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Design, synthesis and evaluation of photoactivatable derivatives of microtubule (MT)-active [1,2,4]triazolo[1,5-*a*]pyrimidines

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ABSTRACT

The [1,2,4]triazolo[1,5-*a*]pyrimidines comprise a promising class of non-naturally occurring microtubule (MT)-active compounds. Prior studies revealed that different triazolopyrimidine substitutions can yield molecules that either promote MT stabilization or disrupt MT integrity. These differences can have important ramifications in the therapeutic applications of triazolopyrimidines and suggest that different analogues may exhibit different binding modes within the same site or possibly interact with tubulin/MTs at alternative binding sites. To help discern these possibilities, a series of photoactivatable triazolopyrimidine congeners was designed, synthesized and evaluated in cellular assays with the goal of identifying candidate probes for photoaffinity labeling experiments. These studies led to the identification of different derivatives that incorporate a diazirine ring in the amine substituent at position 7 of the triazolopyrimidine heterocycle, resulting in molecules that either promote stabilization of MTs or disrupt MT integrity. These photoactivatable candidate probes hold promise to investigate the mode of action of MT-active triazolopyrimidines.

Originally reported as anti-fungal agents, microtubule (MT)-active [1,2,4]triazolo[1,5-*a*]pyrimidines and related heterocyclic molecules[1–5] have since attracted attention as potential candidates for a variety of applications including cancer chemotherapy,[6] as well as neurodegenerative disease treatment.[7–10] The mechanism of action of this class of compounds has been the focus of several studies and appears to be distinct from that of other MT-targeting agents. Competition binding experiments first revealed that cevipabulin[6] (**1**, Fig. 1), a typifying example of the triazolopyrimidine class and a potent anti-cancer compound, competes with vincristine but not taxol or colchicine for binding to MTs, suggesting that compounds from this class may interact with MTs at the binding site of vinca alkaloids. This hypothesis has recently been confirmed by X-ray crystallography and mode- of-action studies[11] in which triazolopyrimidine congener **2** (Fig. 1) was found to bind at the interface between two consecutive longitudinally-associated tubulin heterodimeric subunits, in a region that largely overlaps with the vinca binding site. However, opposite to the activity of vincristine/vinblastine, the binding of **2** to MTs was found to promote MT-assembly and stabilization. Interestingly, the same studies also demonstrated that **2** binds exclusively to MTs and not to unpolymerized tubulin heterodimers.[11]

However, prior investigations with **1** clearly suggested that this compound, like vincristine, can in fact interact with tubulin heterodimers and interfere with the rate of exchange of the guanosine triphosphate (GTP).[12] Furthermore, side-by-side evaluation of the MT-stabilizing properties of different triazolopyrimidines in cell-based assays highlighted important differences in activity depending on whether or not these molecules contained an alkoxide side chain at the 4 position of the fluorinated phenyl ring.[8] Indeed, whereas triazolopyrimidine lacking the alkoxy side chain, such as **3** and **4** (Fig. 1), were found to stabilize MTs and preserve MT integrity, other examples with the side chain, including **1** and **5** (Fig. 1), were found to affect markers of stable MTs with a bell-shaped dose-response relationship, and to produce a proteasomal-dependent degradation of tubulin, thereby leading to an overall disruption of MT integrity.[8] Consistent with this structure-activity relationship (SAR), we confirmed that compound **2**, which

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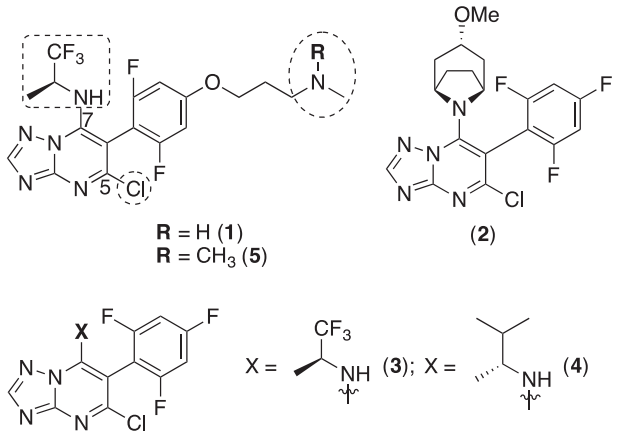

Figure 1. Structures of selected MT-active triazolo-pyrimidines; dotted lines highlight sites chosen for insertion of photoactivatable moieties.

compared to **1** features a relatively bulky bicyclic amine substituent at position 7 but lacks the alkoxy side chain, produces MT-stabilization in HEK-293 cells, as evidenced by an elevation in acetylated α-tubulin, a known marker of stable MTs,[13] without reducing total tubulin levels (see Table 1). Taken together, these observations suggest that different triazolopyrimidine congeners may interact with tubulin/MTs in different manners depending on the particular substitution pattern, thereby producing different effects on MT structure and function. Such differences are likely to have important implications in terms of therapeutic applications of triazolopyrimidines as, depending on the particular clinical indication, only one of these effects (*i.e.*, preservation or disruption of MT integrity) may be desirable.[8] Whereas the X-ray crystallography studies with **2** provide a detailed insight on how MT-stabilizing triazolopyrimidines interact with and stabilize MTs, further investigations are likely needed to fully elucidate the mode of action of other MT-binding triazolopyrimidine congeners that can lead to altered MT morphology and tubulin degradation, such as **1**.

Photoaffinity labeling (PAL) experiments are commonly utilized to identify biological target(s) and/or investigate the mode of action of small molecules, and can be useful to complement other techniques such as NMR or X-ray crystallography.[14] To enable PAL experiments, a series of triazolopyrimidine derivatives bearing known photoreactive moieties were designed, synthesized and evaluated with the goal of identifying candidate probes. In particular, we explored derivatives in which arylazides or diazirines were appended/incorporated at the end of the alkoxy side chain, or at positions 7 or 5 of the triazolopyrimidine core (see Fig. 1). These studies led to the identification of selected photoactivatable derivatives that retain the characteristic biological activity of MT-active triazolopyrimidines that are either lacking or bearing the alkoxy side chain.

The synthesis of test compounds was largely based on strategies that had been previously described by Wyeth[6] for the synthesis of triazolopyrimidine **1** and related derivatives (Scheme 1). Thus, azidated derivatives **6**, **7**,[7] and **8**, in which the photoactivatable group is at C-5 (**7** and **8**) or C-7 (**6**), were accessed respectively from triazolopyrimidine **9**, **10** and **1** upon reaction with sodium azide. Triazolopyrimidine derivative **11**, which comprises an aryl diazirine fragment appended at the terminal amine of the alkoxy side chain, was readily accessed via *N*-acylation of known compound **12**[6] with commercially available diazirine **13**. Finally, triazolopyrimidine **14**–**16**, which incorporate a diazirine ring into the amine fragment linked at C-7, were accessed from dichloride **9** upon reaction with the appropriate diazirine containing amine (**17**–**19**) that were prepared from *N*-Boc protected amino acids (see Supporting Information). Further reaction of **14**–**16** with the appropriate alkoxy side chain resulted in derivatives **20**–**22**.

After synthesis, each of the candidate probes was evaluated in a cell-based MT assay[8] in which acetylated α-tubulin and total α-tubulin levels were determined by ELISA in cell lysates after 4 h of incubation with the test compound at either 1 or 10 μM. As summarized in Table 1, azidated derivatives **6** and **7** were found to be essentially inactive independent of the position of the azide in the triazolopyrimidine heterocycle. Addition of the alkoxy side chain to the fluorinated ring of derivative **7** resulted in a biologically active derivative (**8**) that was considerably less potent than either **1** or **5**, but which at 10 μM compound concentration was able to produce a significant increase in acetylated α-tubulin levels as well as a concurrent reduction in total α-tubulin. These results further confirmed that the addition of the side chain may be critical to impart MT-disrupting activity to triazolopyrimidines, although it is interesting to note that derivative **11**, which carries a modified alkoxide side chain, did not produce a significant reduction in total α-tubulin levels at compound concentrations (10 μM) that are active in the acetylated α-tubulin assay. Among other diazirine containing derivatives, incorporation of the photoactivatable moiety into the amine substituent at C-7 resulted in active derivatives (**14**–**16**) that were similar to **2**–**4** in producing an elevation of acetylated α-tubulin without a significant loss of total α-tubulin,

Table 1
Fold-changes in acetylated α-tubulin and total α-tubulin levels in HEK-293 cells after 4 h incubation with test compounds at either 1 or 10 μM.

Compound	Acetylated α-Tubulin		Total α-Tubulin	
	1 μM	10 μM	1 μM	10 μM
Cevipabulin (1)	7.07** ± 0.36	0.1** ± 0.01	0.51** ± 0.08	0.14** ± 0.03
5	5.83** ± 0.35	0.30** ± 0.15	0.29** ± 0.03	0.14** ± 0.01
2	3.65** ± 0.15	7.43** ± 0.93	1.12 ± 0.08	1.38** ± 0.19
3	2.78** ± 0.17	5.04** ± 0.45	1.18 ± 0.09	1.22 ± 0.18
4	2.14** ± 0.15	2.59** ± 0.15	1.10 ± 0.17	0.90 ± 0.16
6	Inactive	Inactive	ND	ND
8	0.97 ± 0.06	4.99** ± 0.27	0.74** ± 0.14	0.46** ± 0.06
7	Inactive	Inactive	ND	ND
11	1.30 ± 0.28	3.65** ± 0.74	1.31**± 0.13	0.88 ± 0.05
14	ND	1.85** ± 0.08	ND	0.94 ± 0.02
20	6.7** ± 0.45	0.28* ± 0.03	0.69**± 0.05	0.78** ± 0.02
15	1.42 ± 0.08	4.28** ± 0.37	0.92*† ± 0.01	0.89**† ± 0.04
21	21.7** ± 0.4	0.93 ± 0.03	0.73** ± 0.02	1.01 ± 0.05
16	1.56** ± 0.1	3.42** ± 0.06	1.08 ± 0.04	1.03 ± 0.04
22	10.5** ± 0.36	0.29** ± 0.03	0.36** ± 0.04	0.19** ± 0.02

Values represent the average and SD of assays run in triplicate. ND = not determined. *, $p < 0.05$; **, $p < 0.01$ relative to vehicle-treated control cells as determined by one-way ANOVA with Dunnett's post hoc analysis. Assay-to-assay variability suggests that changes of <15% in α-Tubulin are not meaningful.