Alkoxy Chain Length Governs the *In Vitro* and *In Vivo* Potency of 2-Benzylbenzimidazole Opioids Implicated in Human Overdose Deaths

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Illicitly manufactured fentanyl plays a major role in the current opioid crisis, but non-fentanyl mu-opioid receptor (MOR) agonists are emerging in drug markets worldwide. Synthetic 2-benzylbenzimidazole opioids, also known as “nitazenes”, are examples of non-fentanyl MOR agonists linked to human fatalities. Here, we characterized the pharmacological effects of nitazene analogs that differ in their alkoxy chain length, including metonitazene (O-methyl), etonitazene (O-ethyl), protonitazene (O-propyl), isonitazene (O-isopropyl), and butonitazene (O-butyl). We first examined the effects of the analogs in assays measuring MOR binding in rat brain membranes and forskolin-stimulated cAMP accumulation in MOR-transfected human embryonic kidney cells. Next, we examined the antinociceptive, locomotor, and body temperature effects of subcutaneously administered drugs in male C57Bl/6J mice fitted with surgically-implanted temperature transponders. Radioligand binding data demonstrated that all nitazenes display selective affinity for MOR (Ki range=3.6-53.1 nM) versus delta- and kappa-opioid sites. Increasing alkoxy chain length from methyl to ethyl, propyl, and butyl led to a stepwise decrease in MOR affinity. All of the nitazenes were agonists for MOR-mediated inhibition of cAMP accumulation (EC50 range=0.03-0.50 nM), with the O-ethyl compound being the most potent. In the cAMP assay, the O-ethyl, O-isopropyl, and O-propyl analogs were more potent than fentanyl (EC50=0.10 nM) and much more potent than morphine (EC50=1.22 nM). The mouse experiments revealed that nitazenes induce dose-dependent antinociception (ED50 range=0.01-1.4 mg/kg), locomotor stimulation, and hypothermia, with etonitazene having the highest potency. In the hot plate assay, the O-ethyl, O-isopropyl, and O-propyl analogs were more potent than fentanyl (ED50=0.15 mg/kg), and much more potent than morphine (ED50=10.19 mg/kg). Correlation analysis showed that *in vivo* ED50 potencies were positively correlated with the *in vitro* EC50 potencies for inhibition of cAMP but not Ki affinities at MOR. Overall, our findings reveal that alkoxy chain length governs potency of nitazenes *in vitro* and *in vivo*, whereby the ethyl and isopropyl chain lengths provide optimal pharmacological activity at MOR. Three of the tested nitazenes are more potent than fentanyl, and all are much more potent than morphine. Thus, nitazenes may pose serious risks to human users who are unknowingly exposed to the compounds.

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