Journal of
Medicinal Chemistry
Letter

J. Med. Chem. **2009**, *52*, 6523–6526 **6523**
DOI: 10.1021/jm9013407

Mitoxantrone Analogues as Ligands for a Stem–Loop Structure of Tau Pre-mRNA

Yang Liu,[†] Eleanor Peacey,[†] John Dickson,[†] Christine P. Donahue,[†,‖] Suxin Zheng,[‡] Gabriele Varani,[‡,§] and Michael S. Wolfe*[,†]

[†]*Center for Neurologic Diseases, Brigham and Women's Hospital and Harvard Medical School, 77 Avenue Louis Pasteur, H.I.M. 754, Boston, Massachusetts 02115,* [‡]*Department of Chemistry and* [§]*Department of Biochemistry, 109 Bagley Hall, Box 351700, University of Washington, Seattle, Washington 98195.* [‖] *Present address: Archemix Corp., 300 Third Street, Cambridge, Massachusetts 02142.*

Received September 9, 2009

Abstract: A series of mitoxantrone (MTX) analogues have been designed, synthesized, and evaluated for binding to and stabilizing a stem–loop structure that serves as a splicing regulatory element in the pre-mRNA of tau, which is involved in Alzheimer's and other neurodegenerative diseases. Several compounds showed significantly improved binding activity relative to the original screening hit mitoxantrone. These findings establish essential structure–activity relationships to further optimize the activity of this promising class of compounds.

Neuronal filaments of the microtubule-associated protein tau are pathological features in Alzheimer's and other neurodegenerative diseases, and evidence supports a role for aberrant tau in pathogenesis.[1,2] Dominant mutations in the tau gene are associated with familial frontotemporal dementia displaying typical tau pathology.[3] Many of these mutations are silent or intronic, occur near the exon 10-intron 10 border of the tau gene, and increase the inclusion of exon 10 during pre-mRNA splicing to increase the proportion of tau protein containing four microtubule-binding domain repeats (4R tau) to that containing three repeats (3R tau). These mutations were originally postulated to destabilize a stem–loop structure at this exon–intron boundary to regulate exon 10 splicing (Figure 1).[4,5] Our laboratory previously validated this stem–loop as a bona fide structure that can regulate the alternative splicing of exon 10 in cells.[6] Therefore, the tau stem–loop could represent a therapeutic target for treating "tauopathies" such as frontotemporal dementia and Alzheimer's disease: small molecules that could bind to and stabilize the tau pre-mRNA stem–loop would have the opposite effect of disease-causing tau mutations.

Via high-throughput screening, our laboratory recently identified the anticancer drug mitoxantrone as a small molecule that binds to and stabilizes the tau stem–loop (EC_{50} = 0.71 μM).[7] Aminoglycosides such as neomycin are known to bind to duplex RNA and to the tau splicing regulatory element,[8,9] and MTX could effectively compete with fluorescence-labeled aminoglycosides for binding to an oligonucleotide representing the stem–loop RNA. Moreover, MTX substantially increases the melting temperature of the stem–loop RNA, demonstrating its stabilizing effect upon binding. Most recently, we reported the structure of MTX bound to the tau stem–loop as determined by NMR and found that MTX interacts at the base of the stem (Figure 1), intercalating between two G–C base pairs that flank an unpaired ("bulged") adenosine (Figure 2).[10] (Previous biophysical studies have demonstrated that MTX and analogues can also intercalate DNA.[11,12]) The structure also suggested an important role for hydrogen bonding and electrostatic interactions between the side chains and the major groove. Herein, we report MTX analogues that are varied in the aromatic core and the number and type of side chains to better understand the nature of the interaction of MTX with the tau stem–loop, comparing the findings with expectations based on the NMR structure of the complex. These results provide initial structure–activity relationships to direct the further design of this promising class of ligands.

We carried out systematic dissection of or substitutions within MTX to modified compounds that consist of the anthraquinone scaffold with no side chains (**1**–**3**), one side chain (**4**, **5**), two side chains (**6**–**13**), three side chains (**14**), and four side chains (**15**) (Figure 3). These changes also included specific heteroatom replacement (e.g., **7**, **8**) and altered polyamine length (e.g., **10**, **12**). Straightforward amination[13,14] of difluoro-substituted anthraquinone **16** was applied to prepare these various MTX analogues (Scheme 1). Treatment of **16** with an excess of various amines assembled **1**, **20**, and **21**. Boc deprotection of **20** and **21** provided **11** and **12**, respectively. Successive aminations of **16** were carried out to give **5**, **9**, and **19**. Compound **7** was synthesized via a similar method (see Supporting Information). Deprotection of **9** and **19** afforded **8** and **10**, respectively. 2-(2-Aminoethylamino)ethanol was treated with **16** in aqueous butanol to mainly give **6** or mixture **26**/**27** depending on the solvent butanol/water ratio. Mixture **26**/**27** was transformed to **14**, while further amination of **6** afforded **15**. Toward MTX regioisomer 13 (Scheme 2), isopropyl protection and regioselective bromination[15] provided key intermediate **24**, which was followed by amination[16,17] and $AlCl_3$ deprotection.

The capacity for the synthesized MTX analogues to bind the stem–loop was assessed in two ways: a competitive binding assay and an RNA stability assay. The data from these experiments are summarized in Table 1. The EC_{50} values represent the half-maximal effective concentrations for the previously reported competitive binding assay; active compounds increase fluorescence by increasing the level of unbound fluorescent aminoglycoside probe.[7] The IC_{50} values represent the half maximal inhibitory concentrations for the stability assay; active compounds decrease fluorescence by preventing the unfolding of the RNA stem–loop, keeping the fluorophore and quencher on the oligonucleotide termini in proximity. These two complementary assays gave similar overall results: compounds that show higher binding affinity also result in better stabilization of the stem–loop secondary structure. Consistently higher EC_{50} values from the binding assay compared to IC_{50} data from the stability assay likely represent the higher concentration of MTX analogues required to compete with aminoglycoside probe to effectively intercalate into RNA.

No binding or stabilization activity was observed for aliphatic polyamine side chains alone (data not shown; see Supporting Information for structures of side chains tested).

*To whom correspondence should be addressed. Phone: (617) 525-5511. Fax: (617) 525-5252. E-mail: mwolfe@rics.bwh.harvard.edu.

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Published on Web 10/19/2009

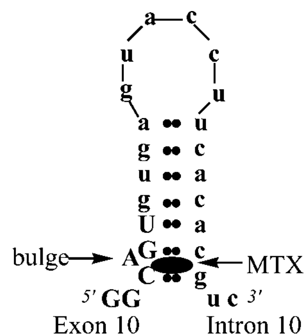


Figure 1. The tau stem−loop and site of MTX binding.

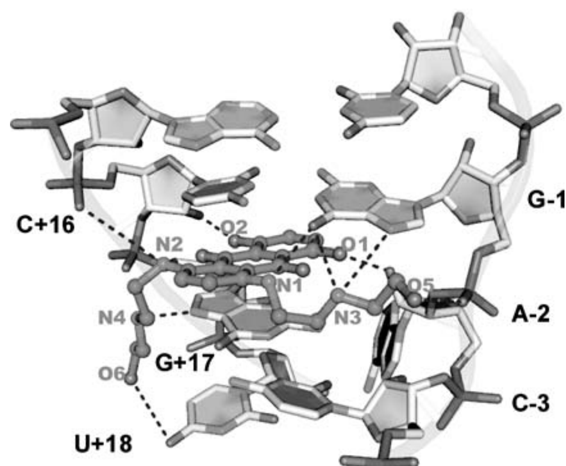


Figure 2. Interaction between MTX and the tau stem−loop structure as deduced by NMR.[10] MTX intercalates between two G−C base pairs that flank the unpaired ("bulged") A-2. The carbon atoms of MTX are shown in light-blue.

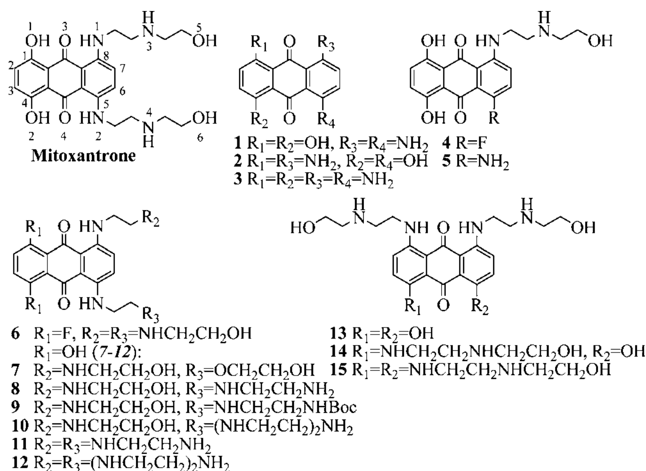
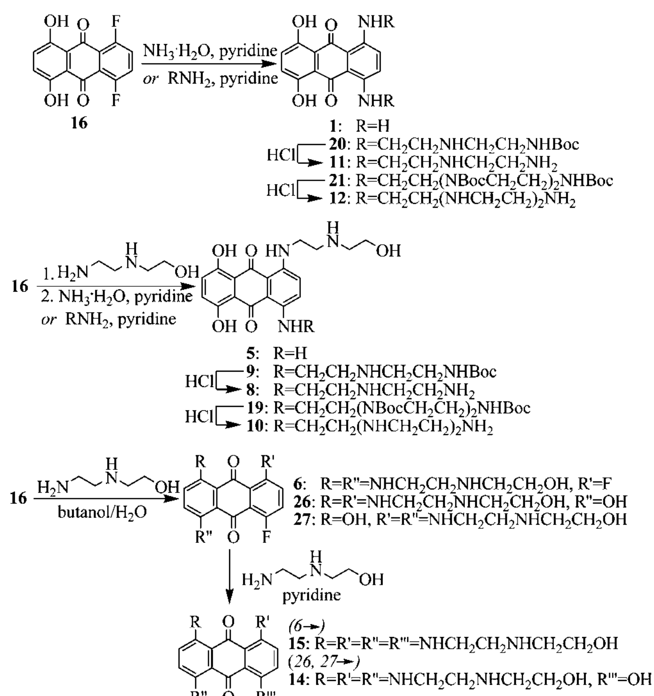


Figure 3. Structures of mitoxantrone and analogues **1**−**15**.

This finding indicates an essential role of aromatic stacking for tau stem−loop binding.

On the other hand, without any aliphatic amine side chains, aromatic anthraquinones **1**−**3** alone cannot bind RNA either. The interaction between these aromatic rings and the two GC base pairs (at the bulge region; see Figure 2) is apparently too weak to allow the aromatic system to effectively intercalate. These data suggest that the aromatic core must cooperate with the aliphatic side chains for binding to the RNA.

Scheme 1. Synthesis of MTX Analogues **1**, **4**−**12**, **14**, and **15** by Amination



Scheme 2. Synthesis of MTX Analogue **13**

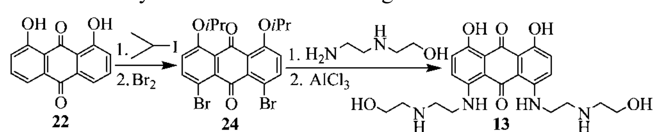


Table 1. Binding and Stabilizing Activity of MTX Analogues

compd	side chain	EC_{50} (μM)	IC_{50} (μM)
MTX	2	0.89	0.46
1	0	> 100[a]	> 100[a]
2	0	> 100[a]	> 100[a]
3	0	> 100	> 100
4	1	75	5.7
5	1	29[a]	5.8[a]
6	2	16[a]	2.3[a]
7	2	14[a]	5.7[a]
8	2	0.31	0.18
9	2	12[a]	2.4[a]
10	2	0.38	0.23
11	2	0.16	0.13
12	2	0.13	0.13
13	2	4.4[a]	1.5[a]
14	3	0.80	0.42
15	4	1.2	0.34

[a] Stock solution of compound in DMSO. The presence of DMSO (2% final concentration) had no effect on background fluorescence.

Introduction of only one $NHCH_2CH_2NHCH_2CH_2OH$ substituent into the aromatic core in **4** and **5** results in measurable activity in the binding and stability assays, although the potency is much weaker than MTX. Thus, the second side chain in MTX contributes substantially to binding (Figure 2). MTX regioisomer **13** is nearly 5 times less potent than MTX in the binding assay and 3 times less potent in the stability assay, indicating that the 5,8 arrangement of the two side chains in MTX is preferable. The substitution of the 1,4

aromatic hydroxyl groups in MTX with $NHCH_2CH_2NH$-CH_2CH_2OH to create structures with three or four side chains (**14** and **15**, respectively) might have led to enhanced binding potency. However, these additional substitutions had little effect on tau stem−loop binding or stabilization. Apparently, one or two additional $NHCH_2CH_2NHCH_2CH_2OH$ substituents installed on the other side of MTX neither sterically hinder formation of the ligand−RNA complex nor build more hydrogen bonds or electrostatic interactions with RNA to enhance binding. However, the retention of binding potency by **14** and **15** suggests that substitution of the aromatic hydroxyl groups at position 1 or 4 of the anthraquinone ring system can be well tolerated in the complex and that attaching additional functionality on MTX in these positions could be advantageous (e.g., to build in additional interaction with the unpaired adenosine located nearby[10]).

MTX analogues **6**−**12** were built with two identical or different side chains. Strengthened by two side chains, **6** shows 2-fold better binding affinity and stabilization activity than **5** (containing one side chain), even though the two electron-withdrawing fluoro atoms of **6** have considerable negative effect on intercalation. This result confirms the importance of having two side chains together interacting with RNA. Compared to MTX, the binding of **6** is decreased ~18-fold and the stabilization effect decreased 5-fold because of the change from two hydroxyls to two fluoro atoms. Besides the decreased aromatic stacking force, possible loss of the suggested hydrogen bonding of aromatic hydroxyls with the 2′ hydroxyl groups of the RNA[10] might also offer some explanation for the low potency of **6**. Replacement of one $NHCH_2CH_2NHCH_2CH_2OH$ substituent in MTX with the ether $NHCH_2CH_2OCH_2CH_2OH$ in **7** results in a 15-fold increase in EC_{50} and a 12-fold increase in IC_{50}. The substantial decrease in binding and stabilization of the stem−loop suggests that the substitution of ether for secondary amine may lead to loss of a critical hydrogen bond or electrostatic interaction and emphasizes that a suitable side chain is a critical factor for binding.

We also replaced the aliphatic OH to NH_2 because NH_2 is likewise a good hydrogen bond donor and could potentially add a new electrostatic interaction with the phosphodiester backbone of the RNA. First, only one hydroxyl was changed to a primary amine (**8**). This modification led to a nearly 3-fold increase in binding potency and stem−loop stabilization for **8** relative to MTX, likely due to a new electrostatic interaction. In contrast, the bulky and uncharged Boc protection group in **9** leads to dramatically decreased potency in the binding and stability assays. We considered that extension of the side chain of **8** by an additional ethylamine (**10**) might further enhance binding and stabilization of the stem−loop, but this change had little effect.

On the basis of the positive results with **8**, both aliphatic hydroxyls of MTX were substituted by NH_2 (**11**), which gave a more than 5-fold improvement in the binding assay and 3-fold improvement in the stability assay compared to MTX. To amplify the electrostatic interaction, two lengthened tetra-amines were incorporated into the aromatic core (**12**), which resulted in a 7-fold increase in binding potency and a more than 3-fold increase in stem−loop stabilization relative to MTX. Additionally, the double tetraamine substitution in **12** led to a 3-fold improvement in binding in comparison with **10**. Interestingly, the overall scaffolds of **8**−**12**, composed of an aromatic moiety and aliphatic polyamine, reflect some similar structural features as "In-PRiNts" (inhibitor of protein−ribonucleotide sequences) reported by Hamy and co-workers.[18]

In summary, a series of MTX analogues were designed, synthesized, and evaluated in vitro for their ability to bind to and stabilize the tau pre-mRNA stem−loop structure, and some of these compounds show substantially higher binding affinity than MTX. The results are largely consistent with the recently reported structure of the MTX−RNA complex elucidated by NMR. We propose a highly synergistic binding mode in which the aromatic ring system and two side chains work together, through intercalation and major groove interaction, respectively, to give strong binding to the tau stem−loop near the bulged adenosine. The structure−activity validation and improved activity provide a design platform for next-generation ligands targeting the tau stem loop. Through iterative design, synthesis, and evaluation, we hope to build in greater potency and specificity to eliminate the cytotoxicity of MTX and identify compounds that work in cells to alter tau pre-mRNA splicing. Such efforts are underway in our laboratory.

Acknowledgment. This work was supported by a Zenith Fellows Award from the Alzheimer's Association to M.S.W.

Supporting Information Available: Experimental details and compound characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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