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RESEARCH PAPER

Identification of Nitazoxanide as a Group I Metabotropic Glutamate Receptor Negative Modulator for the Treatment of Neuropathic Pain: An *In Silico* Drug Repositioning Study

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ABSTRACT

Purpose Drug repositioning strategies were employed to explore new therapeutic indications for existing drugs that may exhibit dual negative mGluR1/5 modulating activities as potential treatments for neuropathic pain.

Method A customized *in silico-in vitro-in vivo* drug repositioning scheme was assembled and implemented to search available drug libraries for compounds with dual mGluR1/5 antagonistic activities, that were then evaluated using *in vitro* functional assays and, for validated hits, in an established animal model for neuropathic pain.

Results Tizoxanide, the primary active metabolite of the FDA approved drug nitazoxanide, fit *in silico* pharmacophore models constructed for both mGluR1 and mGluR5. Subsequent calcium (Ca++) mobilization functional assays confirmed that tizoxanide exhibited appreciable antagonist activity for both mGluR1 and mGluR5 (IC_{50} = 1.8 μM and 1.2 μM, respectively). The *in vivo* efficacy of nitazoxanide administered by intraperitoneal injection was demonstrated in a rat model for neuropathic pain.

Conclusion The major aim of the present study was to demonstrate the utility of an *in silico-in vitro-in vivo* drug repositioning protocol to facilitate the repurposing of approved drugs for new therapeutic indications. As an example, this particular investigation successfully identified nitazoxanide and its metabolite tizoxanide as dual mGluR1/5 negative modulators. A key finding is the vital importance for drug screening libraries to include the structures of drug active metabolites, such as those emanating from prodrugs which are estimated to represent 5–7% of marketed drugs.

KEY WORDS group I metabotropic glutamate receptor (mGluR) · *in silico* drug repositioning · mGluR1 · mGluR5 · nitazoxanide

ABBREVIATIONS

CCDL	Comprehensive clinical drug library
CNS	Central nervous system
GPCR	G-protein coupled receptor
HTS	High-throughput screening
i.p.	Intraperitoneal
mGluR	Metabotropic glutamate receptor
MOE	Molecular Operating Environment
VCDL	Virtual clinical drug library
VS	Virtual screening

INTRODUCTION

Despite enormous technological improvements and continual escalation in spending (www.phrma.org/research/publications/profiles-reports), the productivity of pharmaceutical research and development has remained largely stagnant since the 1990s on the basis of FDA approvals of new chemical or biological entities (1). Drug "repositioning" or repurposing (2), *i.e.*, identifying new indications for existing drugs, is one approach that has gained momentum to address this issue for several diseases, such as Alzheimer's disease (3,4), infectious diseases (5), and

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pain (6). The underlying premise is that well studied pharmacokinetic and toxicity profiles of existing drugs would likely facilitate their entrance into clinical trials for new therapeutic indications and bypass a large fraction of the developmental costs. For the large majority of successful drug repositioning successes thus far, serendipity has played an important role. While good fortune has been rightly credited in the discovery of countless drugs, it is no substitute for rational approaches that actively pursue opportunities for drug repositioning in a broad range of human diseases. Recently several systematic high-throughput screening approaches (7–9) were employed to uncover previously unknown biological activities of existing drugs from in-house chemical libraries, which usually only comprise a fraction of approved drug and clinical candidates. Arguably, broad screening against a comprehensive clinical drug library (CCDL) would reap greater success in finding novel pharmacological functions of existing drugs. However, the CCDL does not exist. The NIH Chemical Genomics Center (NCGC) recently has released the NCGC Pharmaceutical Collection (NPC) to promote drug repositioning and chemical genomics (10). The NPC represents one example of a comprehensive resource of clinically approved drugs around the world, including the US, Canada, UK, EU, and Japan. In spite of its broad coverage on approved drugs, the NPC does not include experimental drugs and clinical candidates. Prohibitive costs and difficulty in acquisition are two major hurdles to assembly a CCDL that truly fulfilled its 'comprehensive' epithet for experimental assessment by the general academic community. With recent advances in information technology and the worldwide network, the instant accessibility of structural and biological information about known drugs and clinical compounds in the public domain (11,12) has facilitated the notion to assemble a virtual clinical drug library (VCDL). For a given therapeutic target, typically a receptor or enzyme, state-of-the-art virtual screening (VS) techniques provide immensely powerful tools to evaluate ligand-protein interactions computationally, thus identifying potential active compounds prior to experiments. Therefore VS is an effective approach to explore new unrelated therapeutic indications for old drugs, *e.g.*, drug repositioning, and/or to extract druggable scaffolds for rapid *in silico* lead hopping, *e.g.*, drug discovery. The entire process of VS would only require the atomic coordinates of existing drugs without physical possession of them. Upon sample acquisition and experimental testing on these virtually identified "hits" to confirm their potential value as therapeutics for new indications, these known drugs can be fast-tracked toward clinical development and commercialization. Therefore, the major aim of the present study was to demonstrate the utility of an *in silico-in vitro-in vivo* drug repositioning protocol to facilitate the repurposing of approved drugs for new therapeutic indications. As an example, herein we describe the identification of nitazoxanide and its metabolite tizoxanide as negative modulators of the Group 1 metabotropic glutamate receptors 1 and 5 (mGluR1/5).

As the most abundant excitatory neurotransmitter in the central nervous system (CNS), glutamate activates metabotropic glutamate receptors (mGluRs) to modulate the activities of many types of synapses (13). There is mounting evidence to support the role of mGluRs in a number of CNS disorders (14) including pain (15,16), anxiety (17), and depression (18), and other neurological impairments (19) such as drug addiction (20) and mental retardation (21). Based on sequence homology, second messenger signaling, and pharmacological functions, mGluRs are divided into three subgroups (22). Group I mGluRs, comprising mGluR1 and mGluR5, have received a significant amount of attention as drug targets. Group I mGluR negative modulators have been found to elicit analgesic effects in diverse models of neuropathic pain (NP), that remains a troublesome illness for which a truly safe and efficacious therapeutic treatment is still lacking. The development of potent mGluR negative modulators as potential therapeutic agents for neuropathic pain has therefore been the focus of intense research activity in many research laboratories and pharmaceutical companies (23–27). In addition to standard drug discovery protocols, drug repositioning presents a promising strategy to meet the need for novel NP therapeutics by targeting group I mGluRs.

The molecular shape of a drug plays a central role in target recognition and, specificity, in its receptor binding to exert a therapeutic benefit. A shape-based VS method, *Shape Signatures*, has recently been introduced to identify lead compounds in drug discovery (28–32). By avoiding description of complex structural queries or molecular alignments, *Shape Signatures* is convenient to use, extremely fast, and amenable to large numbers and types of molecular entities (*e.g.*, fragments/intact molecules, organic/organometallics, neutral/charged species). The method employs a customized ray-tracing algorithm that converts the three-dimensional (3D) shape of a molecule into compact histograms that lend themselves to shape comparison between molecules using trivial arithmetic operations. These histograms can be expanded to incorporate other biorelevant properties such as molecular electrostatic surface potential. Starting with a query compound such as a known drug, *Shape Signatures* rapidly compares its shape signature with those precomputed signatures of database compounds and then displays a ranked list of top scoring compounds retrieved from the database. When employed in concert with other computational and experimental tools, *Shape Signatures* has successfully identified high value bioactive molecules drawn from large databases (33,34).

Pharmacophore mapping matches the geometric arrangement of key structural features of queries associated with tight receptor binding (35). The method has demonstrated its utility as a useful tool, particularly in cases for which there is a

paucity of 3D structural information such as G-protein coupled receptors (GPCRs). Here we describe the construction of an *in silico* drug repositioning scheme, which comprises *Shape Signatures* and a ligand-based pharmacophore model using known allosteric negative modulators of mGluR1 and mGluR5. We then utilize this scheme to search the VCDL for repositioning known drugs as dual mGluR1/5 negative modulators. This process yielded nitazoxanide (Alinia®), an FDA approved drug for the treatment of diarrhea caused by *Cryptosporidium parvum*. We demonstrate that nitazoxanide and its active metabolite tizoxanide are dual mGluR1/5 negative modulators with potent activities *in vitro* and significant *in vivo* efficacy in a rodent model of neuropathic pain.

MATERIAL AND METHODS

Construction of VCDL

Structural information on approved drugs in the VCDL was retrieved from three separate sources: (1) a collection of 1040 drugs that have reached clinical trial stages in the USA compiled by Microsource Discovery Systems, Inc. (Gaylordsville, CT, USA); (2) The DrugBank database (Edmonton, AB, Canada) that includes >1350 FDA-approved small molecule drugs, 71 nutraceuticals, and >3243 experimental drugs (http://www.drugbank.ca/downloads); and (3) the Prestwick chemical library (University of Bologna, Italy) that includes 1117 off-patent drugs selected for structural diversity, collective coverage of multiple therapeutic areas, and known safety and bioavailability in humans. These three collections were cross-checked to remove redundant entries in the VCDL.

Shape Signatures Screening

The *Shape Signatures* tool, developed by Zauhar and Welsh (36), encodes three-dimensional shape-based information of molecules as simple histograms which are probability distributions derived from a ray-trace of the volume enclosed by the solvent accessible surface of the molecule. These signatures lend themselves to rapid comparison between molecules using simple mathematical operations. The three-dimensional structures of the drugs in the VCDL had been pre-processed into shape signatures histograms which are rotationally-invariant descriptors that facilitate rapid comparison between a query molecule and each entry in the VCDL. Histograms of the query molecule and database drugs were compared using the 'chi square' (χ^2) metric. The deviation between the histograms provided a dissimilarity score between the two molecules being compared. The top 100 hits for each query based on 1D score (shape only) were kept for secondary tier screening.

Pharmacophore of mGluR1 and mGluR5

The Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada) was utilized to build and model a collection of known Group I mGluR antagonists from which separate pharmacophore models were generated for mGluR1 and mGluR5 (Fig. 2). The procedure involves three steps: (1) the *flexible alignment* module in MOE was implemented to align the ligands where all-atom flexibility was enabled for conformational searching. Molecular alignment was monitored by the average strain energy (U, in kcal/mol) of the molecules in a particular alignment, the total mutual similarity score of the configuration (F), and the alignment score of the configuration (S) where lower values of S indicate better alignments; (2) pharmacophore sites are created based on the selected molecular alignment by the PCH (polarity-charge-hydrophobicity) pharmacophore scheme in MOE; and (3) common pharmacophores are determined by distance-based inter-site grouping. The resulting pharmacophore models were employed to screen outputs from the *Shape Signatures* screening.

Chemical Similarity Comparison

Hits from the previous two-tier screening were compared for chemical similarity, which was performed in MOE using 2D MACCS Structural Keys to calculate their similarity from the Tanimoto Coefficient.

Chemicals and Reagents

Nitazoxanide and tizoxanide were purchased from Cayman Chemicals (Ann Arbor, MI, USA) and dissolved in DMSO for *in vitro* testing. The agonist (glutamate) employed in the *in vitro* assay was provided by the assay testing company. For the *in vivo* experiments, solid drug samples were dissolved in a mixture of Cremophor and DMSO (*v*/*v*, 4:1) to ensure solubility. Gabapentin, the positive control in the *in vivo* assays, was purchased from Sigma-Aldrich (St. Louis, MO, USA).

In Vitro mGluR1/5 Functional Assay

The *in vitro* activities of hit compounds were tested through a functional assay that utilized an aequorin cell line expressing human recombinant mGluR1 or mGluR5 receptor (Euroscreen, Belgium). Briefly, cell lines that express human receptors were under the control of a promoter induced by doxycycline. Prior to the testing, the mGluR1 or mGluR5 cells were grown for 18 h in the media without antibiotic and supplemented with doxycycline (600 ng/mL). Cells in mid-log phase were detached by gentle flushing with PBS-EDTA (5 mM EDTA). The cells were recovered by centrifugation and resuspended in assay buffer (HBSS, 2.1 mM

$CaCl_2$, 3 μg/mL glutamate-pyruvate transaminase, 4 mM MEM sodium pyruvate, 0.1% BSA protease-free). Coelenterazine-h (Molecular Probes, OR, USA) was incubated with cells at room temperature for at least 4 h. Efflux of calcium ions was measured by detection of light emitted by the luminescent protein aequorin triggered by binding of calcium in the presence of control agonist glutamate. The resulting emission of light was recorded using a Hamamatsu Functional Drug Screening System 6000 (FDSS 6000). The initial screening concentration for test compounds is 10 μM. For mGluR1 and mGluR5 negative modulator testing, 60 μL of the resulting cell suspension containing with test compounds was incubated with 30 μL of glutamate at its EC_{80}, which is the concentration sufficient for a response 80% of the maximal response given by saturating glutamate. We also evaluated the possibility of computational hits identified through screening as mGluR1 or mGluR5 positive modulators. For positive modulator testing, the cell suspension (30 μL) was incubated with test compound or 30 μL of glutamate solution at EC_{20} for at least 3 min then intensity of luminescent light was measured as mentioned before. To standardize the emission of the recorded light (determination of the "100% signal") across plates and across different experiments, some wells contained 100 μM digitonin or a saturating concentration (20 μM) of adenosine triphosphate. For compounds with more than 50% activity at initial screening, dose response curves were determined. For negative modulators, percentages of inhibition were calculated on the basis of the activation induced by the agonist glutamate at its EC_{80} in the assay and represented as mean values for two experiments of three replicates. Dose–response data were analyzed by XLFit (IDBS, London, UK) software using nonlinear regression applied to a sigmoidal dose–response model.

In Vivo Neuropathic Pain Model

The experiments were carried out by Eurofins, USA (Dayton, NJ, USA). Male Sprague–Dawley rats (125–150 g) were received from Ace Animals (Berks, PA, USA). Animals were housed in cages which conform to the size recommendations in the most recent NIH Guide for the Care and Use of Laboratory Animals. The animal room was temperature controlled and had a 12-h light/dark cycle. The animals were acclimated to the facility for 6 days prior to the study. Briefly, the skin and muscles of the thigh of anesthetized rats were reflected to expose the sciatic nerve. About one-third to one-half of the cross-sectional area of the nerve was tightly ligated using a 7–0 nylon suture. The incision was closed in layers (suture for muscle, surgical adhesive for skin). In a sham control group, the surgical procedure was identical to that described above, except that the nerve was not injured. After 3-week recovery, the animals were dosed with nitazoxanide at 12.5 and 50 mg/kg or 100 mg/kg gabapentin *via* the intraperitoneal (i.p.) route. Treated rats were assayed at 1, 3, 5 and 24 h post-dosing for the paw withdrawal thresholds (PWT). The PWT was normalized as percent of pre-injury baseline value for each animal at different post-dosing time points, and data were analyzed by two-way analysis of variance (repeated measures) with Bonferroni tests at individual time points. Vehicle-treated, ligated groups were used as the statistical comparison groups. Data are presented as mean ± SEM from six separate experiments. $P<0.05$ was considered statistically significant.

RESULTS

In Silico Drug Repositioning Protocol

The entire screening process is summarized in Fig. 1. Our current VCDL is composed from three major sources: Microsource USdrug Collection, DrugBank, and Prestwick chemical library. Taken together, the VCDL contained more than one thousand FDA approved drugs and over three thousand experimental drugs. Two tiers of screening were performed to identify Group I mGluR negative modulators from VCDL. First, *Shape Signatures* was employed to assess the molecular similarity between query and library compound based on their three dimensional shape and surface charge distribution. Then, pharmacophore mapping was utilized to select hits that matched the mGluR1 and/or mGluR5 pharmacophore. Given the absence of three-dimensional structural information for mGluR1 and mGluR5, ligand-receptor docking was omitted in the present example. Consequently, two tiers of screening were performed to identify Group I mGluR negative modulators from VCDL.

Six reported Group I mGluR negative modulators (Fig. 2) were used as queries for *Shape Signatures* virtual screening of the VCDL and as a training set for construction of the pharmacophore models. *Shape Signatures* scored the test compounds in the VCDL on a scale ranging from 0.0 (near identity) to 2.0 (little or no similarity). The top-scoring 100 hits (cutoff score <0.1) were selected for each query, thereby yielding a total of 700 compounds for the seven queries.

The mGluR1 antagonist pharmacophore comprised three essential structural features (aromatic center, hydrogen bond acceptor, and hydrophobic center), which defined the critical physiochemical properties on the scaffold of mGluR1 antagonists (Fig. 3a). The constraints imposed on total volume and distance between features improved the quality of the hits emerging from the pharmacophore models. The mGluR5 antagonist pharmacophore (Fig. 3b) consisted of three features, *i.e.*, one hydrogen bond acceptor and two aromatic centers, together with the three constraints on distance between pairs of features. The pharmacophore models of mGluR1 and mGluR5, depicted in Fig. 3, were employed to search the hits

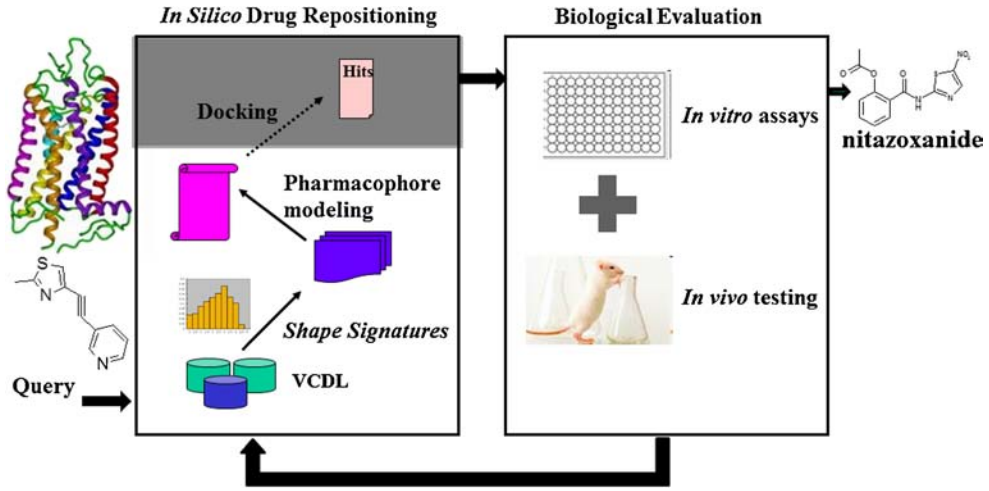


Fig. 1 Detailed illustration of our *in silico* drug repositioning scheme which comprises three tiers of screening. The process starts with the three-dimensional atomic coordinates for small molecule queries. *Shape Signatures* is employed for pre-screening the VCDL by shape comparison with query compounds. Outputs from this first tier screening are sent to the second tier screening that matches the important pharmacophoric features of target binding. If available for the target protein, high resolution structural information can be used in the third tier of this scheme for *in silico* docking and scoring studies to further refine the hit list for biological evaluation. Due to the lack of X-ray crystallographic structures for the Group I mGluR proteins, this third step was omitted in current study (shaded in *gray color*). The chemical structure of a known mGluR5 antagonist MTEP is shown as the query compound. Nitazoxanide is a confirmed hit from this study and its structure is shown also.

retrieved from the VCDL by *Shape Signatures*. As the focus of the present study was on dual mGluR1/5 antagonists, hits were required to satisfy both pharmacophores.

Computational Screening

Among the hits emerging from the two-tier screening, two FDA approved drugs, niclosamide and nitazoxanide, both feature the salicylanilide core that caught our attention (Fig. 4). A separate medicinal chemistry study was performed to investigate structure-activity relationships of various substitutions on this scaffold and their effects on *in vitro* and *in vivo* activities (manuscript under preparation). Nitazoxanide and niclosamide (Fig. 4) both possess the same amide linker between two aromatic rings and a strong electron withdrawing nitro group. However the hydroxyl group of niclosamide is replaced by the acetate group in nitazoxanide. A Tanimoto score of 0.8 based on 2D MACCS structural keys confirmed the high degree of structural similarity between these two compounds. The two-tier process consisting of *Shape Signatures* followed by pharmacophore modeling ensured that hits clearly resembled the query compounds in terms of overall 3D shape, surface charge distribution, and geometric arrangement of key pharmacophoric features. As shown in Fig. 3, the pharmacophore models of both mGluR1 and mGluR5 negative modulators feature a hydrogen bond acceptor which is represented the O atom of the hydroxyl group in the A ring. Modification at this position using medicinal chemistry has demonstrated that the hydrogen bonding capacity of this OH group is an important pharmacophoric feature for receptor binding (manuscript under preparation). Nitazoxanide is a prodrug which converts to its active metabolite tizoxanide *in vivo* by enzymatic hydrolysis of the acetate promoiety to yield a free hydroxyl group in the A ring (37). It is apparent that tizoxanide fits the pharmacophore models for both mGluR1 and mGluR5 activity.

In vitro and *In vivo* Testing

Based on the computational screening results, the biological activity of tizoxanide as a Group I mGluR modulator was tested in a calcium (Ca++) mobilization functional assay. Tizoxanide displayed greater than 50% inhibition of both mGluR1 and mGluR5 receptor activation at 10 μM in the cell-based aequorin assay and was selected to undergo further evaluation. With cells stimulated by an EC_{80} concentration of agonist glutamate, the half-maximal inhibitory concentration (IC_{50}) of tizoxanide was then determined to be 1.8 μM for mGluR1 and 1.2 μM for mGluR5 (Fig. 5). We also tested *in vitro* activities of nitazoxanide against mGluR1 and mGluR5. Since nitazoxanide lacks the essential phenolic hydroxyl group of tizoxanide, it was expected to exhibit little or no activity for mGluR1 or mGluR5. Surprisingly, nitazoxanide did exhibit inhibitory activity for mGluR1 and mGluR5 with IC_{50} values of 6.3 μM and 2.8 μM, respectively (Supplementary Material Figure s1). One possible explanation for this observation is that nitazoxanide is at least partially converted to tizoxanide during the *in vitro* assay. This interpretation was supported by a simple LC-MS experiment on nitazoxanide under assay conditions, which detected the existence of tizoxanide in the presence of the assay buffer alone (data not shown).

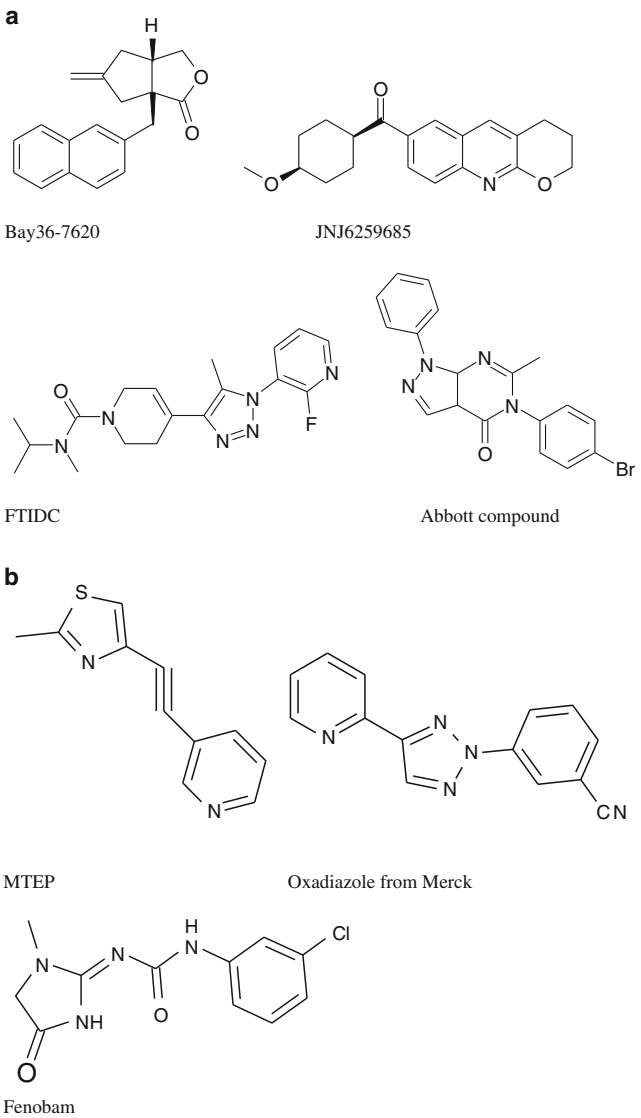


Fig. 2 Chemical structures of known Group I mGluR antagonists. (**a**) mGluR1 negative allosteric modulators. (**b**) mGluR5 negative allosteric modulators.

To determine whether tizoxanide has *in vivo* Group 1 mGluR modulating activity, we tested it in a partial sciatic nerve ligation assay in rat, which is a widely accepted animal model for neuropathic pain. Since nitazoxanide is rapidly converted to its active metabolite tizoxanide *in vivo*, nitazoxanide was administered in the animal study for *in vivo* efficacy. The animals were dosed by intraperitoneal (i.p.) injection with nitazoxanide at 12.5 and 50 mg/kg or with gabapentin at 100 mg/kg (standard dose). Treated rats were assayed at 1, 3, 5, and 24 h using the paw withdrawal threshold (PWT) method (shown in Fig. 6). Systemic administration of gabapentin, a common therapeutic treatment of choice for neuropathic pain, significantly increased PWT to around 85% pre-injury level at 1 h after drug treatment. The ability to reduce mechanical pain hypersensitivity gradually subsided at 3 and 5 h and totally abated at 24 h. For rats treated with nitazoxanide at 50 mg/kg, an antihyperalgesic effect was produced by the drug, which was indicated by the PWT reversal to around 85% percent of pre-injury level observed at 1 h after treatment. This effect was maximized at 3 h and still significant at 5 h, then diminished at 24 h. Low dosage of nitazoxanide (12.5 mg/kg, i.p.) also appeared to reverse mechanical hyperalgesia at 1 h. This analgesic effect peaked at 3 h with more than 90% reversal of pre-injury level for PWT. The delayed onset of activity of low dose of nitazoxanide treatment may stem from the time required to convert the parent prodrug nitazoxanide to its active metabolite tizoxanide. These *in vivo* studies established that systemic administration of nitazoxanide significantly reverses mechanical hyperalgesia caused by peripheral nerve injury in treated rats, which is consistent with antagonistic activities against mGluR1 and mGluR5 of tizoxanide and nitazoxanide in the *in vitro* assays conducted here.

DISCUSSION

Drug repositioning is a promising drug development route through identification of new indications for existing therapeutics, which offer significant time and cost benefits compared with traditional methods. Computational approaches attest to provide substantial advantages in drug repositioning efforts (38), by rapidly screening large, accessible virtual databases based on their chemical-based or disease-based similarity to prioritize compounds for experimental testing. In this study, we described the development of *in silico-in vitro-in vivo* drug repositioning scheme which succeeded in identifying FDA-approved drugs as dual mGluR1/5 negative modulators. Using the subject virtual screening protocol, nitazoxanide was identified as a potential dual mGluR1/5 negative modulator. Tizoxanide, the active metabolite of nitazoxanide, was shown to fit the pharmacophore models of both mGluR1 and mGluR5, and subsequent *in vitro* and *in vivo* experiments confirmed its Group I mGluR modulating activity.

The present results emphasize the need to consider the active metabolites of prodrugs and other parent drugs in studies of this kind. This finding was strengthened by results from a simple LC-MS study to investigate if nitazoxanide can be hydrolyzed to tizoxanide under assay conditions, which indicated that the conversion is possible in the presence of assay buffer alone (data not shown). Metabolites of drugs are important to both activity and toxicity of parent drugs in the body. Many metabolites possess intrinsic biological activities against intended targets, and sometimes even other cellular proteins, therefore suggesting that metabolites may display a different pharmacological profile from their parent drugs. Their structural scaffolds and activities therefore offer unique opportunities for drug discovery. This information is often overlooked,

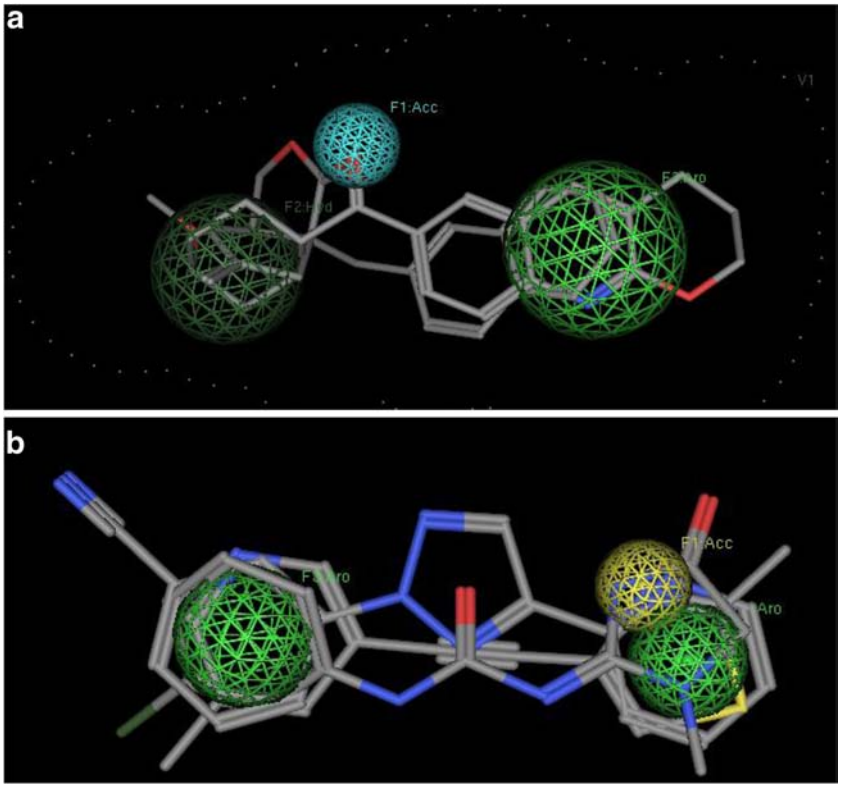
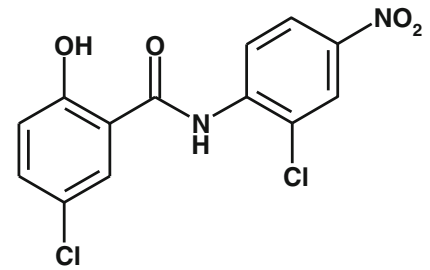


Fig. 3 (**a**) Pharmacophore model for mGluR1 antagonists. This model includes three features: one hydrogen bond acceptor (*cyan sphere*), a hydrophobic center (*dark green sphere*), and an aromatic center (*green sphere*). An additional volume constraint (a contour surface is displayed for clarity) is imposed by grouping together 2 Å radius spheres of all heavy atoms from known mGluR1 modulators. (**b**) Pharmacophore model for mGluR5 antagonists. This model includes three features: one hydrogen bond acceptor (*cyan sphere*), a hydrophobic center (*dark green sphere*), and an aromatic center (*green sphere*).

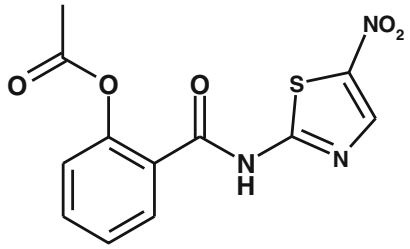
particularly during the construction of experimental screening libraries of drugs. Since a majority of screening efforts are initiated by *in vitro* assays to assess therapeutic potential, the desired biotransformation of drugs and prodrugs to their active metabolites is unlikely to happen and the opportunity for drug repositioning would be missed. It is worth noting that prodrugs account for 5–7% of marketed drugs, consequently the absence of information on their active metabolites would have a significant negative impact on productivity (39). *In silico* drug repositioning is one strategy to obviate this limitation of *in vitro* screening by including related information about metabolites in virtual drug screening libraries for further investigation. During preparation of this manuscript, DrugBank released its latest version, DrugBank 4.0 (12). This major update added more than 1200 drug metabolites and their detailed information, such as structure, abundance, and activity, into the database. This valuable knowledge of metabolites is currently being incorporated into our VCDL to provide more comprehensive coverage of potentially active drugs.

It is worth noting that many hits retrieved from our VCDL are structurally dissimilar to the query compounds and, therefore, might be missed by traditional similarity search methods based on (sub)structure similarity. This positive outcome is a direct consequence of *Shape Signatures*, which was purposely designed to find hits for queries that possess similar shape and biorelevant properties but may differ in chemical structure. The performance of our VS scheme was evaluated in an enrichment study (Supplementary Materials). The encouraging findings of this work establish that *Shape Signatures* and related virtual screening strategies provide a powerful avenue for drug repositioning and related applications. Pharmacophore mapping has already demonstrated its utility in drug discovery and related applications. We also incorporated this method into our *in silico* drug repositioning scheme. Specifically, separate pharmacophore models were developed for mGluR1 and mGluR5 negative modulators. Several studies have suggested there are multiple allosteric sites for modulators interacting with mGluRs; therefore, it is possible to construct multiple pharmacophore models to represent different modes of interactions. Here we only utilize one representative model of Group I mGluR negative modulators to determine the feasibility of the subject *in silico* drug repositioning scheme. Multiple models can be incorporated into the work to discover more drugs with repositioning potential.

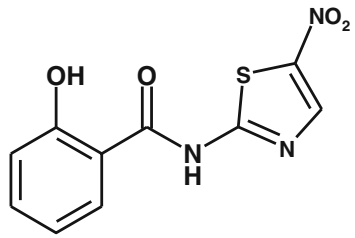
For neuropathic pain, currently there are five widely used drugs to treat this medical condition: pregabalin (*Lyrica*®), duloxetine (*Cymbalta*®), gabapentin (*Neurontin*®), a 5% lidocaine patch (*Lidoderm*®), and an 8% capsaicin patch (*Qutenza*®). Interestingly, pregabalin and gabapentin were previously FDA approved as anticonvulsants. Similarly, duloxetine was prescribed as an antidepressant and lidocaine is an anesthetic and antiarrhythmic drug. Therefore, these known neuropathic pain relievers are themselves examples of drug repositioning. Since they are all associated with a high incidence of side effects (40), there is an urgent need to develop an alternative medicine which can attenuate neuropathic pain



Niclosamide



Nitazoxanide



Tizoxanide

Fig. 4 Chemical Structures of niclosamide,nitazoxanide, and tizoxanide.

effectively. Nitazoxanide is an effective anti-protozoal agent for the treatment of illness caused by *Cryptosporidium parvum* or *Giardia lamblia* infection in immunocompetent adults and children. Recently nitazoxanide was found to treat chronic hepatitis C virus infection (39). A high-throughput screening (HTS)

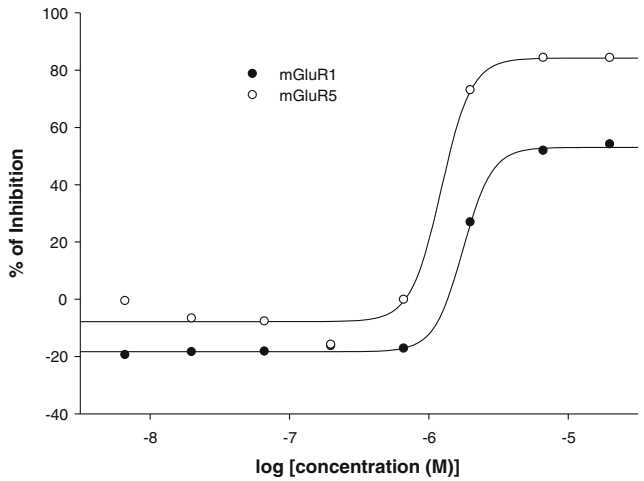


Fig. 5 Dose-dependent negative modulation of tizoxanide at human Group I mGluR expressed cell lines.

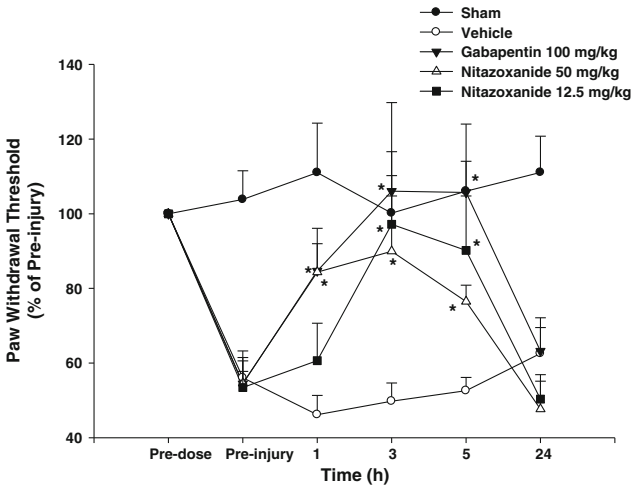


Fig. 6 *In vivo* efficacy data on nitazoxanide for neuropathic mechanical hyperalgesia through i.p. route. * $P < 0.05$ compared with vehicle-treated group, data are presented as mean ± SEM for six animals.

of the Prestwick Chemical Library with >1200 FDA-approved drug and drug-like compounds also identified nitazoxanide with antineoplastic activity through c-Myc activation (8). Together this information should greatly facilitate the development of nitazoxanide as a safe and effective therapeutic agent for other clinical indications such as neuropathic pain and other mGluR1 or mGluR5 mediated medical conditions.

HTS is a powerful tool to identify novel lead compound for drug development from a large chemical library. However, its application to screen drug libraries is hampered by the obstacles in acquiring these drugs, not only FDA-approved ones but also those previously tested for clinical purpose, as well as drug metabolites that may display unique pharmacological profiles from parent drugs. *In silico* screening of the VCDL may be an alternative approach to explore a comprehensive clinical drug library including drug metabolites without the time and resource consuming step involved in organic synthesis. More importantly, this screening platform can be easily tailored to find small molecule modulators for any disease-related targets, which should greatly benefit the discovery of drugs for neglected and orphan diseases.

CONCLUSIONS

In this work we present an integrated *in silico-in vitro-in vivo* screening scheme to accelerate the drug repositioning process on novel therapeutics for neuropathic pain. The applicability of our approach was demonstrated by successful identification of nitazoxanide and its active metabolite tizoxanide from known drug libraries, and their dual mGluR1/5 antagonistic activities were confirmed by *in vitro* functional assay. The *in vivo* efficacy of nitazoxanide was demonstrated in an established

model for mechanical neuropathic pain in rats. In conclusion, we report that nitazoxanide, an FDA approved antiprotozoal agent, is a dual mGluR1/5 antagonist that represents a promising lead compound for the development of novel treatments for neuropathic pain.

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