

Niclosamide is a Negative Allosteric Modulator of Group I Metabotropic Glutamate Receptors: Implications for Neuropathic Pain

Ni Ai¹ · Richard D. Wood² · Eric Yang² · William J. Welsh³

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ABSTRACT

Purpose Novel therapeutics are greatly needed that target specific pathological receptors and pathways involved in Neuropathic Pain (NP). Extending our previous work published in this Journal on Group I metabotropic glutamate receptor (mGluR) modulators, we now investigate the therapeutic potential of niclosamide in modulating aberrant glutamate transmission in NP.

Method Calcium mobilization assays and cross-receptor selectivity experiments are conducted to characterize the pharmacological activity of niclosamide. A focused series of niclosamide analogues is then prepared to elucidate key structural determinants that emerged from computational molecular modeling analysis on drug-receptor interactions. Finally, niclosamide and a carbamate derivative are studied to assess their efficacy in an NP-evoked mechanical hyperalgesia model in rats.

Results Niclosamide is a low-nanomolar allosteric antagonist of Group I mGluRs with high selectivity for Group I over homologous Group III mGluRs. The phenolic hydroxyl group of niclosamide forms a crucial hydrogen bond with mGluR1/5. Its bioactive coplanar conformation is further stabilized by the nitro substituent on the B ring and an

intramolecular bond. Mechanical hyperalgesia in NP rats is reversed by niclosamide through three different dosing routes.

Conclusion To our knowledge, this is the first report of the salicylanilide class of compounds as potential treatments for NP.

KEY WORDS group I metabotropic glutamate receptors · neuropathic pain · niclosamide

ABBREVIATIONS

CNS	Central nervous system
IASP	International Association for the Study of Pain
mGluR	Metabotropic Glutamate receptor
MPEP	2-methyl-6-(phenylethynyl)pyridine
NAM	Negative allosteric modulator
NP	Neuropathic pain
PAM	Positive allosteric modulator
PSN	Partial sciatic nerve
SAM	Silent allosteric modulator

INTRODUCTION

According to the International Association for the Study of Pain (IASP) Taxonomy working group, neuropathic pain (NP) is defined as “pain caused by a lesion or disease of the somatosensory nervous system” (1). About 6–8% of the general population is estimated to be affected by NP (2). NP syndromes can significantly influence the quality of life and debilitate the physical functions of patients. However, treatments for NP are few in number, of limited effectiveness, and beset by side effects. To enhance understanding of the disease and discover new therapeutic options, several pathophysiological mechanisms have been identified to play an important role in NP, such as dysregulated glutamate signaling in the central nervous system (CNS) (3).

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The excitatory amino acid glutamate is ubiquitous in central and peripheral nervous mechanisms. Malfunction of glutamate messaging has been implicated in a variety of CNS and peripheral disorders. Of particular importance is the role of glutamate in pain (4,5). It is known that attenuation of glutamate release, or antagonism of certain glutamate receptors, can modulate pain (6). The metabotropic glutamate receptors (mGluRs), which bind to glutamate then modulate synaptic transmission, are divided into three sub-groups: Group I, II, and III. These mGluRs are members of the class C G-protein coupled receptors (GPCRs) with structures characterized by a seven transmembrane (TM) domain and an extracellular domain responsible for orthosteric ligand binding. Classical pharmacological modulators, which largely target the orthosteric binding site of GPCRs where the endogenous ligand (e.g., glutamate) performs its regulatory role, represent about 40% of currently marketed drugs across all major therapeutic areas. Despite the tremendous record of success in GPCR drug discovery, evolutionary conservation of the orthosteric binding site among GPCRs poses a great challenge for drug selectivity. In addition, chronic activation of certain GPCRs by agonists is not tolerated (7). Since the proposal of allosterism fifty years ago (8), allosteric modulators have attracted considerable interest due to better receptor subtype selectivity compared with compounds binding to the orthosteric site (7,9–11). Moreover, the activity of allosteric modulators is dependent on the presence of endogenous agonist, which can reduce the risk of receptor oversensitization. With respect to their modes of action, allosteric ligands can be separated into three categories: negative allosteric modulators (NAMs) that inhibit the receptor; positive allosteric modulators (PAMs) that activate the receptor; and silent allosteric modulators (SAMs) that exert no effects but physically occupy the allosteric binding site. A plethora of basic and translational discovery research has focused on identification of allosteric modulators of GPCRs, which has led to two approved drugs in the market functioning through this unique mechanism while several more drug candidates are now in the clinical development (11).

Ample studies have confirmed that dysregulated signaling of the Group I mGluRs, which comprise mGluR1 and mGluR5, is observed in many pathological neural processes including NP. Extensive efforts by industry and academic groups have been spent on identification of allosteric modulators of these two receptors as therapeutics for many related

diseases (12,13). Recent results have revealed that Group I mGluR NAMs are effective at relieving NP. The mGluR1 allosteric antagonists developed at Pfizer and the mGluR5 allosteric antagonists SIB-175715 and 2-methyl-6-(phenylethynyl)pyridine (MPEP) (14,15) have demonstrated substantial efficacy in rodent models of NP. These compounds have found utility as research probes, but as yet have fallen short as therapeutic agents. Using an *in silico-in vitro-in vivo* drug repositioning procedure developed by us previously (16), niclosamide and nitazoxanide emerged as two approved drugs with potential Group I mGluR antagonistic activity from an approved drug library. Niclosamide is an off-patent anthelmintic drug with an excellent safety profile, which is indicated for the treatment of intestinal worms by uncoupling mitochondria of the parasitic worm. This drug recently has attracted considerable interest as a treatment for viral infections (17,18), cancers (19–21), Type 2 diabetes (22), and other serious pathologies. However, until now there has been no report of the modulating activity of niclosamide on GPCR functions. Inspired by the excellent safety profile of niclosamide and its modulating effects on Group I mGluRs, we have now characterized the activity profile of niclosamide against six mGluR subtypes and investigated niclosamide in a preclinical model as an oral treatment for NP. In an effort to improve the bioavailability of niclosamide via prodrug design, we also considered a derivative of niclosamide in the animal study. Both niclosamide and its derivative relieved hyperalgesia induced by mechanical injury in NP rats, which attests to its utility as a starting point for the development of NP therapeutics. Additionally, the structure of niclosamide can be a useful pharmacological tool for elucidating the role of mGluR on CNS functions.

METHODS

Chemistry

Commercially available salicylic acids and anilines were coupled in hot xylenes in the presence of PCl_3 to furnish niclosamide analogues (Scheme 1) (23). R_1 – R_4 substituents are defined in Figs. 1, 2, 3 and 4. Reduction of niclosamide with Zn dust in methanol and acetic acid followed by salt formation gave amino salicylanilide hydrochloride **6**. The carbamate derivative of niclosamide was obtained through

Scheme 1 Preparation of 1–5 and 7

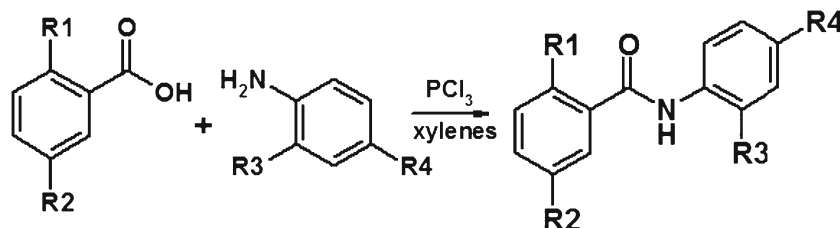
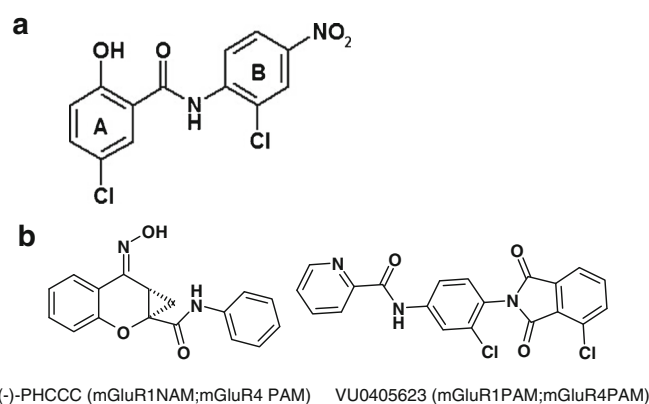


Fig. 1 (a) Chemical structure of niclosamide; (b) Chemical structures of representative modulators with cross-receptor activities between mGluR1 and mGluR4.



treatment of niclosamide with Dimethylcarbonyl chloride to provide acylated derivative **9**. Compound **8** was prepared through the reaction of 3-chlorobenzoyl chloride with 2-chloro-4-nitroaniline in a mixture of THF and CH_2Cl_2 in the presence of *N,N*-diisopropylethylamine (DIEA) and 4-dimethylaminopyridine (DMAP). Detailed information on chemical synthesis was provided in [Supplemental Information](#).

Synthesis of Potential Group I mGluR Modulators

Unless otherwise stated, all solvents and reagents were used as received from vendors. ^1H NMR spectra were measured at either 400 MHz (Varian) or 500 MHz (Varian Inova AS500) in DMSO-d_6 or CDCl_3 . HPLC-MS analyses were carried out with a Shimadzu LC-MS 2020 using a Phenomenex CHO-8463 C18 column (50×3.0 mm) with a gradient of 10% acetonitrile: 90% water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) over 5 min. Retention times (T_R) are reported in minutes (min). Mass spectra (ESI) are reported in positive (m/z^+) and/or negative (m/z^-) mode. The calculated exact mass is denoted as EM. Unless otherwise stated, all

compounds were obtained at $\geq 95\%$ purity (HPLC-MS). Niclosamide was purchased from Sigma-Aldrich (St. Louis, MO, USA).

BIOLOGY

In Vitro mGluR1 and mGluR5 Functional Assay

Preparation of Compounds

For the *in vitro* assays, niclosamide and its analogues were dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 10 mM with dilutions in assay buffer. Positive controls (glutamic acid, JNJ16259685, and MPEP) were provided by the testing company.

EXPERIMENTS

An aequorin cell line expressing the human recombinant mGluR1 or mGluR5 receptor was utilized to test *in vitro*

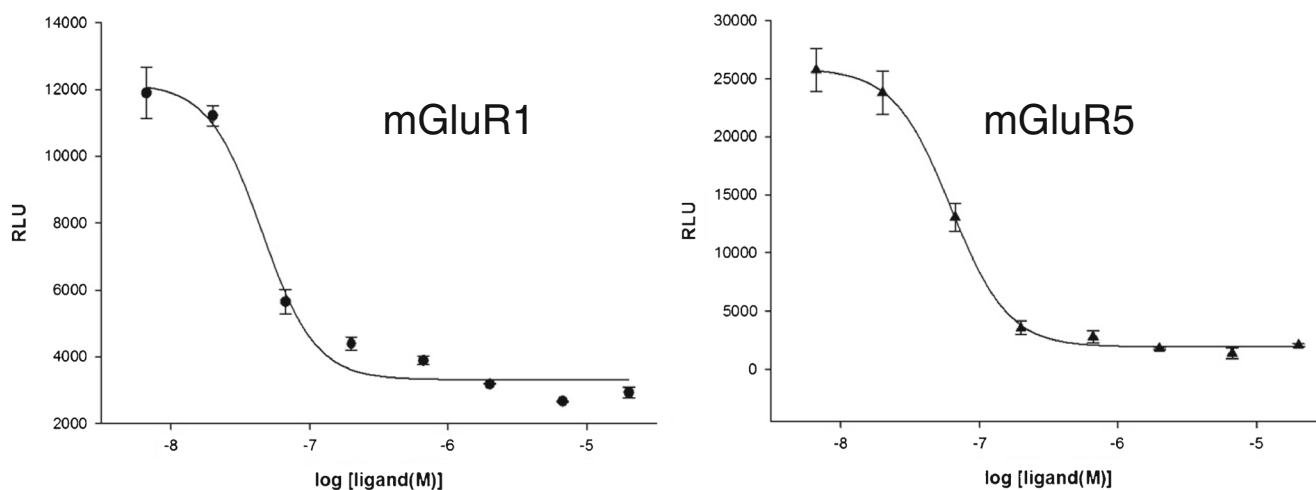


Fig. 2 Dose response curve of niclosamide for group I mGluRs.

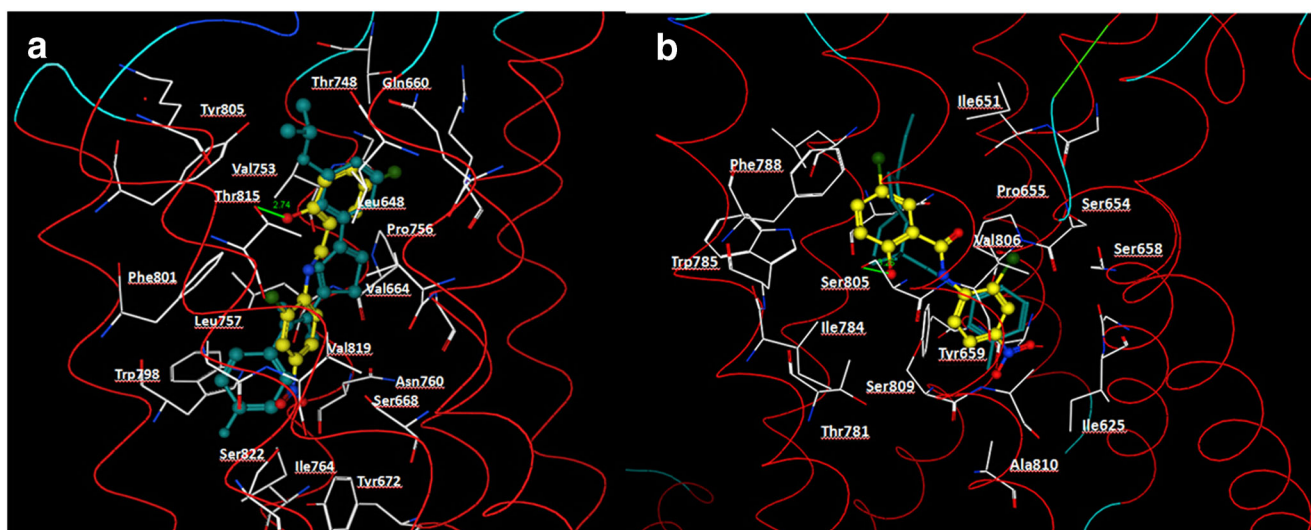
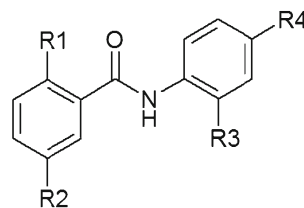


Fig. 3 Detailed interactions of niclosamide with mGluR1 (**a**) and mGluR5 (**b**). The Group I NAM in the crystal structures is colored in olive, and the carbon atoms in niclosamide are colored in yellow.

activities of virtual hit compounds. This functional assay was performed by Euroscreen Inc. (Belgium) and a brief procedure is described as follows. The expression of human mGluR in this cell line was controlled by an inducible promoter through doxycycline. Prior to the test, the mGluR1 or mGluR5 cells were grown in an antibiotic-free media supplemented with doxycycline (600 ng/mL) for 18 h, then detached by gentle flushing with PBS-EDTA (5 mM EDTA). The cells were recovered by centrifugation and re-suspended in assay buffer (HBSS, 2.1 mM CaCl₂, 3 μg/mL glutamate-pyruvate transaminase, 4 mM MEM sodium pyruvate, 0.1% BSA

protease-free). Cells were incubated with the light-emitting coelenterazine-h at room temperature for at least 4 h. Test compound (60 μL) was added into the cell suspension and incubated together for at least 3 min, 30 μL of glutamate solution was then added with a concentration that gives an 80% of maximal response by glutamate saturation (EC₈₀). The resulting emission of light was recorded using a Hamamatsu Functional Drug Screening System 6000 (FDSS 6000). The wells containing 100 μM digitonin or a saturating concentration (20 μM) of adenosine triphosphate were used to standardize the emission of the recorded light

Fig. 4 Structure-activity relationships of niclosamide analogues.



	R1	R2	linker	R3	R4	IC ₅₀ (μM)	
						mGluR1	mGluR5
niclosamide	OH	Cl	amide	Cl	NO ₂	64	38
1	H	Cl	amide	Cl	NO ₂	inactive	inactive
2	OH	H	amide	H	H	inactive	inactive
3	OH	CH ₃	amide	Cl	NO ₂	239	140
4	OH	H	amide	Cl	NO ₂	290	168
5	OH	Cl	amide	Cl	Cl	148	138
6	OH	Cl	amide	Cl	NH ₃	inactive	inactive
7	OH	Cl	amide	H	NO ₂	300	169
8	OH	Cl	reversed	Cl	NO ₂	inactive	inactive
9	(CH ₃) ₂ NC(=O)O	Cl	amide	Cl	NO ₂	inactive	inactive

(determination of the “100% signal”) across plates and across different experiments. Percentages of inhibition were calculated on the basis of the activation induced by the agonist glutamate at its EC_{80} . Each experiment was carried out three times in three replicates. Dose–response data were analyzed with SigmaPlot software using nonlinear regression applied to a sigmoidal dose–response model.

In Vivo NP Animal Model

Animals

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and all protocols were approved by the Product Safety Labs Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (125–150 g weight) were received from Ace Animals (Ace Animals, Inc., Boyertown, PA). The animal room was temperature controlled and had a 12-h light/dark cycle. The animals were fed with Purina Rodent Chow #5012 and acclimated to the facility for 6 days prior to the study. Prior to the experiments, the animals were randomly separated into four groups ($n = 6$) (sham group, vehicle control group, positive control group and dosed group).

Preparation of Compounds and Administration Procedures

Niclosamide was dissolved in the vehicle solution (9% DMSO/27% Cremophor/64% saline) for intrathecal (IT) administration (24) in a 10 μ L injection volume. Then the rat was lightly anesthetized and placed to flex the lower lumbar vertebrae. After palpating the vertebral processes, a 26 gauge needle was inserted through a vertebral interspace. The presence of a tail flick was considered validation of the needle placement; the solution was then injected.

Niclosamide and **9** were dissolved in the vehicle solution (20% DMSO/80% Cremophor) for intraperitoneal (IP) injection in a 5 mL/kg injection volume. The high dose of niclosamide was determined by dose of the drug used in IT route and the low dose was about one-fourth high dose.

Niclosamide and **9** were formulated with 0.5% carboxymethylcellulose for oral dosing in a 10 mL/kg volume. Oral dosage of niclosamide was calculated as three-fold dose used in IP injection, which is approximately one-sixth the reported oral LD₅₀ (2500 mg/kg) of niclosamide in rat and the same concentration was applied to **9** (the carbamate derivative of niclosamide).

Positive control gabapentin was prepared and administered in the same manner as niclosamide in the experiments using different routes of administration. All of the compounds where tested were under blind experimental conditions.

EXPERIMENTS

These experiments were performed by Eurofins Inc. (Dayton NJ, USA). The partial sciatic nerve ligation (25) (PSNL) model of neuropathic pain was used to produce neuropathic hyperalgesia in rats. Partial ligation of the left sciatic nerve is performed on animals under enflurane/O.sub.2 inhalation anesthesia. Following induction of anesthesia, the left thigh of the rat is shaved and the sciatic nerve exposed at high thigh level through a small incision and is carefully cleared of surrounding connective tissues at a site near the trochanter just distal to the point at which the posterior biceps semitendinosus nerve branches off of the common sciatic nerve. A 7-0 silk suture is inserted into the nerve with a 3/8 curved, reversed-cutting mini-needle and tightly ligated so that the dorsal 1/3 to 1/2 of the nerve thickness is held within the ligature. The wound is closed with a single muscle suture (7-0 silk) and a Michelle clip. Following surgery, the wound area is dusted with antibiotic powder. Sham-treated rats undergo an identical surgical procedure except that the sciatic nerve is not manipulated. Following surgery, animals are weighed and placed on a warm pad until they recover from anesthesia. Animals are then returned to their home cages until behavioral testing begins. The animal is assessed for response to noxious mechanical stimuli by determining paw withdrawal threshold (PWT) for the left rear paw of the animal immediately prior to and 1, 3, 6 and 24 h after drug administration. PWT expressed in grams, which is used as an index of mechanical hyperalgesia, was assessed by pressure stimulation method as described by Randall and Selitto. Increasing pressure exerted by Randall Selitto paw pressure device (UGO Basile, SRL Biological Research Apparatus, Italy) is applied to the left hind paw of the rat and withdrawal of left paw or vocalization response was taken as indicator for the nociceptive threshold (see [Supplemental Information for timeline of procedure](#)). To determine oral ED₅₀ value of niclosamide in PSNL model, the drug was administered at increasing doses ranging from 18.8 to 300 mg/kg.

DATA ANALYSIS

All data are presented as mean \pm S.E.M. PWT was normalized as percent of pre-injury baseline value for each animal at different post-injury and post-drug treatment time points then plotted as a function of time. Data analysis was performed using two-way mixed model ANOVA (factors: time/drug treatment, injury group) with Bonferroni post hoc comparison by SigmaPlot 12.3. The AUCs of the antinociceptive effects measured during a 6 h period were calculated by SigmaPlot 12.3 and analyzed using one-way ANOVA with Tukey's honest significant difference (HSD) post hoc test, which is also

used for estimation the oral ED₅₀ of niclosamide by nonlinear regression as described previously (26).

MOLECULAR DOCKING STUDY

Recently the X-ray crystal structures of the seven transmembrane domain of mGluR1 (27) and mGluR5 (28) were reported, both of which contain the allosteric binding site. Structures of mGluR1 (PDB ID: 4OR2) and mGluR5 (PDB ID: 4OO9) were downloaded from the Protein Data Bank (<http://www.pdb.org/pdb/>). The Molecular Operating Environment (MOE) software (Version 2013, Chemical Computing Group, Montreal, Canada) was utilized to prepare and locally energy minimize their X-ray crystal structures for docking studies. The DOCK module of MOE was applied to dock niclosamide into the respective allosteric binding site of each mGluR using default docking parameters in the program.

RESULTS

In Vitro Assays to Evaluate mGluR Subtype Selectivity of Niclosamide

Earlier studies by us have established that both nitazoxanide and its active metabolite (tizoxanide) function as NAMs for Group I mGluRs and show appreciable antinociceptive effects in a NP rodent model (16). The same study also identified the antihelmintic drug niclosamide as a Group I mGluR antagonist at a concentration of 10 μ M *in vitro*. Here we have characterized the pharmacological activity of niclosamide. Due to sequence and geometry similarity shared by the three subgroups of mGluRs, several lines of evidence indicate that many allosteric compounds acting at Group I mGluRs display differential modulating effects on other mGluR subtypes, particularly mGluR4 of the Group III mGluRs (29). Figure 1 shows two representatives of these mGluR4 modulators that share a similar scaffold with niclosamide. Furthermore, Group III mGluR PAMs have been demonstrated to show beneficial effects in related animal models, including NP (30). Therefore, it is possible that niclosamide may function through Group III

mGluRs in addition to Group I mGluRs. To assess whether niclosamide might exert its biological effects through other related mGluRs, we performed an activity profiling study using a cell line expressed with specific human recombinant mGluRs, including mGluR1, mGluR5 or four Group III mGluRs, viz., mGluR4, mGluR6, mGluR7, and mGluR8. For the *in vitro* experiments, three different modes of action were tested for niclosamide at a fixed concentration: agonist (assay buffer only), PAM (cells stimulated by an EC₂₀ concentration of reference agonist) and NAM (cells stimulated by an EC₈₀ concentration of agonist) mode. Dose dependent response of niclosamide against the specific mGluR subtype then would be evaluated if this drug showed activity at a specific mode. At the test concentration (10 μ M), niclosamide was capable of antagonizing about 75% of glutamate calcium mobilization response at a concentration of its EC₈₀ in cells expressing mGluR1 or mGluR5 and ineffective under the other two modes, suggesting it is a NAM for Group I mGluRs. The half maximal inhibitory concentration (IC₅₀) was subsequently determined for this drug to be 38 nM and 62 nM for mGluR1 and mGluR5, respectively (Fig. 2). The selective mGluR1 NAM JNJ16259685 (IC₅₀ = 6.21 nM) and the selective mGluR5 NAM MPEP (IC₅₀ = 28.4 nM) served as positive controls (data not shown). These results compare well with the corresponding reported values of 1.21 nM for JNJ16259685 and 36 nM for MPEP. Our data indicated that negligible *in vitro* activity (<50% activation or inhibition) was induced on the Group III mGluRs by this drug at 10 μ M under all three testing modes (Table I). We conclude that niclosamide does not act through Group III mGluRs at a concentration up to 10 μ M. Taken together, these findings suggest that niclosamide is a selective negative allosteric modulator for Group I over Group III mGluRs.

Computational Modeling to Reveal Structural Determinants of Niclosamide with Group I mGluRs

The *in vitro* cross subtype selectivity assay demonstrated that niclosamide is a dual mGluR1/5 NAM. However, it is not clear how this antihelmintic drug interacts with these two GPCRs to modulate downstream signaling events. Recently published X-ray crystal structures of the transmembrane

Table I Group III mGluR Modulating Activities of Niclosamide at 10 μ M

Testing mode	% of activation or inhibition against Group III mGluRs			
	mGluR4	mGluR6	mGluR7	mGluR8
Agonist	-3.99 \pm 1.71	-0.06 \pm 2.14	32.46 \pm 5.83	-2.08 \pm 0.83
PAM	-13.46 \pm 1.09	-15.34 \pm 5.60	38.77 \pm 4.35	-15.96 \pm 4.34
NAM	21.51 \pm 0.74	19.21 \pm 6.06	-26.40 \pm 8.65	16.77 \pm 3.41

Data are presented as percentage of activity (activation or inhibition) against each mGluR subtype as mean \pm SD for two independent experiments done in triplicate (see Supplementary Information for additional details on biological assays)

domain of both mGluR1 and mGluR5 now make it possible to investigate structural determinants of ligand-receptor interactions of niclosamide at the atomic level of detail. On the basis of structural superimposition of the mGluR1 and mGluR5 crystal structures, the allosteric site is identified for each receptor based on the corresponding co-crystallized NAM, which is formed primarily by transmembrane helices 5, 6, and 7. However, these two sites occupy distinct sites in the TM domain, where the mGluR1 site is more closely positioned to the extracellular domain and the mGluR5 site is more deeply buried inside the TM domain. An overall flat, linear shape is observed for the allosteric binding sites of both Group I mGluRs, which agrees well with the common planar geometry shared by the scaffolds of most Group I NAMs (11). Similarly, the salicylanilide core of niclosamide provides the structural basis for its potent activity against both receptors. Computational docking experiments were then performed on this drug against both mGluR1 and mGluR5. Docking results in mGluR1 revealed that the hydroxyl group in A ring of niclosamide forms a strong (<3.5 Å) hydrogen bond with Thr815, which is the same residue that hydrogen bonds with the co-crystallized mGluR1 NAM FITM (Fig. 3a). The importance of this Threonine residue for ligand binding is further confirmed by other reported mGluR1 NAMs with different scaffolds and by mutagenesis data on the receptor (12). Additionally, extensive hydrophobic contacts with the residues in the mGluR1 TM domain were identified to contribute to the here nanomolar affinity of niclosamide, including Val664, Leu757, Ile764, Phe801, and Val819. The B-ring of the drug was shown to point toward the bottom of the pocket; however, due to the difference in length between niclosamide and FITM, the allosteric binding site of mGluR1 is not fully occupied by niclosamide. Docking of niclosamide in the allosteric binding site of mGluR5 showed near perfect alignment between niclosamide and the co-crystallized ligand mavoglurant (Fig. 3b). In particular, the amide group in niclosamide mimicked the alkyne group in mavoglurant as the linker group, which satisfied the narrow channel shape of mGluR5 allosteric site at this region. Similarly, the interactions of niclosamide with mGluR5 were stabilized by a strong hydrogen bond with Ser805 (O-H \cdots O distance = 3.26 Å) that is known to play a key role in small molecule binding with mGluR5. Interestingly, an intramolecular N-H \cdots O hydrogen bond is found in docked poses of niclosamide in both receptors, which is consistent with a recent report on crystal structure of this drug (31). Taken together, the phenolic hydroxyl group of niclosamide represents an essential structural determinant for interaction with both mGluR1 and mGluR5. The amide linker also serves an important structural role in maintaining the molecular conformation and overall structural rigidity of the drug to satisfy the shape of Group I mGluRs allosteric binding sites.

Medicinal Chemistry Study to Investigate Ligand-Receptor Interactions of Niclosamide

To better understand ligand pharmacology of niclosamide on group I mGluRs, a focused medicinal chemistry effort was carried out to investigate the effects induced by the alteration of substitutions on the rings, especially the phenolic oxygen of the core salicylanilide structure of niclosamide. Structural and activity information of niclosamide analogues were listed in Fig. 4. Removal of the phenolic OH group from the A ring of niclosamide completely abolished activity of compound **1** for both mGluR1 and mGluR5. This finding is consistent with our observations from computational modeling that this OH group played an essential role in determining the binding of niclosamide to both receptors. However, other substituents on the scaffold are also important for binding affinity as suggested by inactive compound **2** in which all other moieties were deleted except the -OH group in A ring. Replacement of the 5-chloro of niclosamide with methyl (**3**) or H (**4**) at A ring moderately decreased activity at both mGluR1 and mGluR5. The effects of modifications of the A ring were more significant on the activities for mGluR1 than for mGluR5, as measured by the greater fold decrease of potency (increase in IC₅₀). The nitro group on the B-ring of niclosamide exerts strong electron withdrawing effects, which contribute significantly to electron delocalization of the conjugated system through the amide linker group. In the docked pose of niclosamide, this electronic substituent is oriented deeply toward the hydrophobic TM in mGluR1 and mGluR5. While replacement of the nitro group at the 4' position by the less electron withdrawing chloro group (**5**) decreased potency for mGluR1 and mGluR5 slightly, replacement by an electron donating amino group (**6**) eliminated mGluR1/5 activity altogether. Likewise, replacement of Cl at the 2'-position with H (**7**) led to a 10-fold and 3-fold loss of activity for mGluR1 and mGluR5, respectively. Alteration of the electron-withdrawing character of the B ring substituents of niclosamide led to a profound effect on its antagonist activity. To assess the significance of the orientation of the amide linker, we synthesized compound **8** in which the amide linker (-C(=O)-NH-O) between the two rings of niclosamide is reversed (-NH-C(=O)-). This compound failed to show antagonist activity in the *in vitro* assays of mGluR1 and mGluR5, which demonstrates that the direction of the amide linker plays a crucial role in receptor recognition of niclosamide.

NP Preclinical Model to Demonstrate *In Vivo* Efficacy of Niclosamide and its Carbamate Derivative

Many years of use of niclosamide attest to its minimal water solubility. This attribute raises little concern for its primary therapeutic indication as an antihelminthic agent in the gut. However, solubility issues are relevant to indications that

require access to the central and peripheral nervous systems. As a proof-of-concept study, niclosamide was administered to partial sciatic nerve (PSN) ligated rats, a reliable preclinical model of NP, to observe its potential to reverse mechanical hyperalgesia through IT route that bypasses the blood–brain barrier (BBB). Gabapentin, an approved standard of care for NP treatment (32) which is known to bind calcium channels and affect release of glutamate in the brain, was selected as the positive control for the current *in vivo* study. Consistent with its inhibitory effects on activation of both mGluR receptors *in vitro*, administration of niclosamide (0.15 μmol , IT) markedly increased the paw withdrawal threshold (PWT) in terms of % of pre-Injury by around 120% after 1 h drug treatment. Niclosamide showed comparable efficacy with gabapentin which was employed as the positive control (% of pre-Injury = 110%, 0.58 μmol , 1 h) (Fig. 5). This effect is still significant at 3 h post-dose but had generally dissipated by 6 h post-dose. These *in vivo* data demonstrated that IT delivery of niclosamide could potentially reduce mechanical hyperalgesia caused by nerve injury, which is consistent with the roles of mGluR1 and mGluR5 in NP.

These encouraging results for niclosamide via IT injection in the NP rat model prompted further *in vivo* evaluations of the antihyperalgesic effects of this drug and its derivative (9) through IP route of administration. Compound 9, a carbamate derivative of niclosamide, lacked mGluR1 and mGluR5 inhibitory activity *in vitro*; however, we reasoned that it would be converted to the biologically active parent niclosamide *in vivo*. The *in vivo* efficacy of the derivative was compared with niclosamide and positive control gabapentin using the PWT model of NP in rat. The activities of these two compounds, administered at low and high doses, to reverse neuropathic mechanical hyperalgesia were evaluated at four different time points (1 h, 3 h, 6 h, 24 h). All of the tested compounds produced a statistically significant reversal of hyperalgesia, at least at one time point (Fig. 6a and b). Dosed at 75 mg/kg, niclosamide produced an antihyperalgesic effect indicated by the PWT to 140% of pre-Injury observed at 1 h after treatment. This effect was still significant at 6 h and abated at 24 h. Niclosamide derivative (9) was effective in the NP animal model with the maximal PWT near 110% of pre-Injury at 3 h. The time delay of the observed analgesic effect for this compound is likely related to cleavage of the pro-moiety to yield bioactive niclosamide *in vivo*. The integrated activities shown by $\text{AUC}_{(0-6\text{h})}$ confirmed that two drugs exhibited potent *in vivo* efficacies at their tested concentrations (Fig. 6c). In summary, the present data indicated that pain in the NP animal is ameliorated by niclosamide and its derivative via IP administration.

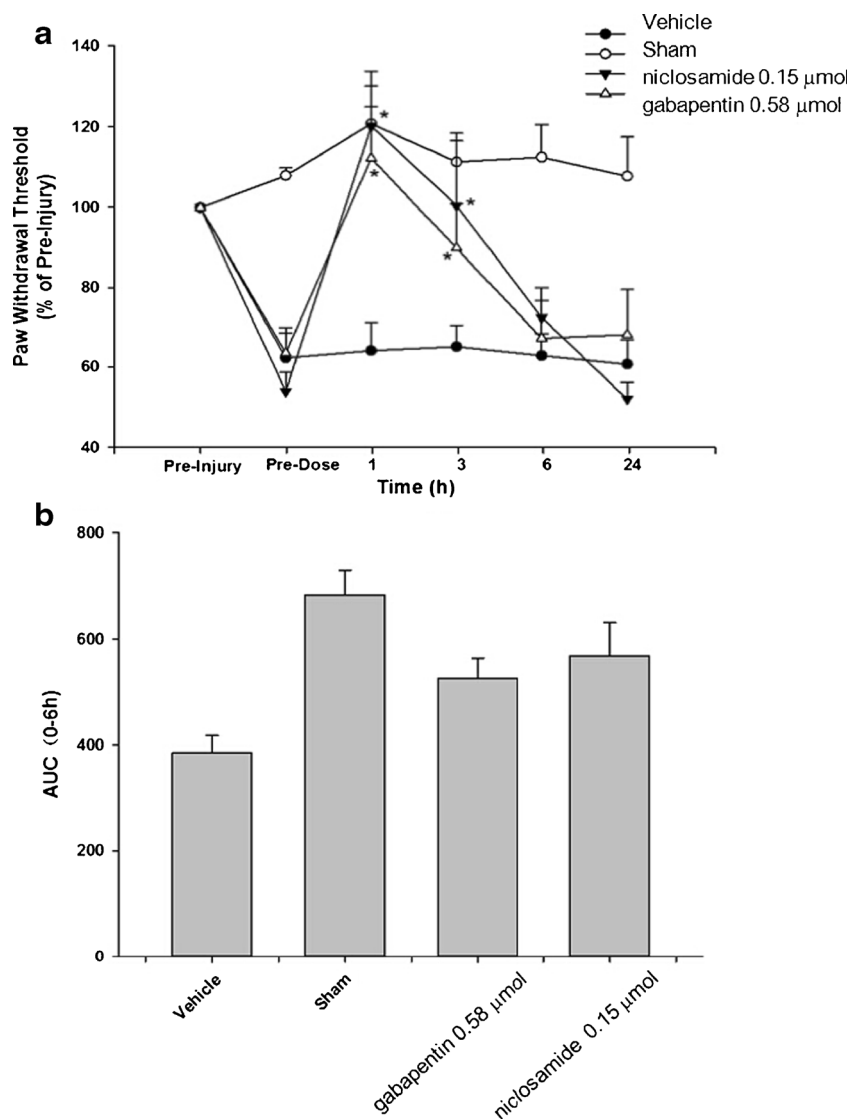
IP injection is an accurate, reliable, and convenient dosing route to test the biological effects of the drugs; however, it is used exclusively on small laboratory animals and is not intended to replicate drug administration in humans. In order

to develop orally active therapeutics for NP, the *in vivo* activities of niclosamide and 9 were then evaluated through oral administration (PO) in the same NP rodent model. Oral gavage of niclosamide reduced sensitivity of mechanical pain in test animals (Fig. 7a). The antihyperalgesic effects in rats treated with niclosamide at 300 mg/kg reached statistical significance at 1 h post-dose. The maximal effect was reached at 1 h post-dose with gradually diminished effects at 3 h and 6 h post-dose. Overall, the results suggest that niclosamide showed substantial *in vivo* efficacy at 300 mg/kg PO. For niclosamide derivative 9, significant antihyperalgesia effects at 300 mg/kg dose were lacking when compared with the vehicle-treated group as indicated by $\text{AUC}_{(0-6\text{h})}$ (Fig. 7b). We also demonstrated that niclosamide dose-dependently and fully relieved PSNL-induced mechanical hyperalgesia following PO administration, with an estimated ED_{50} value of 54 mg/kg (Fig. 7c).

DISCUSSION

Niclosamide is an FDA approved antihelminthic drug to treat tapeworm infection in humans and other animals by uncoupling oxidative phosphorylation in the parasites. Recently, this drug demonstrated beneficial effects against obesity-related type 2 diabetes through the same mechanism in the mitochondria of the mouse liver (22). In addition, other inhibitory mechanisms of action, such as the STAT3 signaling pathway (33) and the Wnt/ β -catenin pathway (34), are also reported for niclosamide to account for its pharmacological activities in cancer cell lines and xenograft models. However, there are no reports of the effect of niclosamide on GPCRs, the group of drug targets mediating a number of important signal transduction and cellular processes. Glutamate plays a key role in pathophysiological conditions in the CNS such as pain and its receptors on synapses. The mGluRs are a group of GPCRs that are widely distributed along the pain neuraxis and modulate cellular mechanisms of nociceptive sensitization. As a consequence, they are considered as attractive drug targets to depress pain transmission. Noncompetitive Group I mGluR antagonists have been shown with great therapeutic potential in several NP animal models. Our preliminary screening effort indicates that niclosamide shows promising inhibitory activities on Group I mGluRs in cell-based assays. Here we further characterized niclosamide *in vitro* and *in vivo*. A major finding from the present study is that niclosamide is a negative allosteric modulator of Group I mGluRs and displays selectivity to Group I over the analogous Group III mGluRs. It is noteworthy, due to the limitation of experimental testing conditions considered herein, the activity of niclosamide on mGluR7 (~30% activation in Table I) requires further attention. Although it is inactive according to our current threshold, secondary assays would be required to confirm the activation of

Fig. 5 (a) *In vivo* efficacy data for niclosamide against neuropathic mechanical hyperalgesia through IT route. (b) The integrated effects of tested drugs that were calculated as AUC for 0 to 6 h post-treatment. * $P < 0.05$ compared with vehicle, data are presented as mean \pm SEM for six animals.



mGluR7 by niclosamide, which would provide in-depth understanding of anti-NP effects of this drug. A limited medicinal chemistry study was carried out to probe structural determinants of niclosamide to group I mGluRs and confirm specific ligand-protein interactions from computational docking of the drug to crystal structures of two receptors. Three essential chemical features are identified for NAM activity of niclosamide, viz., the hydroxy group in the A ring, the strong electron-withdrawing nitro group in the B ring, and the amide linker. Additionally, the nearly flat geometry of this drug imposed by the extended conjugation between the two aromatic rings and amide linker is complementary to the shape of allosteric binding site of Group I mGluRs. Tizoxanide (the bioactive metabolite of prodrug nitazoxanide) which emerged from our previous screening effort (16), also satisfies the same structural characteristics and exhibits weak to moderate micromolar activity on mGluR1/mGluR5. Moreover, CPPHA, a PAM for

Group I mGluRs and Group III mGluR4, also belongs to the salicylanilides with a rather bulky substituent at 3' position of the B ring. These findings suggest that the salicylanilide core is a favorable scaffold recognized by allosteric binding sites of mGluRs. Further SAR studies are required with a larger and more structurally diverse array of salicylanilide analogues and derivatives to more fully discern SAR patterns that will guide structural optimization.

Currently available therapeutic drugs for NP are associated with limited efficacy and a high incidence of side effects (35); therefore, there is an urgent need to develop an alternative medicine which can attenuate neuropathic pain effectively. Results from *in vitro* studies demonstrated that niclosamide allosterically inhibited calcium mobilization induced by endogenous ligand in mGluR1/5. However, the poor solubility of niclosamide may limit its therapeutic potential in the clinic. A carbamate derivative was synthesized to overcome this

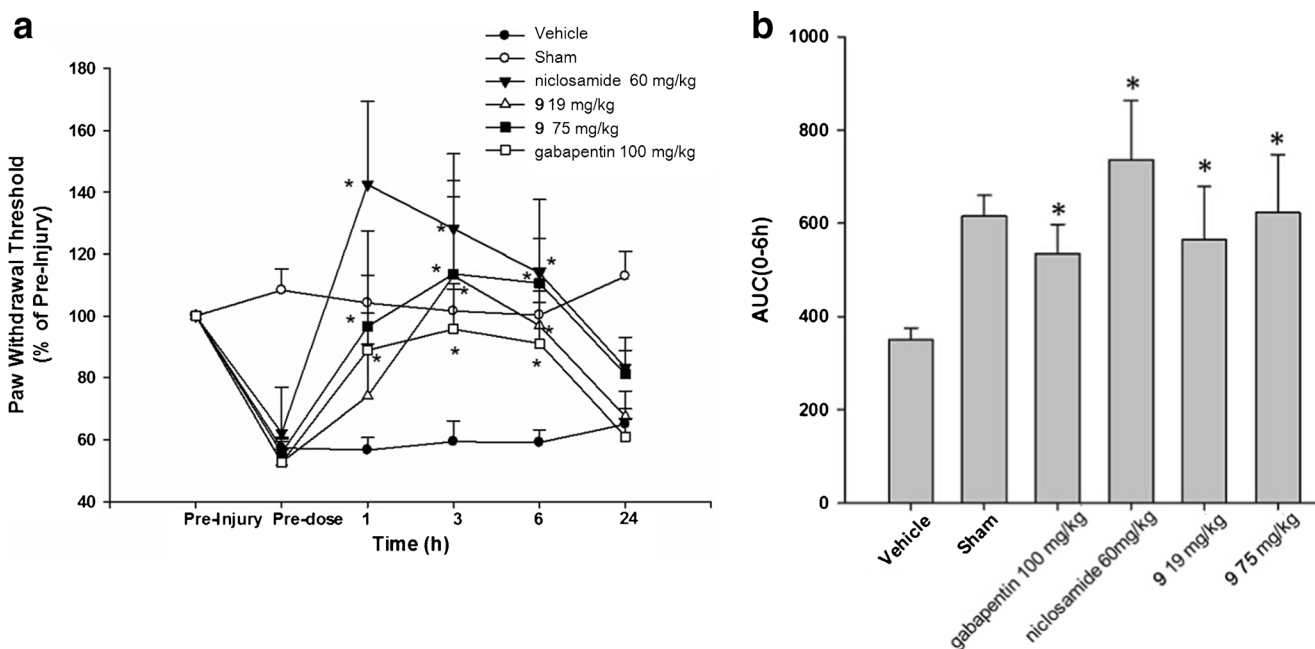


Fig. 6 (a) *In vivo* efficacy data for niclosamide and its derivative **9** against neuropathic mechanical hyperalgesia through IP route. (b) The integrated effects of the two compounds were calculated as AUC for 0 to 6 h post-treatment. * $P < 0.05$ compared with vehicle, data are presented as mean \pm SEM for six animals.

issue. This derivative was inactive against both receptors *in vitro*. In order to evaluate the capacity of niclosamide to reverse neuropathic hyperalgesia, it was tested in a preclinical model of mechanical NP using PSN ligation on the rear limb of rats. Three different routes of drug administration (IT, IP, and PO) were tested to assess *in vivo* efficacies of niclosamide and its carbamate derivative. Niclosamide was found to be substantially active in blocking the mechanical hyperalgesia associated with nerve injury through all three dosing routes. The *in vivo* activity of the carbamate prodrug of niclosamide (**9**) was influenced by drug dosing forms which affect its absorption and, consequently, the systemic concentration of bioactive niclosamide. Compound **9** produced significant anti-hyperalgesic effects *in vivo* when administered IP but only minimally when administered PO. A plausible explanation for the ineffectiveness of **9** when administered via PO is poor oral bioavailability through the GI tract with the current formulation, although this notion would not explain the modest effectiveness of **9** for inflammatory pain (Fig. 1s). The carbamate pro-moiety of **9** can be released by hydrolytic reaction to yield bioactive niclosamide *in vivo*, as demonstrated by effective attenuation of NP in animal models with **9** through IP injection. Differences in bioavailability between the PO and IP routes more likely stem from poor oral solubility in the digestive tract, poor membrane permeability, or a combination of both phenomena that would impair systemic exposure of the drug that is needed to produce significant *in vivo* effects. Formulation is known to be a possible approach to overcome the issue of

bioavailability for certain drugs. We tested niclosamide carbamate derivative with a different vehicle formulation in another preclinical pain model, the peripheral inflammatory pain model induced by injection of complete Freund's adjuvant (CFA) on the hind paw of rats, in which some NAMs of group I mGluRs are shown to attenuate the enhanced nociception and noxious stimulus-induced glutamate release in rats with inflammation pain *in vivo* (36). Our preliminary results indicated that the mechanical hyperalgesia in animals with inflammatory pain was significantly ameliorated by the prodrug of niclosamide (**9**) with new formulation (Supplemental Information). Bioanalytical experiments need to be conducted to compare the pharmacokinetic profiles of niclosamide with different formulations. The findings from our two pain-related preclinical models not only provide further evidence for *in vivo* efficacy of niclosamide through modulating aberrant glutamate transduction by antagonizing Group I mGluRs, but also suggest that proper formulation is required to fully realize the therapeutic potential of this poorly soluble drug.

Since niclosamide is a dual mGluR1/mGluR5 NAM, it would be interesting to compare its *in vivo* efficacy with other selective NAMs, such as MPEP. A substantial body of structural and neurophysiological evidence and *in vivo* studies using animal models suggests that the effects of mGluR1 and mGluR5 modulation would be similar and perhaps complementary (11,37). For example, a recent study that compared the effects of mGluR1-specific NAM (JNJ1656708) and mGluR5-specific NAMs (MTEP and MPEP) in two

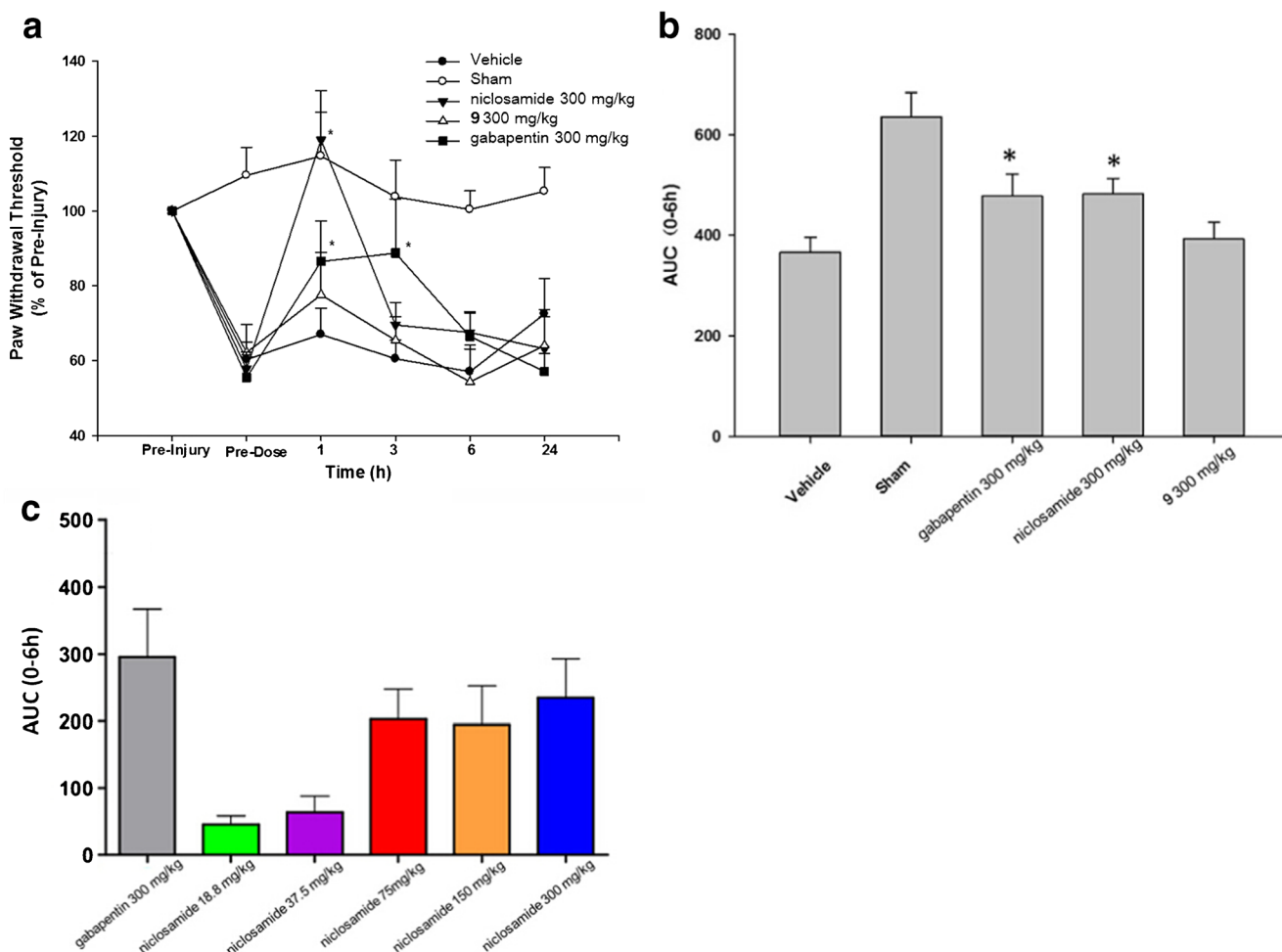


Fig. 7 (a) *In vivo* efficacy data for nicosamide and **9** against neuropathic mechanical hyperalgesia through PO route. (b) The integrated effects of the two compounds were calculated as AUC for 0 to 6 h post-treatment. (c) The AUC values of nicosamide at five different concentrations. * $P < 0.05$ compared with vehicle, data are presented as mean \pm SEM for six animals.

behavioral screening experiments showed very similar activity profiles among these agents (38). In addition, the recent study by Caraci *et al.* (39) demonstrates dual mGluR2/3 receptor agonist LY379268 is neuroprotective against synthetic β -amyloid protein (A β) induced toxicity; however, selective potentiation of the mGluR2 receptor enhances neuronal vulnerability to A β . Thus nicosamide found in the present study would be a valuable pharmacological tool as a dual mGluR1 and mGluR5 NAM with low nanomolar activity to examine specific or complementary role of these two receptors in NP and other related neurological diseases.

The toxicity profiles of nicosamide are well known, and the therapeutic dosage of nicosamide is 2000 mg/day in adults and 1000–1500 mg/day in children when given orally. However, a complete toxicological profile of nicosamide and **9** remains to be determined, especially for those related to neurological and psychiatric adverse effects, due to the important role of Group I mGluRs played in glutamatergic transmission.

CONCLUSION

Taken together, the present study reveals that nicosamide is a potent Group I mGluR NAM, which may serve as a promising starting point for subsequent development of effective therapeutics for NP. To our best knowledge, this report together with a concomitant publication by the authors (16) represents the first instance of nicosamide and its structurally related analogues and derivatives as potential treatments for pain.

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Contributed new reagents or analytic tools: R.D. Wood and W.J. Welsh.

Performed data analysis: N. Ai, R.D. Wood, E. Yang, and W.J. Welsh.

Wrote or contributed to the writing of the manuscript: N. Ai and W.J. Welsh.

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