# Supporting Information

# Structure Activity Relationship and Mechanism of Action Studies of Manzamine Analogues for the Control of Neuroinflammation and Cerebral Infections

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Content:

**Table 1.** *In vitro* activities against two *Plasmodium falciparum* strains.

| Compounds | *P. falciparum* (D6 clone) | *P. falciparum* (W2 clone) | Cytotoxicity (Vero) |
|---|---|---|---|
| | $IC_{50}$ (ng/mL) | $IC_{50}$ (ng/mL) | $IC_{50}$ (μg/mL) |
| Manzamine A (**1**) | 4.5 | 8.0 | 0.2 |
| 8-OH-Manzamine A (**2**) | 6.0 | 8.0 | 1.1 |
| 9-*N*-methylmanzamine A(**6a**) | 1900 | 1000 | NC |
| 9-*N*-ethylmanzamine A (**6b**) | 140 | 470 | 1.5 |
| 9-*N*-propylmanzamine A (**6c**) | 690 | 740 | 3.7 |
| 9-*N*-butylmanzamine A (**6d**) | 330 | 1300 | 2.0 |
| 9-*N*-isobutylmanzamine A (**6e**) | 39 | 78 | 0.4 |
| 9-*N*-isopentylmanzamine A (**6f**) | 2300 | 3800 | NC |
| 9-*N*-neopentylmanzamine A (**6g**) | 2400 | 2800 | 0.7 |
| 9-*N*-dodecylmanzamine A (**6h**) | NA | NA | NC |
| 9-*N*-(4-methylcarboxybutyl) manzamine A (**6i**) | 2000 | 2000 | NC |
| Tetrahydromanzamine A (**3**) | 82 | 1500 | NT |
| Dihydromanzamine A (**5**) | 200 | 1800 | NT |
| Tetrahydro-8-hydroxy-manzamineA (**4**) | 90 | 620 | NT |
| Dehydromanzamine A (**10**) | 120 | 2200 | NT |
| Dehydro-8-acetoxy-manzamine A (**11**) | 2500 | 3800 | NT |
| 8-Acetoxymanzamine A (**9**) | 29 | 30 | 670 |
| Manzamine D (**20a**) | 90 | 88 | NC |
| 2*N*-methylmanzamine D (**20b**) | 310 | 440 | NC |
| 6-Methoxymanzamine D (**20c**) | 57 | 64 | 0.2 |
| Methyl manzamine A-3-carboxylate (**21b**) | 11 | 15 | 0.2 |
| Methyl manzamine D-3-carboxylate (**20d**) | 100 | 170 | 1.0 |
| 6-Methoxymanzamine A (**21a**) | 28 | 58 | 0.5 |
| 30-(3-Methoxyphenyl)manzamine F (**17a**) | NA | NA | NC |

| | | | |
|---|---|---|---|
| 30-(4-Methoxyphenyl)-manzamine F (**17b**) | NA | NA | NC |
| 30-(4-Nitrophenyl) manzamine F (**17c**) | NA | NA | NC |
| 30-(2,6-Dichlorophenyl) manzamine F (**17d**) | NA | NA | NC |
| 30-(3,4-Dimethoxyphenyl) manzamine F (**17e**) | NA | NA | NC |
| 30-(4-Bromophenyl) manzamine F (**17f**) | NA | NA | NC |
| 30-(4-Fluorophenyl) manzamine F (**17g**) | NA | NA | NC |
| Manzamine F-31-hydrazone (**15**) | 29 | 38 | NC |
| 31-Ethylmanzamine F (**18**) | 77 | 86 | NC |
| 31-Hydroxymanzamine F (**16**) | NA | NA | NC |
| Manzamine A-*N*-Oxides (**13**) | 17 | 67 | NC |
| 6-Nitromanzamine A (**8**) | 18 | 28 | 270 |
| 8-Nitromanzamine A (**7**) | 310 | 410 | NC |
| Chloroquine | 16 | 155 | NT |
| Artemisinin | 13 | 8 | NT |

NA = Not active; NC = no cytotoxicity at 4.6 μg/mL; NT = not tested.

**Table 2.** *In Vitro* antibacterial and antifungal data

| Compounds | *M. tuberculosis* (H37Rv) | *C. albicans* | *C. neoformans* | *M. intracellulare* |
|---|---|---|---|---|
| | MIC (μg/mL) | $IC_{50}$ (μg/ml) | $IC_{50}$ (μg/ml) | $IC_{50}$ (μg/ml) |
| Manzamine A (**1**) | 1.53 | 2.0 | 1.0 | 0.35 |
| 8-Hydroxy-manzamine A (**2**) | 0.9 | 3.5 | 2.0 | 0.1 |
| 9-*N*-methylmanzamine A (**6a**) | >64 | >20 | >20 | >20 |
| 9-*N*-ethylmanzamine A (**6b**) | >128 | 20 | 10 | 0.50 |
| 9-*N*-propylmanzamine A (**6c**) | 37 | >20 | >20 | 2.0 |
| 9-*N*-butylmanzamine A (**6d**) | 128 | >20 | >20 | >20 |
| 9-*N*-isobutylmanzamine A (**6e**) | 4.6 | 10 | 3 | 1.0 |
| 9-*N*-isopentylmanzamine A (**6f**) | 128 | >20 | >20 | 6.5 |
| 9-*N*-neopentylmanzamine A (**6g**) | 4.0 | 7.0 | 4.0 | 0.50 |
| 9-*N*-dodecylmanzamine A (**6h**) | NT | >20 | >20 | >20 |
| 9-*N*-(4-methylcarboxybutyl) manzamine A (**6i**) | NT | >20 | 15 | 20 |
| Tetrahydromanzamine A (**3**) | 50 | 7.5 | 0.9 | 0.45 |
| Dihydromanzamine A (**5**) | 66 | 9.5 | 1.5 | 0.6 |
| Tetrahydro-8-hydroxy-manzamine A (**4**) | 24 | 6 | 1.5 | 0.1 |
| Dehydromanzamine A (**10**) | 46 | 9.5 | 4.5 | 0.4 |
| Dehydro-8-acetoxy-manzamine A (**11**) | >64 | >20 | >20 | 10 |
| 8-Acetoxymanzamine A (**9**) | NT | 1.5 | 1.5 | 0.1 |
| Manzamine D (**20a**) | 0.99 | 15 | 0.8 | 0.1 |
| 2*N*-methylmanzamine D (**20b**) | >64 | >20 | 6.0 | 0.7 |
| 6-Methoxymanzamine D (**20c**) | >64 | >20 | 2.0 | 0.2 |
| Methyl manzamine D-3-carboxylate (**20d**) | >64 | >20 | 3.5 | 0.06 |
| 6-Methoxymanzamine A (**21a**) | >64 | 5.0 | 3.0 | 0.8 |

| | | | | |
|---|---|---|---|---|
| Methyl manzamine A-3-carboxylate (**21b**) | >64 | 20 | 2.0 | <0.02 |
| 31-(3-Methoxyphenyl) manzamine F (**17a**) | >64 | >20 | >20 | >20 |
| 31-(4-Methoxyphenyl) manzamine F (**17b**) | >64 | >20 | >20 | >20 |
| 31-(4-Bromophenyl) manzamine F (**17f**) | >64 | >20 | >20 | >20 |
| 31-(2,6-Dichlorophenyl) manzamine F (**17d**) | >64 | >20 | >20 | >20 |
| 31-(4-Fluorophenyl) manzamine F (**17g**) | NT | >20 | >20 | >20 |
| 31-(4-Nitrophenyl) manzamine F (**17c**) | >64 | >20 | >20 | >20 |
| 31-(3,4-Dimethoxyphenyl) manzamine F (**17e**) | >64 | >20 | >20 | >20 |
| Manzamine F-31-hydrazone (**15**) | 1.9 | >20 | 1.0 | 0.09 |
| 31-Ethylmanzamine F (**18**) | 1.9 | 2.5 | 6.0 | 0.25 |
| 31-Hydroxymanzamine F (**16**) | 1.7 | 15 | 10 | 1.5 |
| Manzamine A-*N*-oxide (**13**) | NT | >20 | >20 | 10 |
| 6-Nitromanzamine A (**8**) | 1.6 | >20 | 2.5 | 0.15 |
| 8-Nitromanzamine A (**7**) | NT | >20 | 4.0 | 0.25 |
| Manzamine F (**14**) | NT | 15 | 6.5 | NT |
| Amphotericin B | NT | 0.20 | 0.75 | NT |
| Ciprofloxacin | NT | NT | NT | 0.30 |
| Rifampin | 0.09 | NT | NT | NT |

NT = not tested.

**Table 3.** *In Vitro* anti-neuroinflammatory activity*

| Compounds | $O_2^-$ | $TXB_2$ | LDH |
|---|---|---|---|
| | $IC_{50}$(μM) | $IC_{50}$(μM) | $LDH_{50}$(μM) |
| Manzamine A (**1**) | 0.1 | <0.1 | >10 |
| 8-Hydroxy-manzamine A (**2**) | >10 | ±0.5 | >10 |
| Ircinal (**19**) | >10 | 0.33 | 4.6 |
| 9-*N*-methylmanzamine A (**6a**) | >10 | >10 | >10 |
| 9-*N*-ethylmanzamine A (**6b**) | 9.7 | >10 | >10 |
| 9-*N*-propylmanzamine A (**6c**) | 3 | >10 | >10 |
| 9-*N*-butylmanzamine A (**6d**) | 0.12 | 0.4 | >10 |
| 9-*N*-isobutylmanzamine A (**6e**) | 5.7 | >10 | >10 |
| 9-*N*-isopentylmanzamine A (**6f**) | 3.2 | 3.2 | >10 |
| 9-*N*-neopentylmanzamine A (**6g**) | 0.07 | 0.07 | >10 |
| Tetrahydromanzamine A (**3**) | >10 | >10 | >10 |
| Tetrahydro-8-hydroxy-manzamine A (**4**) | >10 | 1.97 | >10 |
| Manzamine D (**20a**) | 2.45 | 2.1 | ±1 |
| 2*N*-methylmanzamine D (**20b**) | >10 | 12.2 | ±8 |
| 6-Methoxymanzamine D (**20c**) | 2.2 | 1.65 | ±3 |
| Methyl manzamine D-3-carboxylate (**20d**) | 8.6 | 4.9 | ±5 |
| 6-Methoxymanzamine A (**21a**) | 0.1 | 0.11 | >10 |
| Methyl manzamine A-3-carboxylate (**21b**) | 0.03 | 0.11 | >10 |

*Anti-neuroinflammatory assay: effect of compounds on rat microglia PMA [1 μM]-stimulated release of $O_2^-$, $TXB_2$ and LDH. Data shown corresponds to 2-7 independent experiments and is expressed as $IC_{50}$ (μM) for $O_2^-$ and $TXB_2$. $LDH_{50}$ (μM) is the compound's concentration causing 50% percent of maximal LDH release triggered by treating microglia with 0.1% Triton X-100.

**Manzamine A blood-brain barrier (BBB) crossing assay**

**Sample preparation.** A stock solution of manzamine A was prepared by dissolving in DMSO. The standard solutions were diluted with 4% DMSO, and 2% cyclodextrin in HBSS at concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 5.0 μg/mL. Samples from the BBB assay were directly injected into LC/MS.

**LC/MS analysis**. Agilent HP1100 with Bruker micro-TOF was used. The analysis was performed on a $C_8$ column (4.6 mm×150 mm, 5 μm, Phenomenex Luna) using a gradient of MeOH – 2 mM $NH_4Ac$ (0.05% formic acid) from 40:60 to 80:20 over 8 minutes at a flow rate of 0.8 mL/min. A flow splitter with a ratio of 1:5 was used. The mass spectrometer was operated in positive mode. The nebulizer pressure was 2 Bar, the source temperature was 200º, the drying gas flow was 4 L/min, the capillary exit voltage was set at 90 V, and m/z = 549.4 was extracted.

**LC/MS quantification.** The standard curve was $y = 419157x - 44745$ ($R^2 = 0.9975$, $n = 3$), which was linear over the range of 0.05-5 μg/mL. The precision was tested at concentrations of 0.05, 0.5, 2.0 μg/mL (Table 7), and the values were 3.85%, 3.21%, and 2.85%, respectively. The recovery was performed by adding standard solution at concentrations of 0.05, 0.5, 2.0 μg/mL to a known-concentration solution (Table 8), results were 109.0%, 94.71%, and 101.2%, respectively. The limit of detection (counted as S/N = 3) was 100 pg.

**Table 7.** Precision of manzamine A LC/MS quantification linearity (n=3)

| Concentration (μg/mL) | RSD (%) |
|---|---|
| 0.05 | 3.85 |
| 0.5 | 3.21 |
| 2.0 | 2.85 |

**Table 8.** Recovery of manzamine A (n=3)

| Known concentration (μg/mL) | Added (μg/mL) | Determined (μg/mL) | Recovery (%) | RSD (%) |
|---|---|---|---|---|
| 0.21 | 0.05 | 0.26 | 109.0 | 4.21 |
| 0.20 | 0.5 | 0.67 | 94.71 | 3.31 |
| 0.23 | 2.0 | 2.25 | 101.2 | 2.59 |

**Cytotoxicity:**

To help elucidate the mechanism of cytotoxicity, cell viability was evaluated by treating HeLa cells with manzamine A (**1**) and compound **6e** for 48 h. As shown in Figure 5, the $IC_{50}$ for manzamine A (**1**) was 2 $\mu$g/mL while compound **6e** did not reach an $IC_{50}$ value within the tested dose range (>30 $\mu$g/mL), indicating that the free NH group is also an essential moiety for cytotoxicity. Recent studies have demonstrated that a synthetic manzamine A (**1**) analogue inhibits human cancer cell proliferation by impairing cell cycle progression.[1] To determine whether manzamine A (**1**) also affects cell cycle progression, HeLa cells were treated with manzamine A (**1**) for 24 h. As shown in Figure 6 and Table 9, manzamine A (**1**) at 3-10 $\mu$g/mL slows S phase progression and leads to the reduction of G2/M cell population. At a concentration of 20 $\mu$g/mL of manzamine A (**1**), the drug arrests the cell cycle at the G1 phase. The effect of compound **6e** on cell cycle progression was also evaluated. Similar to manzamine A (**1**), compound **6e** slightly increases S phase population in a dose-dependent manner. These results demonstrated that manzamine A (**1**) and 9-*N* alkylated manzamine derivatives impede cell cycle progression at S phase but at a significantly higher concentration than that at which they are effective as antimalarial or anti-inflammatory agents.

Manzamine A (**1**) was also evaluated for its effect on tubulin polymerization *in vitro* at 30 °C in two steps. In the first step (0-30 min) it was obvious that manzamine A (**1**) was not able to induce tubulin polymerization and thus did not show a paclitaxel-like effect (Figure 7). In the second step (30-60 min), after addition of GTP and glutamate, manzamine A (**1**) showed that it is not a tubulin polymerization inhibitor such as colchicine or vinblastine (Figure 7). From the above two steps it is obvious that manzamine A (**1**) neither stabilizes nor destabilizes microtubules. This is further reflected by the fact that manzamine A (**1**) does not lead to G2/M arrest as this would be expected from microtubule-interfering compounds.

The 9-*N*-alkylation of manzamine A (**1**) resulted in a reduction of its antiparasitic activity but improved its anti-inflammatory activity. The cytotoxic activity of manzamine A (**1**) is clearly not mediated through microtubule function but appears to be a function of DNA interaction with the β-carboline group. The manzamine analogue (**8**) is therefore an interesting new lead compound for the potential control of neuro-inflammation and appears to have a novel mechanism of action and a reasonable therapeutic index *in vitro*. It is clearly revealed in this study that it is possible to delineate cytotoxicity from antimalarial, Mtb and anti-inflammatory activity for this class of marine alkaloids.

Cell Growth Inhibition Assays: Cells were seeded at appropriate density and grown in the continuous presence of drugs for 48 h. At the end of incubation, viable cell numbers were determined by trypan blue dye exclusion and counted with a hemocytometer.

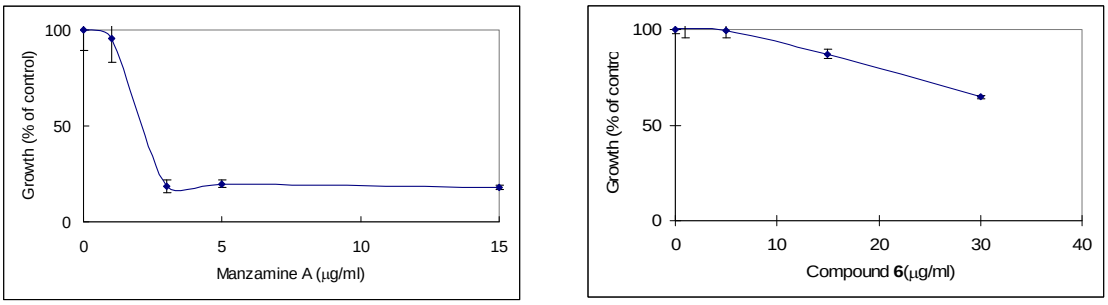
Cell Cycle Analysis: Cells were treated with drugs for 24 h. To prepare for analysis, the cells were pelleted and resuspended in 100 $\mu$l citrate buffer (250 mM sucrose; 20 mM trisodium citrate; 5% DMSO pH 7.6) and stored at –70°C prior to further analysis. The cells were thawed and treated as follows: addition of 0.45 mL of solution I (0.5 mM Tris; 10 mM spermine tetrahydrochloride; 0.1 % NP 40; 0.03 mg/mL trypsin pH 7.6) at room temperature (RT), each sample was mixed thoroughly and incubated for 10 min at RT. Next, 0.375 mL of solution II (0.5 mM Tris; 10 mM spermine tetrahydrochloride; 0.1% NP40; 0.05 mg/mL trypsin inhibitor; 0.001 mg/mL ribonuclease A, pH 7.6)

was added and the mixture incubated for 10 min at RT. Finally, 0.375 mL of solution III (0.5 mM Tris; 50 mM spermine tetrahydrochloride; 0.1% NP40; 0.1 mg/mL propidium iodide, pH 7.6) was added and the mixture was incubated for another 10 min at RT. The cells were then analyzed on a FACS Calibur flow cytometer (Becton Dickinson) and the data was analyzed using Cell Quest software.

Tubulin Polymerization Assays: Pure tubulin was isolated from pig brain according to the method[2] in which tubulin (1 mg/mL) was incubated in BRB80 buffer at 30 °C with the test compounds. A vehicle control (DMSO) and a positive control (paclitaxel) were included. After 30 min, 0.5 mM GTP and 04 M glutamate were added to initiate tubulin assembly. The assay was carried out in triplicate in a 96-well format and polymerization was followed turbidimetrically at 340 nm in a Genios Pro (Tecan, Switzerland) spectrophotometer.

**Table 9.** The effect of manzamine A (**1**) and 9*N*-isobutylmanzamine A (**6e**) on cell cycle progression

| Manzamine A (**1**) | | | | 9*N*-isobutylmanzamine A (**6e**) | | | |
|---|---|---|---|---|---|---|---|
| $\mu$g/mL % | G0/G1 | S | G2/M | $\mu$g/mL % | G0/G1 | S | G2/M |
| 0 | 56.5 | 14.5 | 19.9 | 0 | 56.7 | 14.6 | 18.8 |
| 3 | 56.2 | 24.2 | 13.0 | 5 | 54.2 | 16.6 | 19.2 |
| 5 | 56.3 | 23.8 | 14.3 | 10 | 54.9 | 16.4 | 17.8 |
| 10 | 59.1 | 20.0 | 13.6 | 24 | 51.1 | 19.5 | 17.0 |
| 20 | 67.2 | 11.7 | 11.8 | 30 | 52.3 | 19.1 | 17.4 |




A. Manzamine A (**1**) B. 9*N*-isobutylmanzamine A (**6e**)

**Figure 5**. HeLa cell growth inhibition assay of Manzamine A (**1**) and 9*N*- isobutylmanzamine A (**6e**)

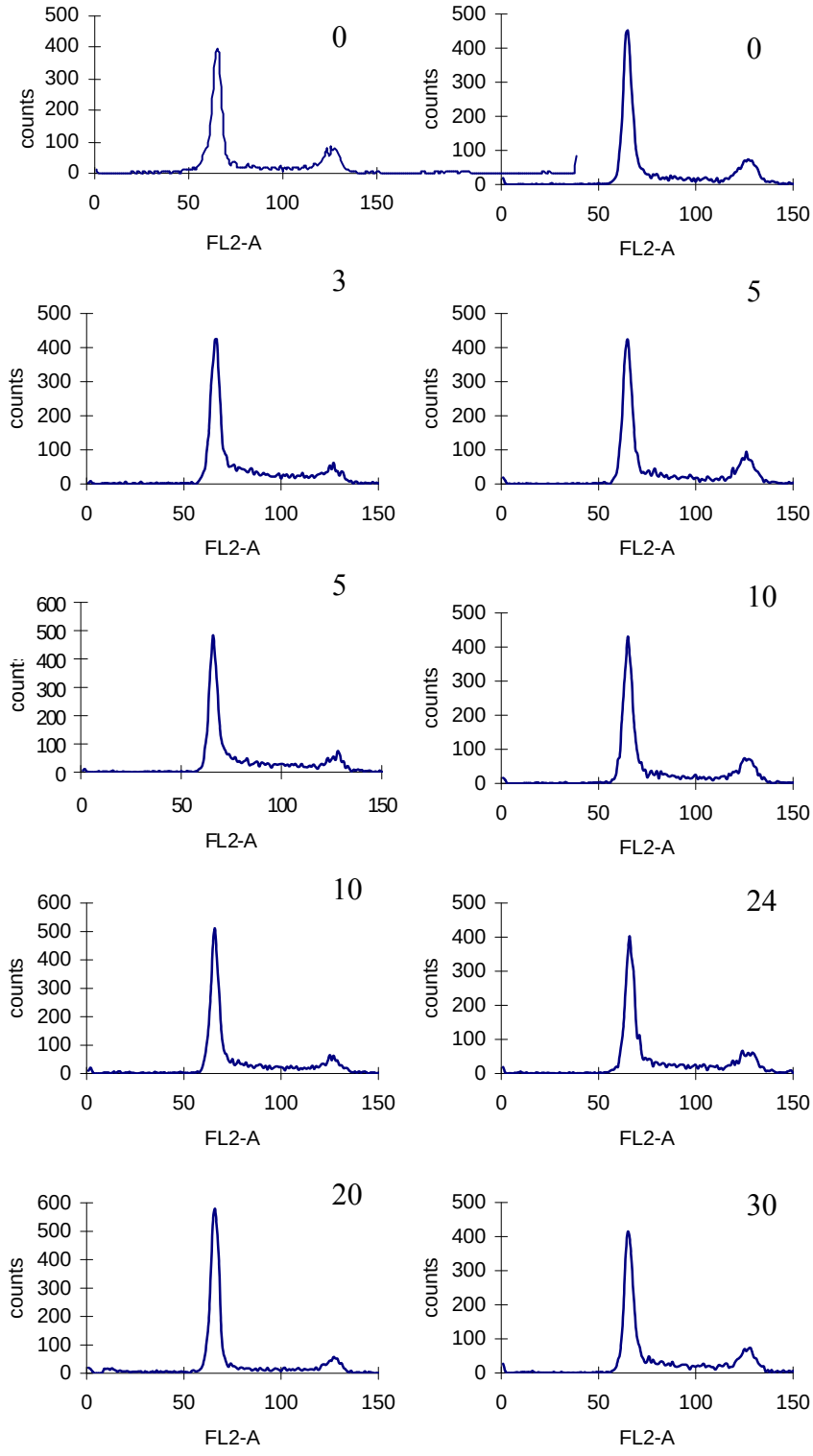



**Figure 6**. HeLa cell cycle profiles after Manzamine A (**1**) and 9*N*-isobutylmanzamine A (**6e)** treatments.

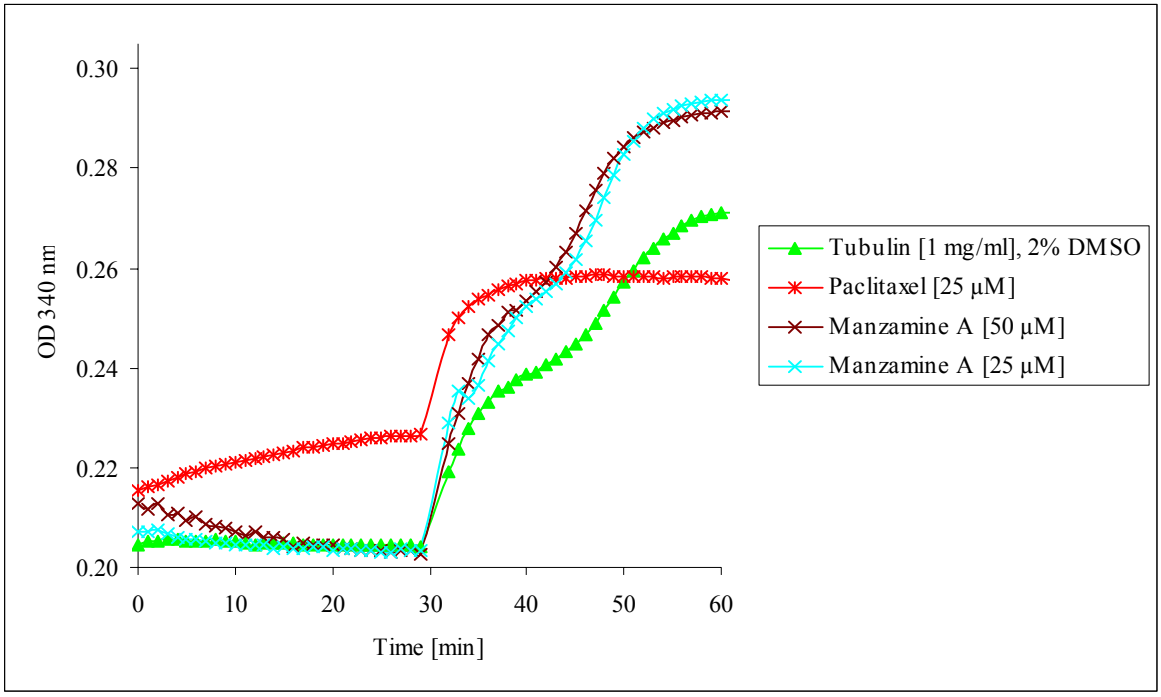



**Figure 7**. In vitro activity of Manzamine A (**1**) on tubulin polymerization

References:

(1) Tu, L. C.; Chou, C.-K.; Chen, C.-Y.; Chang, Y.-T.; Shen, Y.-C.; Yeh, S.-F. Characterization of the cytotoxic mechanism of Mana-Hox, an analog of manzamine alkaloids. *Biochim. Biophys. Acta, Gen. Subj.* **2004,** *1672*, 148-156.

(2) Castoldi, M.; Popov, A. V. Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr. Purif.* **2003**, *32*, 83-88.