Chemical, Pharmacological, and Toxicological Assessment of 6-Methylnicotine

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Abstract

There is interest in nicotine-related alkaloids for both recreational use and pharmaceutical applications such as smoking cessation and central nervous system disorders conditions such as Parkinson's, Tourette's, ADHD. Nicotine is one of many alkaloids produced by the tobacco plant (*Nicotiana tabacum* species) and more recently synthesized for commercial use. The compound 6-methylnicotine (CAS# 101540-79-8) has been identified as a nicotine analog of interest based on its chemical structure, sensorial properties, and commercial availability. Chemical, pharmacological, and toxicological assessments were conducted on 6-methylnicotine and compared to pharmaceutical grade (*S*)-nicotine. Samples of 6-methylnicotine analyzed included both freebase and salt forms, as well as in e-liquid formulations containing propylene glycol (PG) and vegetable glycerin (VG) for use in an electronic nicotine delivery system (ENDS). Chemical analysis confirmed the sample was 6-methylnicotine, racemic, and ~98% pure utilizing ^{1}H NMR, chiral UPLC-UV, and GC-MS. The aerosol transfer efficiency of 6-methylnicotine was similar to that of nicotine (82.5 ± 0.6 % vs. 85.6 ± 2.9 % for freebase forms). Archival pharmacological data indicates that 6-methylnicotine is similar in potency and binding affinity to that of (*S*)-nicotine in *in vivo* and *ex vivo* models. Regulatory *in vitro* toxicology testing (Neutral Red, Ames, and Micronucleus) demonstrated 6-methylnicotine salt e-liquid formulations have similar cellular cytotoxicity and mutagenicity/genotoxicity responses to the analogous (*S*)-nicotine salt e-liquid formulation. The totality of available evidence indicates that 6-methylnicotine has comparable chemical, pharmacological, and toxicological properties to the more widely used nicotine.

Introduction

(S)-nicotine is the primary active ingredient in a range of tobacco and nicotine consumer products and in smoking cessation drug therapies such as Nicorette™ gum, lozenge, and mini-lozenges. Since April 2022, when the "synthetic nicotine loophole" was closed by US Congress, all nicotine-containing products are now required to submit a PMTA to FDA to receive marketing approval, regardless of the nicotine source (tobacco-derived or synthetic). This process is a costly, time-consuming, unpredictable, and uncertain. Consequently, there is interest in identifying alternative agents that act in a manner similar to nicotine. One such molecule, 6-methylnicotine, was identified during tobacco industry research conducted between 1977 and 1982 as having similar pharmacological effects in animal models, though it was not incorporated into any marketable products. Recent publications[1-3] seem to indicate that interest in alternate nicotine analogs is rising again. Herein, we present the results of chemical, pharmacological, and toxicological assessments of 6-methylnicotine conducted to fill the existing knowledge gap.

Materials & Methods

Materials

- All nicotine and 6-methylnicotine-containing materials were donated by SS Vape Brands, with the exception of freebase nicotine which was sourced from MilliporeSigma (St. Louis, MO). ENDS devices used for aerosol studies were also provided by SS Vape Brands.

Methods

- GC-MS EI: Agilent HP-5ms, 15 m × 250 µm × 0.25 µm; 70 to 300 °C, 20 °C/min.
- Chiral UPLC-UV: AM-271, AZYP NicoShell SPP, 100 mm × 4.6 mm, 2.7 µm; 0.2 % NH_4HCO_2 in methanol.
- ^{1}H NMR: Bruker NanoBay AVANCE III 400 MHz NMR spectrometer, conducted at the Virginia Commonwealth University (VCU) NMR Center.
- Aerosol Transfer Efficiency: *Devices* – Vaporesso® Tarot Nano (tank-based) for freebase formulations; Vaporesso® Zero (pod-based) for benzoate salt formulations; *Collection* – ISO 20768 conditions, pad collection and extraction into isopropanol; *Analysis* – AM-224, GC-FID, Restek Stabilwax-DA, 30 m × 320 µm × 1 µm, 80 to 240 °C, 20 °C/min.
- Neutral Red Uptake: AM TOX-002, based on ISO 10993:2009 and OECD Guideline 129 (2010); tested in BALB/c 3T3 (mouse fibroblast) and A549 (human lung epithelial) cell lines.
- Bacterial Reverse Mutation Assay (Ames): AM TOX-003, based on OECD Guideline 471 (2020; tested in TA98, TA100, TA102, TA1535, and TA1537 strains of *S. Typhimurium.*
- Micronucleus Test: AM TOX-020, based on OECD Guideline 487 (2023); tested in human lymphoblast TK6 cells; scored using flow cytometry (MicroFlow *in vitro* 250/50 Kit, Litron).

Chemical Characterization

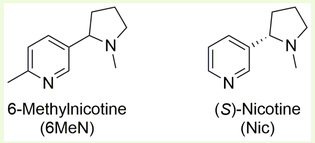

Figure 1. Molecular structures of 6MeN and Nic.

- Predicted physicochemical properties[4] for 6MeN and Nic were found to be comparable (Table 1).
- GC-MS analysis (Figure 2) confirmed nicotine methylation, with ^{1}H NMR indicating methyl group was in the pyridyl 6-position (Figure 3). Latter also showed 6MeN purity to be >98%
- Chiral analysis showed the received 6MeN was racemic (i.e. 1:1 (*R*)/(*S*)-enantiomers) and Nic was >99% (*S*)-nicotine.
- Aerosol transfer efficiency from 4.8% e-liquid formulations (freebase and benzoate salts) was assessed by GC-FID, showing comparable behavior between 6MeN and Nic for both forms (Table 2).

Table 1. Predicted physicochemical properties comparison.

	Predicted Average Values	
Physicochemical Property	**6-MethylNicotine[4]**	**(S)-Nicotine[5]**
Physical State	Liquid	Liquid
Melting Point (°C)	20.9	18.1
Boiling Point (°C)	259	246
Density (g/cm^3)	1.01	1.03
Vapor pressure (mm Hg)	7.30×10^{-3}	2.39×10^{-2}
Partition coefficient, log K_{ow}	1.42	0.928
Solubility in water (mol/L)	0.849	2.18
Surface tension (dyn/cm)	37.4	38
Flash point (°C)	112	98.6
Viscosity (cP)	11.8	7.28

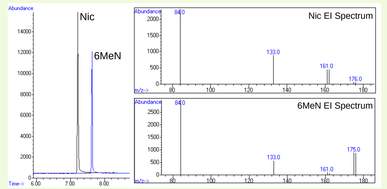

Figure 2. GC-MS analysis of 6MeN and Nic: (left) overlapped SIM traces (*m/z* 84); (right) individual EI mass spectra.

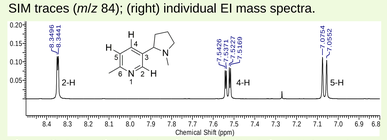

Figure 3. Aromatic proton region of the 6MeN ^{1}H NMR spectrum.

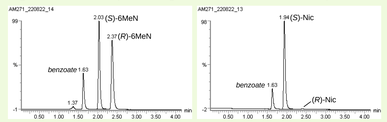

Figure 4. Chiral UPLC-UV analysis of 6MeN (left) and Nic (right) (4.8% benzoate salt e-liquids were analyzed).

Table 2. Aerosol transfer efficiency comparison between 6-MeN and Nic. Values are average ± standard deviation (n = 3).

		"Nicotine" Conc. (mg/g)		
Form	**Active Agent**	**E-Liquid**	**Aerosol**	**Transfer Efficiency (%)**
Freebase	Nic	43.0 ± 0.0	36.8 ± 1.2	85.6 ± 2.9
	6MeN	41.5 ± 0.1	34.2 ± 0.2	82.5 ± 0.6
Benzoate	Nic	40.4 ± 0.1	36.4 ± 0.8	90.2 ± 2.1
	6MeN	42.3 ± 0.1	37.4 ± 2.0	88.3 ± 4.6

In Silico Toxicology and Pharmacological Review

- *In silico* toxicological models were used to predict the toxicity of 6MeN and compare to Nic. Five quantitative structure-activity relationship (QSAR) models were used: (1) ICH M7, (2) Derek, (3) Sarah, (4) VEGA (incl. Toxtree), and (5) OECD Toolbox.
- Overall, toxicity predictions were nearly identical for 6MeN and Nic across all models.
- A review of industry-funded research available within the Truth Tobacco Industry Documents repository[6] was conducted.
- Limited industry-sponsored pharmacological studies on nicotine analogs were conducted by the Institut für Biologische Forschung (INBIFO) between 1977 and 1982.
- The *in silico*-predicted similarity was supported by the similar pharmacology shared by the two compounds (Table 2)
- However, caution should be taken since these studies are not peer-reviewed and used potentially outdated methodologies
- For instance, a 3-fold stronger affinity for rat brain nicotinic receptors was shown for 6MeN,[7] but a more recent peer reviewed study indicated it was actually slightly weaker[2].

Table 2. Relative affinity and potency of 6-methylnicotine compared to (*S*)-nicotine.

Affinity Experiments (Rat Model)	**Relative Affinity (Higher = stronger)**	**Ref.**
Brain	3.03	[7]
Nic	0.38	[7]
ACh - K_i (relative)	0.08	[7]
nAChR radioligand binding (rat brain)	0.70	[2]
Functional Experiments	**Relative Potency (Higher = more potent)**	**Ref.**
LD50 (mice)	3.83	[8]
ED50 (mice; effect: convulsions)	4.22	[8]
Model 03 Guinea Pig Ileum	1.96	[8]
Model 05 Rat Phrenic Nerve-Diaphragm	0.46	[8]
Model 06 Guinea Pig Auricle	1.47	[8]
Model 09 Rabbit Aortic Strip	1.10	[9]
Blood Pressure (Increase by 25%)	0.42	[7]
Drug Discrimination	1.09-2.19	[7]
Prostration	1.08	[7]

Toxicology

Test Samples

All toxicological testing was performed on 4.8% benzoate salt e-liquid formulations in PG-VG (1:1) with tobacco flavoring.

Cytotoxicity – Neutral Red Uptake (NRU) Assay

- Identifies cytotoxic agents via their ability to impair a cell's ability to incorporate the neutral red dye into lysosomal compartments.
- Dosing 0.08 to 5 mg/mL e-liquid (10 mg/mL dose excluded due to >30% osmolality change); cells incubated for 48 h.
- No substantial cytotoxicity observed and IC_{50} values could not be determined for either formulation (Figure 5).

Mutagenicity – Bacterial Reverse Mutation Assay (Ames Test)

- Identifies mutagenic agents via restoration of histidine-independency to histidine-dependent bacterial test strains.
- Dosing 0 to 5000 µg/plate; incubated for 48–72 h.
- No dose-response behavior observed in any test strain for both agents (Figure 6).
- Revertant formation is comparable to the vehicle control (0 µg/mL dosage) at all doses.
- Both 6MeN and Nic show no mutagenic activity.

Genotoxicity – *in vitro* Micronucleus (MN) Test

- Identifies agents that induce cytogenetic damage via the observation of micronuclei in daughter cells.
- TK6 cells treated with serial dilutions (7200 to 847 µg/mL e-liquid) under three conditions:
  - Schedule (i): short term (4 h) with no metabolic activation
  - Schedule (ii): short term (4 h) with metabolic activation.
  - Schedule (iii): long-term (22 h) with no metabolic activation
- Both formulations were determined to be negative for genotoxicity under all conditions (Figure 7).

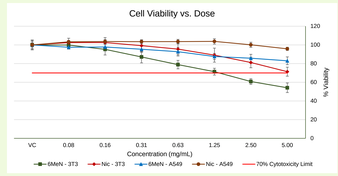

Figure 5. Neutral Red Uptake results for 6MeN and Nic e-liquid formulations (n = 9).

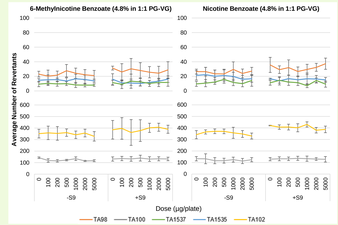

Figure 6. Ames Test results for 6MeN (left) and Nic (right) e-liquid formulations (n = 6).

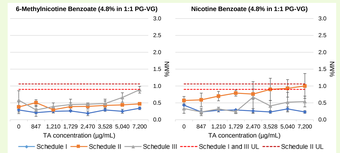

Figure 7. Micronucleus Test results for 6MeN (left) and Nic (right) e-liquid formulations (n = 4)

Summary

- 6-Methylnicotine has similar chemical characteristics to (*S*)-nicotine and behaves similarly in ENDS devices.
- *In silico* toxicological and historical pharmacological studies suggest somewhat comparable activity.
- 6-Methylnicotine exhibits comparable toxicological behavior to (*S*)-nicotine with no mutagenic or genotoxic activity and limited cytotoxicity.
- While initial experiments suggest 6-methylnicotine could be a suitable replacement for nicotine, pharmacological and epidemiological studies are needed.

References

[1] Dukat , M. et al., *Synthesis, receptor binding and QSAR studies on 6-substituted nicotine derivatives as cholinergic ligands*. *Eur. J. Med. Chem*., 1999, 34(1), 31-40.
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[3] Qi, H., et al., *Comparative analyses of transcriptome sequencing and carcinogenic exposure toxicity of nicotine and 6-methyl nicotine in human bronchial epithelial cells. Toxicol. In Vitro*, 2023, doi: 10.1016/j.tiv.2023.105661; available online 14 August, 2023.
[4] Source: EPA CompTox Chemicals Dashboard, accessed September 11, 2023. Data Quality Level 4: "Programmatically curated from ChemID, unique chemical identifiers have no conflicts in PubChem." 6MeN predictions based on CAS# 13270-56-9, (*S*)-6-methylnicotine, since no information is available for racemic 6MeN (CAS# 101540-79-8.
[5] Source: EPA CompTox Chemicals Dashboard, accessed September 11, 2023. Data Quality Level 1: "Expert curated, highest confidence in accuracy and consistency of unique chemical identifiers."
[6] Truth Tobacco Industry Documents, https://www.industrydocuments.ucsf.edu/tobacco, accessed 11 September, 2023.
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