Spring 3-9-2017

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# Tetramethylenedisulfotetramine Neurotoxicity: *In vivo* Validation of *In Vitro* Screen to Identify Potential Countermeasures

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Poster ID 1735

## ABSTRACT

Tetramethylenedisulfotetramine (TMDT), a synthetic neurotoxin, induces a seizure syndrome by blocking $Cl^-$ influx through the $GABA_A$ channel. This process leads to uncontrolled depolarization followed by excessive $Ca^{2+}$ entry into neurons and potential excitotoxicity. No standardized, effective treatment for TMDT poisoning currently exists. Primary neuronal cultures were used to screen candidate countermeasures for alleviation of TMDT-provoked hyperexcitability by monitoring changes in intracellular $Ca^{2+}$ levels ($[Ca^{2+}]_i$). Agents antagonizing NMDA or β-adrenergic receptors reversed TMDT-induced increases in $[Ca^{2+}]_i$ and displayed the best counteracting potential. We have commenced testing these *in vitro* leads *in vivo*. Adult male mice were injected with 0.25 mg/kg TMDT subcutaneously followed by intraperitoneal monotherapy immediately after the first clonic seizure observed. They were continuously monitored over 1 hr, for the occurrence and severity of clonic and tonic-clonic seizures, and for 24-hr mortality. Our current results indicate that MK-801 is superior, completely eliminating tonic-clonic seizures and 24-hr mortality. At 40 mg/kg, memantine decreased mortality by 75%, however delayed tonic-clonic seizures were observed. Although both procyclidine and ketamine prevented tonic-clonic seizures at higher doses (60 and 70 mg/kg, respectively), they were not as effective in preventing TMDT-induced lethality. Propranolol was the least effective at reducing seizure severity and mortality rate. Altogether, our *in vitro* assay provides a useful screen to identify potential countermeasures against TMDT neurotoxicity. Positive leads are being tested and show activity *in vivo,* supporting utility of the screen.

## INTRODUCTION

TMDT is a synthetic neurotoxic chemical formerly used worldwide as a rodenticide. Though its production, sale, and use are now banned, it is widely available, particularly in mainland China. TMDT is water soluble, odorless and tasteless, stable and persistent in the environment, making it an effective adulterant of food and water. TMDT mediates its neurotoxic effects by binding inside the $GABA_A$ channel and thus blocking the hyperpolarizing chloride ion influx. This leads to neuronal hyperexcitability and calcium dependent excitotoxicity due to an excessive $Ca^{2+}$ influx into the cell via $Ca^{2+}$calcium-permeable channels. Among these, the NMDA receptor (NMDAR) represents one of the main regulators of $Ca^{2+}$ influx during (patho)physiological conditions.

Since currently there is no standardized and effective treatment against TMDT and other $GABA_A$ receptor poisons, we tried to develop a medium throughput screening for faster identification of potential countermeasures against TMDT. For this purpose, we use dissociated primary cortical neurons, as an *in vitro* functional assay of neuronal excitability and neuronal cytotoxicity preserving sufficient biological context to allow identification of compounds with potential to prevent neurotoxicity. The mature (14-16 days *in vitro* (DIV)) neuronal culture exhibits spontaneous $Ca^{2+}$ oscillations, which correspond to synchronized bursts of action potentials with attendant release of neurotransmitters. These oscillations can be altered by addition of different neurotoxins including TMDT, to the cells, to simulate *in vivo* changes resulting in $[Ca^{2+}]_i$ flux in neuronal network with convulsive and often lethal outcomes. Here we aimed to validate several drug candidates from our *in vitro* screen to test them *in vivo* based on their ability to reduce TMDT-induced increase of $[Ca^{2+}]_i$.

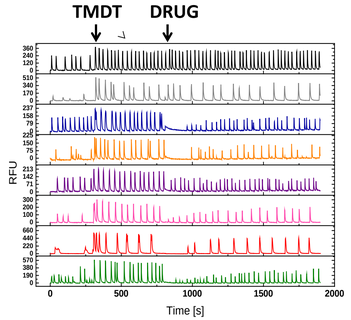
### Material and Methods

Cortical neurons were isolated from Sprague-Dawley rat embryos (E18) and cultured in Neurobasal medium + 2% B27 + 0.5mM GlutaMax on poly-D-lysine coated 96-well plates. The cell culture between 14-16 DIV was used to screen the effect of TMDT on $[Ca^{2+}]_i$ flux. Neurons were loaded with Calcium 6 fluorescent dye (Molecular Devices) and after 1-hr incubation monitored for $Ca^{2+}$ oscillations using FlexStation 3 (Molecular Devices). Various drugs were tested to evaluate their counteracting efficacy against TMDT-induced changes in $Ca^{2+}$ oscillations. OriginPro software was used to quantify the area under the curve per 100 seconds for each time interval (baseline, following TMDT injection and after each treatment).

Simultaneously, in another set of experiments, 14-day old cultures were incubated with different concentrations of TMDT and kainic acid (positive control) to evaluate the neuronal cell death. Following 24-hr incubation with TMDT or kainic acid, the cell vitality was analyzed with the Alamar blue test. Relative fluorescence intensity was measured with FlexStation 3 every hour during a 4-hr time interval. GraphPad Prizm was used for statistical evaluations. Similarly, neuronal cell death was evaluated *in vivo* in brain sections from 24-hrs survivors of TMDT or kainic acid intoxication using FluroJade C staining.

To validate our *in vitro* screening method and efficacy of drug candidates to ameliorate TMDT toxidrome, (seizure severity and lethality), adult male C57BL6 mice were injected with 0.25 mg/kg of TMDT s.c. Following occurrence of the first clonic seizure mice received a single treatment (i.p.) and observed for 1 hr for development of further seizures and for 1- and 24-hr lethality. Severity score was calculated for each mouse, drug and dose and graphed using GraphPad Prism. A significant difference between the each treatment vs the vehicle (sterile water) control was calculated using Student's t-test.

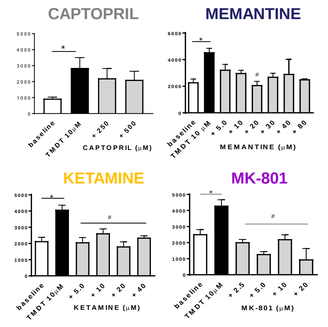
## RESULTS

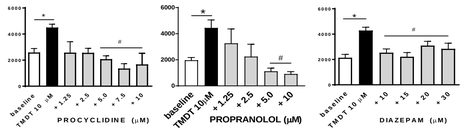



**Effect of different drugs on TMDT-induced increase of $[Ca^{2+}]_i$**

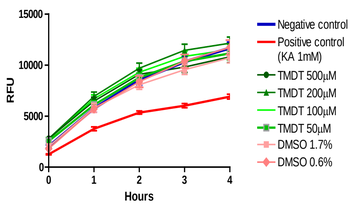
Treatment of 14-16 DIV cortical cultures with 10 µM TMDT increased peak amplitude but not frequency of $[Ca^{2+}]_i$ oscillations. Subsequent injection of vehicle (Hank's solution) to the wells did not modify this effect, whereas certain pharmacological agents significantly altered TMDT-induced changes in these oscillations. NMDAR blockers (MK-801, ketamine, memantine, procyclidine) displayed a similar pattern of changes and led to a more persistent alteration of TMDT effect. Diazepam, a $GABA_A$ receptor antagonist and propranolol, a β-adrenergic receptor blocker also showed significant improvements upon reduction of $Ca^{2+}$ influx. Angiotensin-converting enzyme inhibitor, captopril, was not effective even at high concentrations used. Dose response analysis and evaluation of area under the curve showed that all the drugs tested, except captopril, reduced TMDT-induced raise in $[Ca^{2+}]_i$, thus suggesting their potential efficacy in preventing TMDT-induced toxidrome *in vivo*.

**AREA UNDER THE CURVE ANALYSIS**

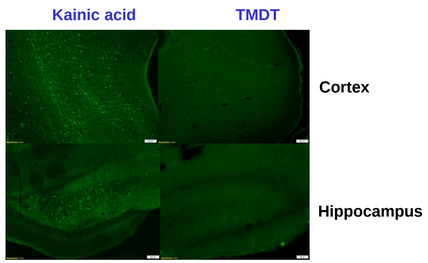



**Alamar Blue Test (Cell vitality test)**




**FluoroJade C Staining**



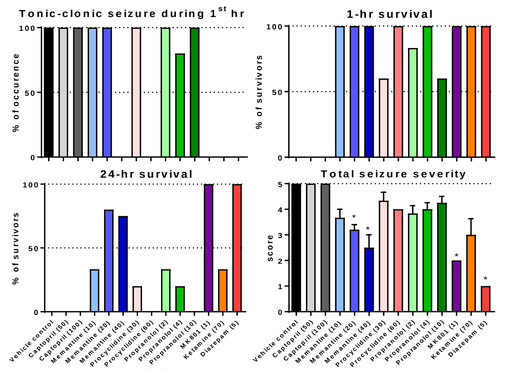

**Effect of TMDT on Neuronal Cell Death**

No significant alteration in cell vitality was observed after 24-hr incubation of neurons with 50-500 µM TMDT *in vitro* as compared to untreated or vehicle-treated ($H_2O$ or DMSO) groups. Kainic acid at 1mM (positive control) induced a significant impairment of cell viability, as expected. Similarly, no cell death was observed *in vivo* 24 hrs after s.c. injection of sublethal dose of TMDT to rodents, in spite of occurrence of severe tonic-clonic seizures. In contrast, the treatment with 12.5 mg/kg of kainic acid i.p. (positive control) induced a visible neuronal cell death within the rodent cortex and hippocampus 24 hrs later. These data suggest different cellular responses after TMDT treatment as compared to kainic acid and no production of neuronal cell death by TMDT *in vitro* or *in vivo.*

## RESULTS

**_In vivo_ validation of candidates from _in vitro_ screening against TMDT-induced seizures and lethality**

In adult male mice, TMDT induced symptoms in the following sequence: myoclonic whole body twitches, clonic seizures, and tonic-clonic seizure resulting in death within 1 hr. Vehicle or the treatment was given after the first clonic seizure and animals were observed up to 1 hr for seizures, and for lethality at 1- and 24-hr. Severity score which encompases both seizure severity and lethality was calculated for each animal, drug and dose: 1 for mice displaying one clonic seizure (complete prevention of neurotoxic effects), 2 – multiple clonic seizures, 3 – tonic-clonic seizure, 4 – lethality within 24 hrs, 5 – lethality within 1 hr (no efficacy).




All candidates except captopril (negative control) reduced 1-hr lethality comparing with the vehicle. However, only memantine, procyclidine, MK-801, ketamine and diazepam prevented tonic-clonic seizures. From among these drugs, MK-801 and diazepam completely rescued from lethality within 24 hrs. Severity scores confirmed statistically significant counteracting potential of memantine (20 and 40 mg/kg), MK-801 (1 mg/kg) and diazepam (5 mg/kg) against TMDT toxidrome in the following order diazepam>MK-801>memantine. Diazepam appeared to be superior in preventing all symptoms of intoxication scored.

## CONCLUSIONS

- *In vivo* validation showed that all positive leads from *in vitro* screen significantly reduced 1-hr lethality and thus extended the time window for further intervention.
- Diazepam, a $GABA_A$ receptor positive modulator, and MK-801, an NMDAR antagonist, reversed both TMDT-evoked increase in $[Ca^{2+}]_i$ *in vitro* as well as completely prevented occurence of tonic-clonic seizures and 24-hr lethality *in vivo,* thus representing superior treatments compared with other drugs tested.
- Drugs of the same pharmacological class, such as NMDAR antagonists (MK801, memantine, ketamine, procyclidine), displayed differing efficacies in reversing the features of the TMDT toxidrome (tonic-clonic seizures vs lethality), likely due to different pharmacokinetics and pharmacodynamics including NMDAR binding properties.
- No neuronal death has been observed 24 hrs following TMDT treatment *in vitro* or *in vivo.*
- Our *in vitro* monitoring of $Ca^{2+}$ fluxes represents a valid and useful medium-throughput screening tool to evaluate the neurotoxic effects and for a rapid identification of potential treatment candidates against TMDT, and most probably against other neurotoxins inducing lethal convulsions with concurrent rise of $[Ca^{2+}]_i$.
- These results demonstrate reliability and validity of our *in vitro* method, serving as a very effective low-cost and time-saving alternative to animal experimentation during a search for an effective treatment. Nevertheless, *in vivo* testing is still needed to evaluate efficacy of positive hits against all of the symptoms of TMDT toxidrome, and to establish accurate dosing.
- Exploitation of our *in vitro* approach may be particularly significant in cases of sudden accidental or intentional exposure to various neurotoxic agents inducing lethal seizures with concurent rise of $[Ca^{2+}]_i$ and the need to promptly identify effective treatments.

**Acknowledgements:** NIH CounterACT Program (1R21NS084900) for financial support and Drs. Janet Alder and Smita Varia of Robert Wood Johnson Medical School, Rutgers University, for assistance with tissue dissections.

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