

Ethanol and cocaine increases microtubule stability and decreases [¹¹C]MPC-6827 uptake in SH-SY5Y cells

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Short Report

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Abstract

Microtubules (MTs) are structural units in the cytoskeleton. In brain cells they are responsible for axonal transport, information processing, and signaling mechanisms. Proper function of these processes is critical for healthy brain functions. Alcohol and substance use disorders (AUD/SUDs) affects the function and organization of MTs in the brain, making them a potential neuroimaging marker to study the resulting impairment of overall neurobehavioral and cognitive processes. Our lab reported the first brain-penetrant MT-tracking Positron Emission Tomography (PET) ligand [^{11}C]MPC-6827 and demonstrated its *in vivo* utility in rodents and non-human primates. To further explore the *in vivo* imaging potential of [^{11}C]MPC-6827, we need to investigate its mechanism of action. Here, we report preliminary *in vitro* binding results in SH-SY5Y neuroblastoma cells exposed to ethanol (EtOH) or cocaine in combination with multiple agents that alter MT stability. EtOH and cocaine treatments increased MT stability and decreased free tubulin monomers. Our initial cell-binding assay demonstrated that [^{11}C]MPC-6827 selectively bound to free/unbound tubulin units. Consistent with this mechanism of action, we observed lower [^{11}C]MPC-6827 uptake in SH-SY5Y cells after EtOH and cocaine treatments (e.g., fewer free tubulin monomers). We are currently performing *in vivo* PET imaging and *ex vivo* biodistribution studies in rodent and nonhuman primate models of AUD and SUDs.

Background

Cytoskeletal defects, including alterations in microtubule stability, axonal transport and actin dynamics, have been characterized in several psychiatric and neurodegenerative disorders, including alcohol and substance use disorders (AUD/SUDs) and Alzheimer's disease, suggesting they are a common feature contributing to neurodegeneration. An organized neuronal cytoskeleton is required for nervous system development, maintenance, and regenerative processes after injury. Its three components intermediate filaments, actin filaments, and microtubules (MTs), all play a vital role in neurological processes. MTs are critical to cellular structure; as neuronal backbones they facilitate cell division, axonal transport, and neurotransmission. MTs are hetero-dimer units formed from α - and β -tubulin monomers.[1] Essential biophysical functions, including cellular signaling and axoplasmic transport, depend on the structural integrity of MTs i.e., polymerization with bound and free tubulin units and MT integrity is heavily dysregulated in AUD and SUDs.[2-5] Addictive behaviors lead to many adaptations in postsynaptic spine structure that result in profound alterations in synaptic transmission.[6] At the molecular level, synaptic activity triggers diverse signaling pathways, which, in turn regulate and reorganize cytoskeleton-associated proteins. For example, repeated cocaine administration has been shown to change stathmin, a regulatory protein crucial to MT dynamics,[7,6] causing morphologic changes.[8]

Neuronal structural changes may contribute to the progression of AUD and SUDs.[9] Chronic ethanol (EtOH) exposure significantly stabilizes neuronal and acetylated MTs in hepatic PC12 cells,[10] increases dendrite lengths and neurite outgrowth and causes aberrant sprouting of hippocampal neurites.[11] Loss of α and β free tubulin units in the caudate nucleus, cortex, and cerebellum was noted in post-mortem

brain samples from individuals diagnosed with AUD. Repeated exposure to drugs of abuse like alcohol and cocaine induces structural plasticity[12,13] in many brain circuits and changes in the density and morphology of dendritic spines.[5,14,15] These alterations have significant consequences including cognitive deficits and neurodegeneration.[16,17] Prolonged SUD is also associated with brain injury characterized by impaired synaptogenesis, cellular migration, and neurogenesis—all of which require proper MT functioning.[18] [19] MT agents (MTAs), believed to work primarily by altering MT network integrity, are widely being investigated as drug candidates to treat cancer, brain disorders, and cardiovascular diseases. Thus, MT integrity is important to many neurochemical pathways commonly associated with SUD. However, studies of cytoskeleton-dependent structural plasticity resulting from alcohol and cocaine use have focused predominantly on actin and filament dynamics; molecular level MT impairments remains largely unexplored. Positron Emission Tomography (PET) imaging is a sensitive modality to examine and quantify *in vivo* MT-based changes in the neurochemical cascades of SUD.

MPC-6827 is a small molecule MTA that causes mitotic arrest and cell death. It exerts antitumor (glioblastoma) properties by binding to β -tubulin sites. We reported the automated radiochemical synthesis of [^{11}C]MPC-6827 as the first brain-penetrating, MT-tracking PET ligand and imaged it *in vivo* in normal rodents and non-human primates.[20] [21] To establish the potential of [^{11}C]MPC-6827 as a PET imaging ligand for various neurological disorders, we need to investigate its mechanism of action. Here, we report our preliminary *in vitro* evaluations of [^{11}C]MPC-6827 in SH-SY5Y neuroblastoma cells[22-24] with (a) two different abused drugs (alcohol and cocaine), and (b) various MT stabilizing and destabilizing agents.

Methods

To investigate the effects of cocaine and alcohol on tubulin dynamics, we performed a MT-based assay (Cytoskeleton, Inc., Denver, CO, USA)[25-27] in SH-SY5Y neuroblastoma cells treated with 100 mM EtOH[10] and 1 mM cocaine[8] (n=6/group) for 3 days. This commercially available kit separates large complexes of polymerized MTs attached to nuclei and Golgi bodies into bound and non-polymerized free tubulins. After differential centrifugation, the high-speed pellet supernatant and low-speed pellet samples were isolated and used for western blot analysis. An enhanced Chemiluminescence kit was used to visualize the tubulin bands,[12,11] (**Fig. 1a** and **2a**). Bound/stabilized MT content was significantly higher and unbound/free α/β MTs lower in EtOH-treated cells than control cells treated with PBS. Cells treated with cocaine showed no significant difference in bound MTs and slightly fewer free α/β MTs than untreated cells possibly due to the accrued rate of MT polymerization with substances. Therefore, both EtOH and cocaine compromise MT integrity i.e., increase in bound and decrease in free tubulin units. Having demonstrated these drug-induced changes in MT integrity in SH-SY5Y cells, we next aimed to determine whether [^{11}C]MPC-6827 could also detect similar MT alterations using the same cells.

We performed cell binding assays *in vitro* in SH-SY5Y cells with [^{11}C]MPC-6827 following our previously published protocols.[28-30] The cells were treated with 100 mM EtOH or 1 mM cocaine[8] (n = 6/group)

for 3 days. We then measured radiotracer binding by adding [^{11}C]MPC-6827 (1-2 $\mu\text{Ci}/\text{well}$) and incubating the cells for 5, 30, 60, and 90 min at room temperature ($n = 6/\text{time point}$). To demonstrate tracer specificity, a subgroup of cells ($n = 3$) was pre-treated with non-radioactive MPC-6827 (1.0 μM), adding radiotracer 60 min later and incubating for 30 min. To demonstrate tracer sensitivity to length of drug exposure, cells were treated with 100 mM EtOH or 1 mM cocaine for 1 h, 1 day, or 3 days and incubated with [^{11}C]MPC-6827 for 30 min at room temperature. All the cells were then washed with PBS and lysed with 1N NaOH. Finally, the lysate from each well was g-counted (PerkinElmer, Waltham, MA, USA) and counts-per-minute (cpm) values were normalized to the amount of radioactivity added to each well. Cpm values were then matched with the protein concentration per well, and the data expressed as %ID/mg of protein present in each well.

EtOH- (**Fig 1b**) and cocaine-treated (**Fig 2b**) cells demonstrated an $\sim 30(\pm 2)$ and $\sim 24(\pm 6)$ percent decrease respectively in radioactive uptake versus non-treated controls over the 30-90 min incubation times. Additionally, uptake in EtOH-treated and cocaine-treated cells increased $\sim 13(\pm 3)$ and $\sim 12(\pm 2)$ percent from 5 to 30 min of incubation times respectively and decreased $\sim 53(\pm 2)$ and $\sim 19(\pm 3)$ percent by 90 min in EtOH- and cocaine-treated cells; thus demonstrating favorable pharmacokinetics. For the self-blocking assays (**Fig 1b**), uptake was $\sim 78(\pm 1)$ percent lower after addition of nonradioactive MPC-6827, demonstrating high specificity. Radioactive uptake was decreased $\sim 21(\pm 1)$ and $28(\pm 1)$ percent from 1 h to 3 days EtOH and cocaine exposures (**Fig 3**) respectively. Therefore, [^{11}C]MPC-6827 uptake decreased selectively with increased exposure to EtOH or cocaine. Moreover, since no significant decrease in radioactivity was observed after 3 days of drug exposure we used the same 3 days exposure in all our assays. MPC-6827 primarily targets the β tubulin site at pharmacological doses.[31-33] The lowered radioactive uptake in EtOH- and cocaine-treated SH-SY5Y cells indicates that [^{11}C]MPC-6827 uptake correlate well with observed bound/free tubulin changes and may be tracking free β tubulin units, as both substance treatments decreased free tubulin content in the same cells.

MTAs are categorized as either stabilizing agents (paclitaxel, laulimalide, and EpoD),[34-36] which favor polymerization of tubulin units and inhibit cell proliferation, or destabilizing agents (vinblastine and mertasine),[37-40] which increase free/unbound tubulins and promote apoptotic cell death. To distinguish their effect on MT integrity in SH-SY5Y cells, we performed the same tubulin-based western blot assays on paclitaxel- and vinblastine-treated cells.[41] The paclitaxel-treated cells had more bound/stabilized MTs, and vinblastine-treated cells had more unbound/free MTs than the untreated cells (**Fig 4a**). To confirm the free tubulin-based binding mechanism of [^{11}C]MPC-6827, SH-SY5Y cells were pretreated with different MTAs at 1.0 μM concentration, 3.0 h prior to addition of [^{11}C]MPC-6827. Paclitaxel, laulimalide and EpoD decreased radioactive uptake by $\sim 58(\pm 3)$, $\sim 40(\pm 4)$, and $\sim 66(\pm 7)$ percent respectively, while vinblastine, and mertasine increased it by $\sim 77(\pm 6)$, and $64(\pm 5)$ percent respectively (**Fig 4b**), confirming that [^{11}C]MPC-6827 primarily targets primarily free tubulin units.

Results

Results of the preliminary [^{11}C]MPC-6827 *in vitro* assays with EtOH and cocaine treatments at different incubation times in SH-SY5Y cells indicate that radioactive uptake decreases with increased drug exposure. Tests with various MTAs demonstrate that [^{11}C]MPC-6827 preferentially binds to free/unbound tubulin units with high selectivity. The radioactive uptake results are well-corroborated with observed changes in bound and free tubulin expressions in SH-SY5Y cells with EtOH and cocaine treatments. Taken together, these studies confirm that [^{11}C]MPC-6827 has great potential as an MT imaging agent for defining MT-based mechanisms that underlie the development of alcohol and cocaine addiction. We are currently characterizing its complete *in vivo* and *ex vivo* imaging properties in both rodent and nonhuman primate models of AUD/SUDs.

Sources: Cocaine hydrochloride and other reagents were purchased from Sigma Aldrich; MPC 6827 hydrochloride was purchased from Tocris a biotech brand; SH-SY5Y cells was purchased from ATCC (American Type Culture Collection) and the precursor for [^{11}C]MPC-6827 was purchased from ABX Inc supplies.

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Figures

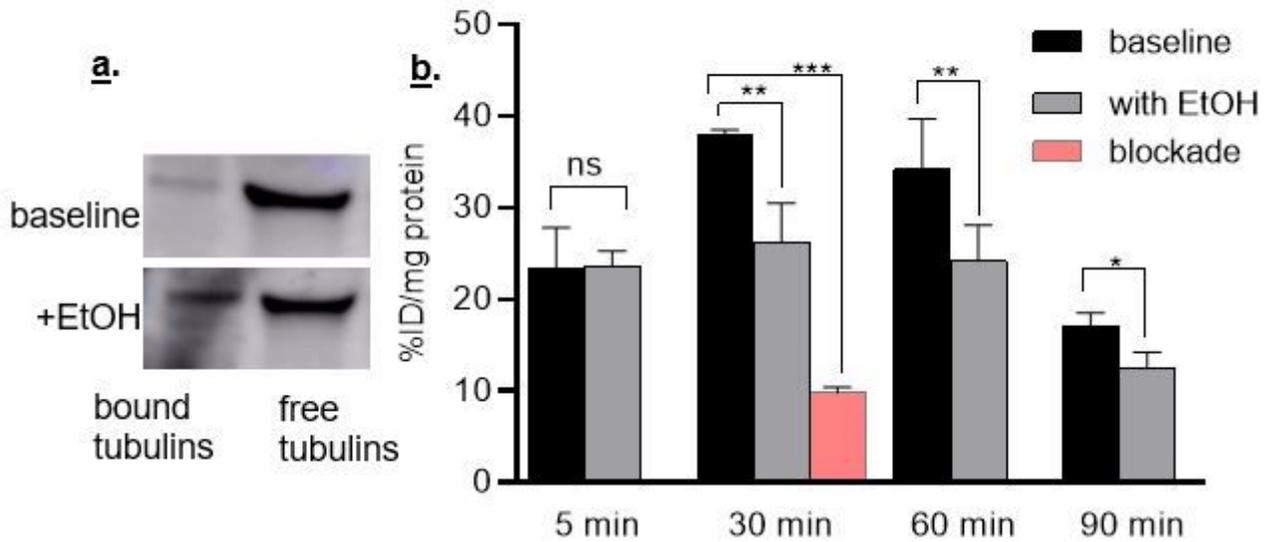


Figure 1

Representative bound and free tubulin a. western blots and b. [11C]MPC-6827 uptake in vitro at different incubation times in SH-SY5Y cells with EtOH (100 mM/3 days) and without EtOH treatment (n=6/group); *p<0.05 and ns: non-significant

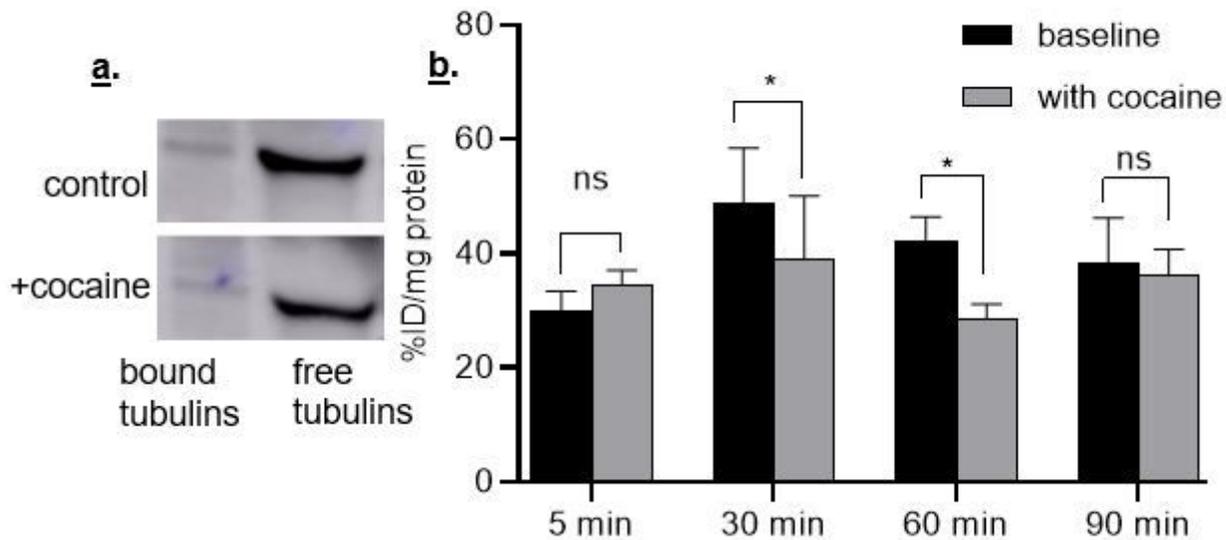


Figure 2

Representative bound and free MT a. western blots and b. [11C]MPC-6827 uptake in vitro at different incubation times in SH-SY5Y cells with cocaine (1 mM/3 days) and without cocaine treatment (n=6); *p<0.05 and ns: non-significant

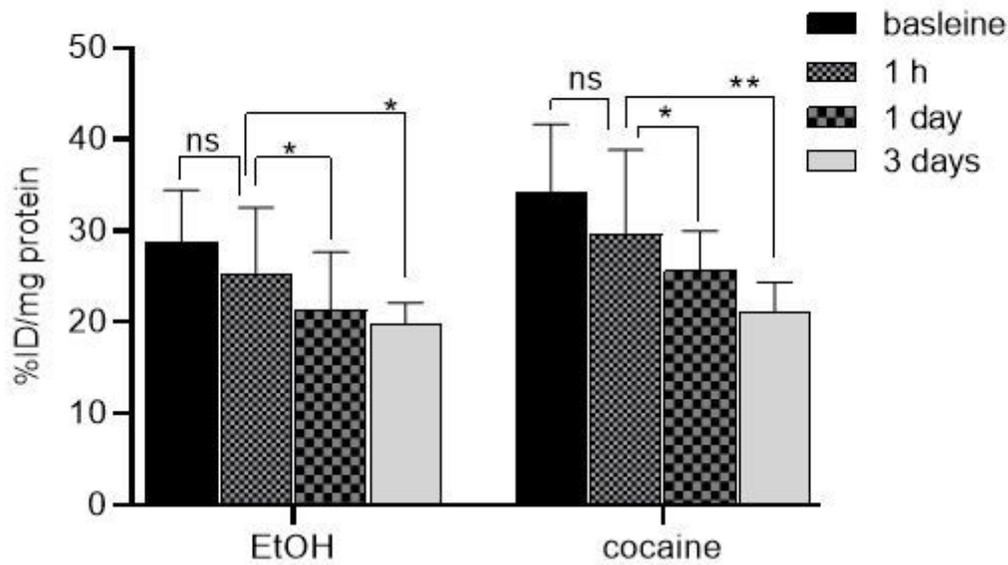


Figure 3

[11C]MPC-6827 uptake in vitro with EtOH (100 mM) and cocaine (1 mM) for 1h, 1 day and 3 day exposures in SH-SY5Y cells (n=6); *p<0.05 and ns: non-significant

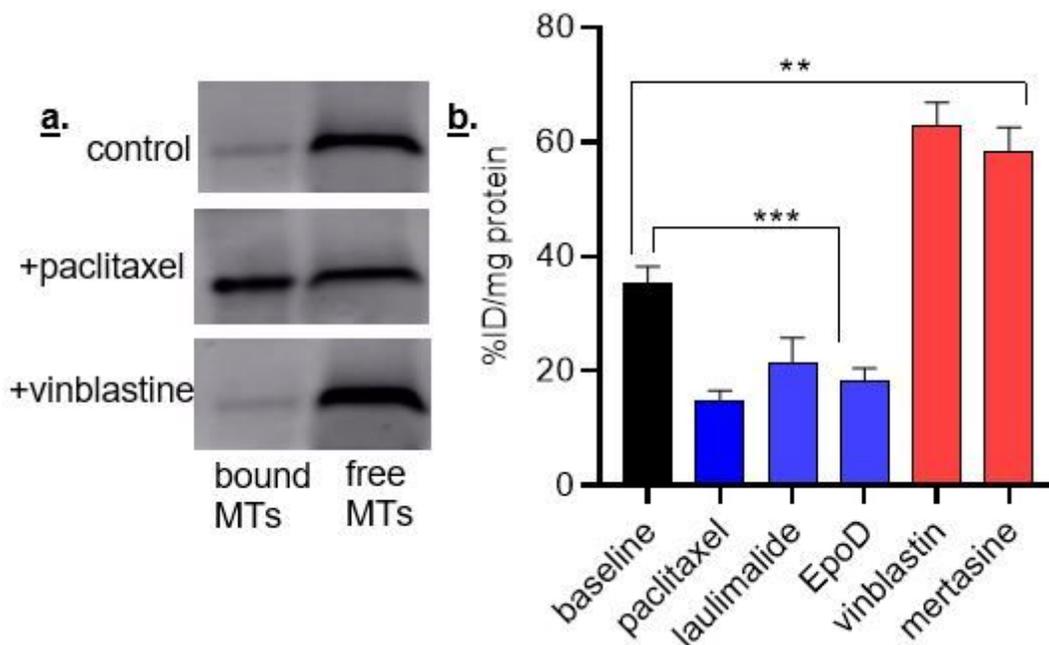


Figure 4

Representative bound and free tubulin a. western blots and b. [11C]MPC-6827 uptake in vitro with different MT agents (1 μM/60 min) in SH-SY5Y cells (n=6); *p<0.05