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Synthesis and SAR of pyrrolotriazine-4-one based Eg5 inhibitors

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Abstract—Synthesis and SAR of substituted pyrrolotriazine-4-one analogues as Eg5 inhibitors are described. Many of these analogues displayed potent inhibitory activities in the Eg5 ATPase and A2780 cell proliferation assays. In addition, pyrrolotriazine-4-one analogue **26** demonstrated in vivo efficacy in an iv P388 murine leukemia model. Both NMR and X-ray crystallographic studies revealed that these analogues bind to an allosteric site on the Eg5 protein.

Antimitotic agents such as taxol and the vinca alkaloids have been successfully used for the treatment of human cancer.[1] These agents bind to the tubulin component of the mitotic spindle microtubules, disrupting microtubule dynamics, inducing mitotic arrest, and inhibiting tumor cell proliferation.[2,3] Because microtubules play important roles throughout the cell cycle, disruption of microtubule dynamics by taxol and vinca alkaloids also produces undesirable side effects, such as toxicity in non-dividing cells like peripheral neurons, leading to peripheral neuropathy in patients.[4]

Antimitotic agents that specifically bind and modulate the activity/function of other components of the mitotic spindle apparatus may provide a novel mechanism by which to target the clinically validated mitotic spindle and reduce undesirable side effects. Human Eg5, a Kinesin 5 family member (also known as KIF 11 or KSP), is a motor protein that specifically localizes to the mitotic spindle apparatus and plays an important role in formation and function of the bipolar mitotic spindle.[5–7] Inhibition of Eg5 activity induces the formation of an aberrant mitotic spindle (monopolar spindle or monoastral array of spindle microtubules), induces cell cycle arrest at M-phase, and inhibits cell proliferation.[8–10] Small molecule inhibitors of the ATPase activity of the human Eg5 (KSP) motor domain have recently been reported.[10–12]

Herein, we report on the synthesis of pyrrolotriazin-4-one based Eg5 inhibitors and SAR as shown in Figure 1.

The 5-chloropyrrolotriazine analogue **7** was prepared starting from 3-chloropyrrole methylester **1**[13] which was aminated using 2,4-dinitrophenoxyamine[14] to afford

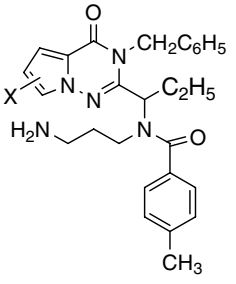

Figure 1. General structure of the pyrrolotriazine-4-one analogues.

Keywords: Eg5; Pyrrolotraizine; Microtubule; Kinesin; Anti-tumor; Cytotoxic.
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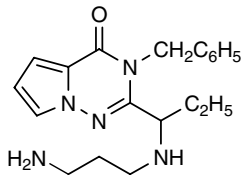
2 (Scheme 1). Reacting aminopyrrole **2** with acetonitrile followed by cyclization and benzylation led to pyrrolotriazine-4-one **4**. Conversion of the 2-methyl group to the 2-formyl group was achieved via enamine formation and oxidative cleavage using $NaIO_4$ to obtain 2-formylpyrrolotriazin-4-one **5**. Reaction of compound **5** with ethyl magnesiumbromide followed by chlorination using thionyl chloride provided the 2-chloromethylpyrrolotriazine-4-one intermediate **6**. Coupling of **6** with *N*-Boc-1,3-diaminopropane, acylation with *p*-toluoyl chloride, and deprotection provided 5-chloropyrrolotriazine-4-one **7** as a HCl salt.

The 6-chloropyrrolotriazine-4-one analogue **14** was prepared starting from 4-chloropyrrole ester **9**[15] (Scheme 2). The amination product of **9** was reacted with Fmoc-protected thioamide **A**[16] to afford benzylaminobutylidene adduct **10**. Thermal cyclization of **10** afforded a mixture of 6-chloropyrrolotriazine-4-one **11** and triazepine **12** which was easily separated by flash column chromatography on silica gel. Deprotection of the Fmoc of **11** followed by reductive alkylation, acylation with *p*-toluoyl chloride, and deprotection of the *N*-Boc group afforded 6-chloropyrrolotriazine-4-one **14**.

The 7-chloro, 5,7-dichloro, and des-chloropyrrolotriazine analogues **18**, **19**, and **20** were prepared following the similar procedures described in Scheme 1 starting from 5-chloro and 3,5-dichloropyrrole esters **16** and **17**[17] (Scheme 3). Dehalogenation of 7-chloro-pyrrolotriazine-4-one **18** using PtO_2 in MeOH–HOAc under 1 atm of H_2 gas afforded des-chloro-pyrrolotriazine-4-one **20**.

The binding site on Eg5 for this series of compounds was determined initially by NMR studies of **21**, a truncated analogue of **20**.[18]

Although resonance assignments for the protein were not determined, intermolecular nuclear Overhauser effects (NOEs) observed between labeled alanine, isoleucine, and threonine ('AIT') Eg5 protein and **21** were tentatively assigned to amino acid type based on typical

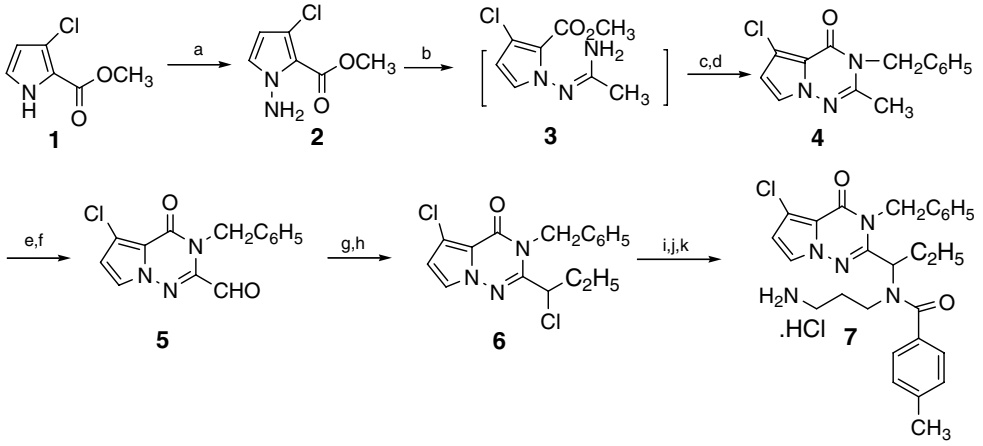


21

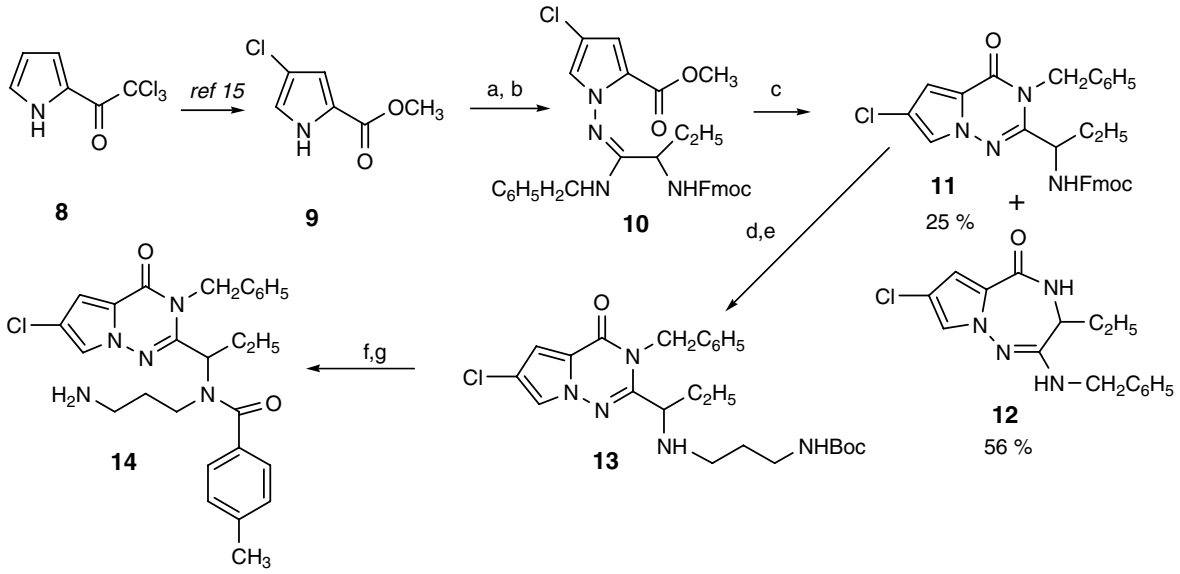
IC_{50} = 100 μM, ATPase assay

^1H and ^{13}C chemical shifts of Ala, Ile, and Thr residues.[19] Intermolecular NOEs were observed between protons on the pyrrolotriazine-4-one ring and a single Ala methyl group and a single Ile δ-methyl group. No Ala or Ile residues were within 5 Å of the ADP pocket, eliminating this pocket as a potential binding site for **21**. Using the NOE information, we identified 4 possible binding sites where an Ala and Ile were proximal and could accommodate **21**. The preferred candidate binding site formed a pocket where numerous protein:ligand interactions could occur and a model of Eg5:compound **21** using the NMR experimental data led to the tentative assignments of the NOEs to Ala218 and Ile136.

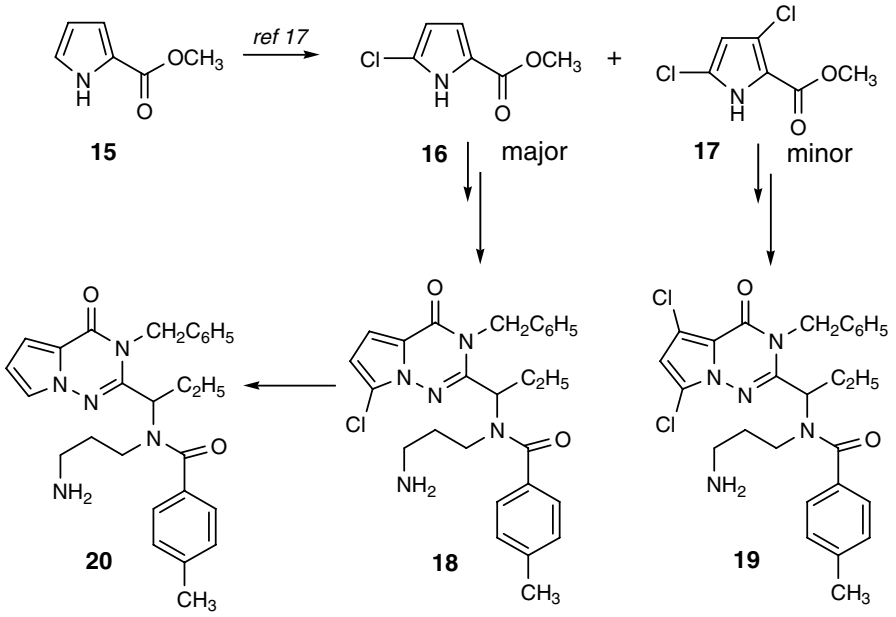
The X-ray crystal structure[20] of the ternary complex of Eg5, compound **24**, and ADP confirmed these assignments from the NMR studies. This series of compounds binds to the allosteric site (Fig. 2), which has also been seen by others with monastrol,[12a] dihydropyrazoles,[21] and tetrahydroisoquinolines.[22] The binding of ligands to the allosteric site causes considerable change in the tertiary structure of Eg5. The center-to-center distance between compound **24** and the ADP is ~13 Å and the closest approach between atoms is the amino nitrogen to a phosphate oxygen distance that is greater than 7 Å, indicating that beyond some possible long distance electrostatic interactions, compound **24** and the ADP do not directly interact with each other. The X-ray crystal structure shows the inhibitor binds the protein in a folding conformation where two side-chain phenyl rings interact with each other in a face-to-edge fashion.[23] These two phenyl rings and the cyclopropyl interact



Scheme 1. Reagents and conditions: (a) 2,4-dinitrophenoxyamine, NaH, DMF, rt; (b) CH_3CN, HCl, dioxane, 82 °C; (c) CH_3CN, Et_3N, 85 °C; (d) Cs_2CO_3, BnBr, dioxane, 95 °C; (e) DMF dimethylacetal, $MgSO_4$, DMF, 146 °C; (f) $NaIO_4$, THF, pH 7 buffer, rt; (g) ethyl magnesiumbromide, CH_2Cl_2, −78 to −50 °C; (h) $SOCl_2$, pyridine, CH_2Cl_2, 0 °C to rt; (i) *N*-Boc-1,3-diaminopropane, EtOH, reflux temp; (j) *p*-toluoyl chloride, Et_3N, CH_2Cl_2, rt; (k) 4 M HCl/dioxane, rt.



Scheme 2. reagents and conditions: (a) 2,4-dinitrophenoxyamine, NaH, DMF, rt; (b) BnNHC(S)CH(NHFmoc)Et (**A**), EDCI·HCl, CH_2Cl_2, rt; (c) xylene, 130 °C, 7 h; (d) piperdine, DMF; (e) (3-oxo-propyl)-carbamic acid *tert*-butyl ester, $NaBH(OAc)_3$, HOAc, THF, rt; (f) *p*-toluoyl chloride, Et_3N, CH_2Cl_2, rt; (g) TFA, CH_2Cl_2, rt.



Scheme 3. Synthetic steps similar to those outlined in Scheme 1.

with Trp127, Tyr211, Pro137, and Arg119 of the protein. The pyrrole ring makes van der Waals contacts with the surface of the pocket formed by Ile136, Leu214, Phe239, and Leu160 of the protein.

Compound **24** inhibits the ATPase activity of Eg5, an apparent consequence of changes in Eg5 tertiary structure that result from the binding of compound **24** (Table 2). Optimization of this series through the development of structure–activity relationships monitored the activity of new analogues in a microtubule-dependent Eg5 ATPase assay.[25] The cytotoxic activity of new analogues on human tumor cell lines was also determined. Inhibition of A2780 ovarian carcinoma cell proliferation is reported here.[26]

Analogues having a chlorine substituent on the pyrrole ring at either C-5 or C-7 (**7** and **18**) retained the same activity as non-substituted analogue (**20**) both in ATPase and human ovarian carcinoma A2780 cell line cytotoxicity assays (Table 1). However, chlorinated analogues produced generally more favorable pharmacokinetic profiles than the non-chlorinated analogue. While one chlorine substituent at either C-5 or C-7 is tolerated, bis-chlorination at C-5 and C-7 (**19**) leads to a reduction in potency, possibly because compound **19**

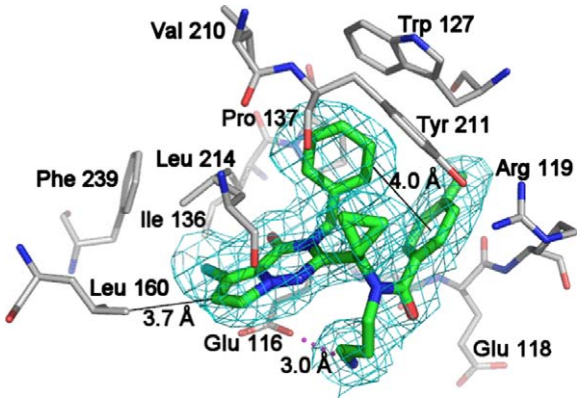
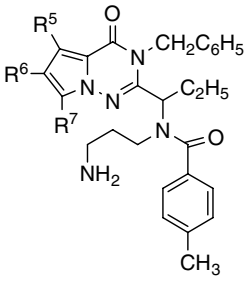


Figure 2. The binding site for compound **24** on Eg5. Compound **24** is shown with thick rods and the protein is shown with thinner rods. The initial $2F_o - F_c$ electron density map contoured at 1σ is shown in cyan mesh. Atomic coloring (N, blue; O, red; Cl, cyan) is used with green for carbon atoms of the compound and gray for carbon atoms of the protein. Figure created using PyMol.[24]

Table 1. SAR of the pyrrole ring



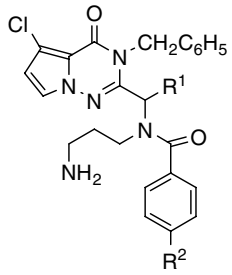
Compound	R^5	R^6	R^7	IC_{50}[a] (μM)	
				ATPase	A2780 cytotoxicity
7	Cl	H	H	0.07 (0.01)	0.14 (0.02)
14	H	Cl	H	0.33 (0.04)	0.98 (0.01)
18	H	H	Cl	0.06 (0.01)	0.13 (0.03)
19	Cl	H	Cl	0.29 (0.06)	0.47 (0.03)
20	H	H	H	0.08 (0.01)	0.11 (0.004)

[a] IC_{50} values are reported as means of three independent determinations with standard deviations shown (in parentheses).

loses the flexibility to adjust itself to the compact space of the hydrophobic pocket. Chlorination at C-6 (**14**) also resulted in reduction in potency. The pyrrole ring's C-6 is directed toward Leu160 (see Fig. 2) and the distance from the side chain of Leu160 to C-6 of pyrrole ring is only 3.7 Å which would cause a steric clash when C-6 is substituted with chlorine.

Substitution of ethyl or cyclopropyl for the C-2 methyl side chain produced analogues (**7**, **22**, and **24**) which displayed potent enzyme and cell activity, while non-substituted or vinyl-substituted analogues **23** and **25** possess significantly less activity (Table 2). Loss of activity of the vinyl analogue **25** is surprising in view of the fact that ethyl and cyclopropyl analogues are potent inhibitors. Generally, analogues with a *p*-methyl substituent on the phenyl ring were slightly more potent than the *p*-chloro-substituted analogues (**7** vs **22**).

Table 2. SAR of C-2 methyl substituent (R^1) and phenyl substituent (R^2)



Compound	R^1	R^2	IC_{50}[a] (μM)	
			ATPase	A2780 cytotoxicity
7	C_2H_5	CH_3	0.07 (0.01)	0.14 (0.02)
22	C_2H_5	Cl	0.19 (0.03)	0.44 (0.01)
23	H	Cl	>25	>10
24	Cyclopropyl	CH_3	0.10 (0.02)	0.18 (0.01)
25	Vinyl	CH_3	1.92 (0.12)	2.79 (0.47)

[a] IC_{50} values are reported as means of three independent determinations with standard deviations shown (in parentheses).

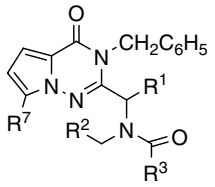
Analogues with a basic amine function on the C-2 side chain displayed the best enzyme inhibitory activity (**26**, **28** vs **27**, **31**, Table 3). This is explained based on the observation that the ammonium ion of the inhibitor interacts with Glu116 (3.0 Å, see Fig. 2). A relatively small space in this region disfavors the gem-dimethyl substituted amines (**29**, **30**) or bulkier amine (**32**).

As shown in Tables 1–3, inhibition of Hs Eg5 ATPase activity in vitro correlates with inhibition of A2780 human ovarian carcinoma cell proliferation in a 72 h cytotoxicity assay. Examination of treated cells at earlier time point shows a significant increase in the percentage of cells arrested in mitosis compared to untreated controls (3% mitotic cells). For example, treatment of A2780 cells with compound **26** for 17 h, corresponding to approximately one cell cycle, results in 65% of the cell population in mitosis.[27] The induction of mitotic arrest is consistent with an Eg5 mechanism of action for the cytotoxic activity of compound **26** in human cancer cell lines.

In order to determine the in vivo antitumor effects of pyrrolotriazine-4-one based Eg5 inhibitors, compounds were evaluated in the iv P388 murine leukemia model.[28] Compounds were evaluated for their effect upon lifespan, compared to untreated iv P388 control mice. Compound **26**, which has a good V_{ss} with a 4.5 h half-life (Table 4), was active in an iv P388 murine leukemia model (Table 5, where T/C > 125% is considered active). At its MTD of 20 mg/kg/inj, when given by intravenous injection daily for five consecutive days (5× qd, iv), a maximum increase in lifespan corresponding to a T/C of 163% was achieved.

In summary, potent pyrrolotriazine-4-one based Eg5 inhibitors were synthesized and were shown to have potent activity in ATPase and cell proliferation assays. Compound **26** was efficacious in an iv P388 leukemia

Table 3. SAR of amine side chain (R^2)



Compound	R^7	R^1	R^2	R^3	IC_{50}[a] (μM)	
					ATPase	A2780 cytotoxicity
26	Cl	Cyclopropyl	$CH_2CH_2NH_2$	4-$C_6H_5CH_3$	0.06 (0.003)	0.05 (0.025)
27	Cl	Cyclopropyl	$CH_2C(O)NH_2$	4-$C_6H_5CH_3$	0.98 (0.11)	0.33 (0.044)
28	Cl	C_2H_5	$CH_2CH_2NH_2$	4-C_6H_5Cl	0.06 (0.006)	0.16 (0.01)
29	H	C_2H_5	$CH_2C(CH_3)_2NH_2$	4-C_6H_5Cl	0.65 (0.08)	1.14 (0.03)
30	H	C_2H_5	$C(CH_3)_2NH_2$	4-C_6H_5Cl	3.72 (0.93)	2.72 (0.33)
31	H	C_2H_5	CH_2CH_2OH	4-$C_6H_5CH_3$	6.88 (0.33)	2.86 (0.003)
32	H	C_2H_5	4-Piperdyl	4-$C_6H_5CH_3$	6.06 (1.02)	4.46 (0.71)

[a] IC_{50} values are reported as means of three independent determinations with standard deviations shown (in parentheses).

Table 4. Summary of pharmacokinetic parameters of compound **26** (10 mg/kg in 50% PEG400/50% water) in mice ($n = 3$)

Clearance (mL/min/kg)	V_{ss} (L/kg)	MRT (h)	$t_{1/2}$ (h)
16 ± 2.7	5.9 ± 0.92	6.5 ± 2.3	4.5 ± 1.6

Table 5. Summary of compound **26** in vivo antitumor activity against P388 murine leukemia

Dose (mg/kg)	Route of administration	Schedule	% T/C
20	iv	5× qd	163

model. NMR and X-ray crystallographic studies revealed that these analogues bind to an allosteric site on the Eg5 protein.

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