional paraformaldehyde/methanol method, and stained immunologically. Mitotic chromatin was stained by anti-phospho-Histon H3 (Ser10) antibody (Upstate). Microtubules and DNA/chromatins were stained by monoclonal anti-a-tubulin (DM1A) antibody (Sigma) and DAPI, respectively. Fluorescent images were observed under Olympus IX71 microscope with DP30BW CCD camera.