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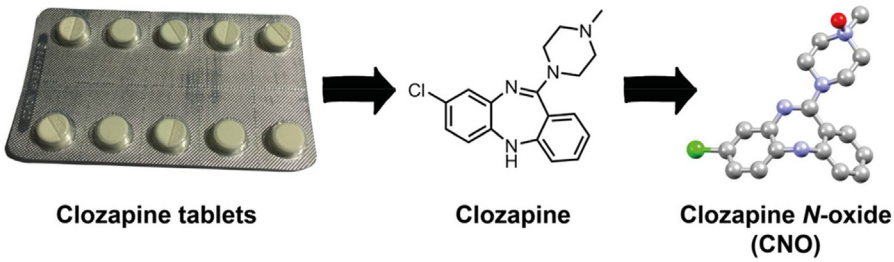
Method Article

Gram scale preparation of clozapine *N*-oxide (CNO), a synthetic small molecule actuator for muscarinic acetylcholine DREADDs

Phillip L. van der Peet[a], Christian Gunawan[a], Alaa Abdul-Ridha[b], Sherie Ma[b,c,1], Daniel J. Scott[b,c,d], Andrew L. Gundlach[b,c], Ross A.D. Bathgate[b,c,d], Jonathan M. White[a], Spencer J. Williams[a,*]

[a] *School of Chemistry and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Victoria 3010, Australia*
[b] *The Florey Institute of Neuroscience and Mental Health, Parkville, Victoria 3052, Australia*
[c] *Florey Department of Neuroscience and Mental Health, The University of Melbourne, Victoria 3010, Australia*
[d] *Department of Biochemistry and Molecular Biology, The University of Melbourne, Victoria 3010 Australia*

GRAPHICAL ABSTRACT



ABSTRACT

Chemogenetics uses engineered proteins that are controlled by small molecule actuators, allowing *in vivo* functional studies of proteins with temporal and dose control, and include Designer Receptors Exclusively Activated by Designer Drugs (DREADDs). One major class of DREADDs are mutated muscarinic receptors that are unresponsive to acetylcholine, and are activated by administration of clozapine *N*-oxide (CNO). However, CNO is available in only small amounts and large scale studies involving animals and multiple cohorts are

* Corresponding author.
E-mail address: sjwill@unimelb.edu.au (S.J. Williams).
[1] Present address: Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia.

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prohibitively expensive for many investigators. The precursor, clozapine, is also expensive when purchased from specialist suppliers. Here we report:

- A simple extraction method of clozapine from commercial tablets;
- A simple preparation of CNO from clozapine, and for the first time its single-crystal X-ray structure; and
- That the CNO prepared by this method specifically activates the DREADD receptor hM3Dq *in vivo*.

This method provides large quantities of CNO suitable for large-scale DREADD applications that is identical to commercial material.

ARTICLE INFO

Method name: Preparation and application of clozapine N-oxide (CNO)
Keywords: G-protein-coupled receptor (GPCR), Chemical-genetics, Chemical synthesis
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Specifications Table

Subject area	*Pharmacology, Toxicology and Pharmaceutical Science*
More specific subject area	*Chemogenetics*
Method name	Preparation and application of clozapine *N*-oxide (CNO)
Name and reference of original method	D. Bender, M. Holschbach, G. Stocklin, Synthesis of n.c.a. carbon-11 labelled clozapine and its major metabolite clozapine-*N*-oxide and comparison of their biodistribution in mice, Nucl. Med. Biol. 21 (1994) 921-5, doi:10.1016/0969-8051(94)90080-9.
Resource availability	*Not applicable*

Method details

Overview

DREADD (designer receptors exclusively activated by designer drugs) technology is a chemical-genetic approach that posits the development of mutated G-protein-coupled receptors (GPCRs) that no longer respond to a receptor-specific drug or the endogenous ligands, but which respond exclusively to a designer drug that is otherwise inert and inactive [1,2]. Mutant DREADD receptors are expressed within cells or organisms and provide a powerful way to exert transient and repeatable control of receptor function. DREADDs were initially developed for the muscarinic acetylcholine receptor family [3], but have since been applied to a range of GPCRs, including β_2-adrenergic receptor [4], κ-opioid receptors [5] and 5-HT_{2A} receptors [6]. A range of mutant muscarinic acetylcholine receptors have been developed [2], including axonally-targeted activating and silencing subtypes [7], as well as arrestin-biased receptors [8]. Muscarinic DREADD receptors contain two mutations of conserved orthosteric site residues that cause loss of responsiveness to their native ligand acetylcholine, and respond specifically upon treatment with the small molecule clozapine *N*-oxide (CNO) [3]. While initially it was believed that CNO was the activating ligand, recent studies suggest that CNO does not enter the brain; rather metabolic conversion yields clozapine, which readily enters the brain and occupies DREADDs expressed within the central nervous system [9]. CNO therefore may function as a precursor to deliver 'sub-threshold' clozapine that selectively activate DREADDs. Nonetheless, CNO does provide activating effects and remains an important reagent for *in vitro* and *in vivo* studies of brain function, provided that well-controlled experimental design is used to control for effects of clozapine beyond those at DREADDs [10].

CNO is commercially available, but its high cost has limited its application for *in vivo* animal studies where multiple animal cohorts and extended dosing regimens require sizeable (multigram) quantities