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Small molecule inhibitors of PSD95-nNOS protein–protein interactions as novel analgesics

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ABSTRACT

Aberrant increases in NMDA receptor (NMDAR) signaling contributes to central nervous system sensitization and chronic pain by activating neuronal nitric oxide synthase (nNOS) and generating nitric oxide (NO). Because the scaffolding protein postsynaptic density 95kDA (PSD95) tethers nNOS to NMDARs, the PSD95-nNOS complex represents a therapeutic target. Small molecule inhibitors IC87201 (EC_{50}: 23.94 μM) and ZL006 (EC_{50}: 12.88 μM) directly inhibited binding of purified PSD95 and nNOS proteins in AlphaScreen without altering binding of PSD95 to ErbB4. Both PSD95-nNOS inhibitors suppressed glutamate-induced cell death with efficacy comparable to MK-801. IC87201 and ZL006 preferentially suppressed phase 2A pain behavior in the formalin test and suppressed allodynia induced by intraplantar complete Freund's adjuvant administration. IC87201 and ZL006 suppressed mechanical and cold allodynia induced by the chemotherapeutic agent paclitaxel (ED_{50}s: 2.47 and 0.93 mg/kg i.p. for IC87201 and ZL006, respectively). Efficacy of PSD95-nNOS disruptors was similar to MK-801. Motor ataxic effects were induced by MK-801 but not by ZL006 or IC87201. Finally, MK-801 produced hyperalgesia in the tail-flick test whereas IC87201 and ZL006 did not alter basal nociceptive thresholds. Our studies establish the utility of using AlphaScreen and purified protein pairs to establish and quantify disruption of protein–protein interactions. Our results demonstrate previously unrecognized antinociceptive efficacy of ZL006 and establish, using two small molecules, a broad application for PSD95-nNOS inhibitors in treating neuropathic and inflammatory pain. Collectively, our results demonstrate that disrupting PSD95-nNOS protein–protein interactions is effective in attenuating pathological pain without producing unwanted side effects (i.e. motor ataxia) associated with NMDAR antagonists.

1. Introduction

Chronic pain is a devastating clinical problem resulting from nerve injury, disease states (e.g. diabetes or cancer) or toxic challenges. It is the most common cause of long-term disability, and fewer than 50% of patients receive adequate pain relief (Steglitz et al., 2012). Alterations in the properties of peripheral nerves by inflammation-associated changes in the chemical environment of the nerve fiber has been implicated in peripheral sensitization (Basbaum et al., 2009). In addition to peripheral mechanisms, central sensitization, a process which establishes hyperexcitability in the central nervous system (CNS), leads to enhanced processing of nociceptive messages, thus contributing to both the development and maintenance of chronic pain (Basbaum et al., 2009). One of the mechanisms involved in central sensitization is through excessive glutamatergic signaling and overactivation of the *N*-methyl-D-aspartate receptor (NMDAR) (Latremoliere and Woolf, 2009; South et al., 2003; Woolf, 1983). Overactivation of NMDARs

Nonstandard abbreviations: NMDAR, N-methyl-D-aspartate receptor; nNOS, neuronal nitric oxide synthase; PSD95, postsynaptic density 95; CFA, complete Freund's adjuvant.

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leads to increased calcium influx in the postsynaptic cell and, consequently, increases downstream signaling events critical for the development and maintenance of chronic pain (Latremoliere and Woolf, 2009). NMDAR antagonists (e.g. ketamine, MK-801, and memantine) produce antinociceptive efficacy in various animal pain models (Zhou et al., 2011); however, direct antagonism of NMDAR produces adverse pharmacological effects (e.g. motor impairment, memory impairment, dissociative effects) due to the broad involvement of NMDAR signaling in numerous physiological functions (Zhou et al., 2011).

We hypothesized that disrupting protein–protein interactions that mediate downstream signaling events critical for pronociceptive NMDAR activation may suppress pain without triggering unwanted side effects mediated by direct antagonism of NMDAR (Florio et al., 2009). NMDAR activation leads to production of nitric oxide (NO), which, when produced in excess, is implicated in pathological processes including inflammation and pain (Miclescu and Gordh, 2009). The production of NO is linked to NMDAR activation through neuronal nitric oxide synthase (nNOS), an enzyme which is tethered to NMDAR by the scaffolding protein postsynaptic density 95 kDA (PSD95) (Christopherson et al., 1999; Luo and Zhu, 2011; Sattler et al., 1999). This NMDAR-PSD95-nNOS complex is required for NMDAR mediated NO production and has thus been implicated in neurological diseases and neuronal excitotoxicity (Courtney et al., 2014; Li et al., 2013; Toro and Deakin, 2005). IC87201 (see Fig. 1), the first small molecule disruptor of PSD95-nNOS interaction, produces antinociceptive efficacy following intrathecal administration in a manner mimicked by the fusion protein TAT-nNOS (Florio et al., 2009). ZL006 (see Fig. 1) is a second small molecule disruptor of PSD95-nNOS interaction that shows therapeutic efficacy in models of stroke and depression (Doucet et al., 2013; Zhou et al., 2010). Whether ZL006 produces antinociceptive efficacy in models of pathological pain remains unknown. Disruption of the NMDAR-PSD95 complex with a peptide inhibitor, TAT-NR2B9c, also produces antinociception in a model of neuropathic pain induced by traumatic nerve injury (D'Mello et al., 2011). These observations provide evidence that targeting the NMDAR-PSD95-nNOS complex downstream of NMDAR signaling pathway may be effective in blocking the NMDAR triggered central sensitization. Thus the PSD95-nNOS protein–protein interface represents a potential target for treating pain and other disease states involving aberrant NMDAR signaling.

ZL006 inhibits glutamate-induced cell death and glutamate-induced increases in nNOS-PSD95 protein complex in primary neuronal cells (Zhou et al., 2010). However, it is unclear whether these effects occur through direct disruption of the PSD95-nNOS complex because there is no evidence that ZL006 inhibits this complex *in vitro*. We, therefore, examined the ability of small molecule inhibitors ZL006 and IC87201 to disrupt binding between purified PDZ domains of nNOS and PSD95 using an amplified luminescence proximity homogeneous assay (AlphaScreen). In addition, we characterized the effect of IC87201 and ZL006 on the binding of PSD95 to receptor tyrosine-protein kinase ErbB4 to assess specificity of these inhibitors for disrupting PSD95-nNOS protein–protein interactions. To characterize the cell-based efficacy of these small molecules, we evaluated whether these inhibitors protect cortical neurons from glutamate-induced excitotoxicity. We also characterized the antinociceptive efficacies of these small molecule disruptors in mouse models of inflammatory and neuropathic pain. Intrathecal administration of IC87201 has previously been shown to attenuate both NMDA-induced hyperalgesia and neuropathic pain induced by chronic constriction injury (CCI) (Florio et al., 2009). However, whether IC87201 shows antinociceptive efficacy in chronic pain models following systemic administration remains unknown. Here, we used two small molecule PSD95-nNOS inhibitors to determine whether disruption of PSD95-nNOS interactions produces broad spectrum antinociceptive efficacy in models of inflammatory pain induced by intraplantar administration of either formalin or complete Freund's adjuvant (CFA) administration and neuropathic pain induced by treatment with the chemotherapeutic agent paclitaxel. Our studies provide evidence that ZL006 directly disrupts the PSD95-nNOS complex and provides the first characterization of the antinociceptive efficacy of ZL006 in pain models. Our studies also extend findings of Florio (2009) by evaluating efficacy of IC87201 in models of inflammatory pain and toxic neuropathy and by employing a systemic (i.p.) route of drug administration. The formalin test is a model of tonic inflammatory pain and the second phase of formalin-evoked nociceptive behaviors have been linked to NMDAR-dependent central sensitization (Tjolsen et al., 1992). The CFA model is a pervasive inflammatory pain model which has been linked to activation of nNOS (Chen et al., 2010; Chu et al., 2005). Thus, these two inflammatory pain models were used, together with two small molecule inhibitors (i.e. IC87201 and ZL006) to ascertain whether nNOS-PSD95 inhibitors suppress inflammatory pain. Chemotherapeutic agents, such as paclitaxel, induce peripheral neuropathy in mice and rats, similar to that observed in cancer patients undergoing chemotherapy treatment (Windebank and Grisold, 2008). Thus, we asked whether nNOS-PSD95 inhibitors disrupt mechanical and cold allodynia (Jaggi et al., 2011) induced by paclitaxel treatment in mice. We also employed the tail immersion test to assess whether small molecule inhibitors themselves produce analgesic effects in the absence of a pathological pain state. Comparisons in all studies were made with the NMDAR antagonist MK-801, used as a reference compound. Finally, we compared side-effect profiles of PSD95-nNOS inhibitors with MK-801 using the rota-rod test of motor ataxia. Our studies demonstrate that ZL006, like IC87201, directly disrupts the PSD95-nNOS complex and reveal previously unidentified anti-allodynic properties of ZL006. Our *in vivo* studies also suggest that IC87201

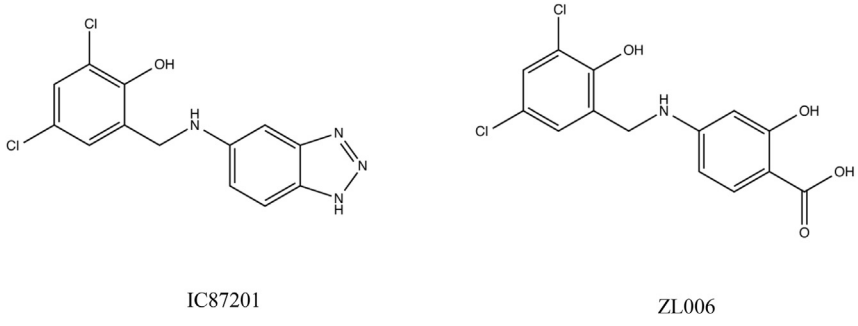

Fig. 1. Chemical structures of small molecule PSD95-nNOS inhibitors IC87201 and ZL006.