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Evidence that tricyclic small molecules may possess Toll-like receptor and MD-2 activity

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Abstract

Opioids have been discovered to have Toll-like receptor (TLR) activity, beyond actions at classical opioid receptors. This raises the question whether other pharmacotherapies for pain control may also possess TLR activity, contributing to or opposing their clinical effects. We document that tricyclics can alter TLR4 and TLR2 signaling. *In silico* simulations revealed that several tricyclics docked to the same binding pocket on the TLR accessory protein, MD-2, as do opioids. Eight tricyclics were tested for effects on TLR4 signaling in HEK293 cells over-expressing human TLR4. Six exhibited mild (desipramine), moderate (mianserin, cyclobenzaprine, imipramine, ketotifen) or strong (amitriptyline) TLR4 inhibition, and no TLR4 activation. In contrast, carbamazepine and oxcarbazepine exhibited mild and strong TLR4 activation, respectively, and no TLR4 inhibition. Amitriptyline but not carbamazepine also significantly inhibited TLR2 signaling in a comparable cell line. Live imaging of TLR4 activation in RAW264.7 cells and TLR4-dependent interleukin-1 release from BV-2 microglia revealed that amitriptyline blocked TLR4 signaling. Lastly, tricyclics with no (carbamazepine), moderate (cyclobenzaprine), and strong (amitriptyline) TLR4 inhibition were tested intrathecally (rats) and amitriptyline tested systemically in wildtype and knockout mice (TLR4 or MyD88). While tricyclics had no effect on basal pain responsivity, they potentiated morphine analgesia in rank-order with their potency as TLR4 inhibitors. This occurred in a TLR4/

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MyD88-dependent manner as no potentiation of morphine analgesia by amitriptyline occurred in these knockout mice. This suggests that TLR2 and TLR4 inhibition, possibly by interactions with MD2, contributes to effects of tricyclics *in vivo*. These studies provide converging lines of evidence that several tricyclics or their active metabolites may exert their biological actions, in part, via modulation of TLR4 and TLR2 signaling and suggest that inhibition of TLR4 and TLR2 signaling may potentially contribute to the efficacy of tricyclics in treating chronic pain and enhancing the analgesic efficacy of opioids.

Keywords

(+)-naloxone; hotplate; *in silico* docking; tricyclic anti-depressants; cytokine; innate immunology

Spinal cord glia are importantly involved in the creation and maintenance of diverse enhanced pain states. Spinal cord microglia are generally considered to be the first glial cell activated in response to inflammatory or traumatic injuries to bodily tissues, including peripheral nerve injury leading to neuropathic pain (Milligan and Watkins, 2009). A class of receptors expressed by microglia that enable them to sense that peripheral nerve injury has occurred is via Toll-like receptors (TLR) such as TLR2 (Kim et al., 2007) and TLR4 (Tanga et al., 2005, Hutchinson et al., 2008c, Watkins et al., 2009). TLR2 and TLR4 are best known as the receptor that immune cells, including microglia, use to detect yeast cell wall (zymosan) and lipopolysaccharide (LPS) of gram-negative bacteria, respectively. Activation of either TLR2 or TLR4 results in proinflammation via MyD88 dependent signaling. However, it is now recognized that TLR2 and TLR4 are more promiscuous, as its signaling is also activated by substances released by stressed and damaged cells (Miyake, 2007). Such substances have been coined as “alarmins” or “endogenous danger signals” (Miyake, 2007), and are thought to be the source of TLR activation under conditions of neuropathic pain (Tanga et al., 2005, Kim et al., 2007, Hutchinson et al., 2008c, Watkins et al., 2009). Activation of TLR2 or TLR4 signaling, whether by components of invading pathogens or endogenous danger signals, leads to the production and release of proinflammatory cytokines (Miyake, 2007), which are intimately linked to the glial contribution to neuropathic pain (Milligan and Watkins, 2009).

Spinal cord glia also become activated in response to opioids, such as morphine (Song and Zhao, 2001, Watkins et al., 2005). Opioid-induced glial activation suppresses acute opioid-induced analgesia, enhances the development of opioid analgesic tolerance, dependence, and reward, and contributes to negative side effects of opioids such as respiratory depression (Hutchinson et al., 2007, Hutchinson et al., 2008a, Hutchinson et al., 2008b, Hutchinson et al., 2009, Hutchinson et al., 2010). Importantly, one of the ways that opioids exert such effects is via the activation of, at minimum, TLR4 (Hutchinson et al., 2007, Watkins et al., 2009, Hutchinson et al., 2010). Parallel research also implicates opioid-TLR2 activity, but the behavioral ramifications of this signaling cascade are not clear yet (Li et al., 2009, Li et al., 2010). Thus, opioids, components of invading pathogens, and endogenous danger signals may act as activators of TLR signaling.

Opioid activation of TLR2 or TLR4 provides evidence that TLRs can recognize and become activated in response to select xenobiotics; that is, chemicals found in an organism but not normally produced or expected to be present at elevated levels. Given this, it is important to define whether other clinically relevant, small molecule pharmacotherapies also possess unrealized TLR actions. Hence, the present series of studies explored whether a group of structural analogues that share a tricyclic motif (amitriptyline, imipramine, desipramine, mianserin, cyclobenzaprine, carbamazepine, oxcarbazepine) may also alter TLR signaling. Tricyclics were the primary focus here given the use of members of this class of compound in the treatment of chronic pain (Saarto and Wiffen, 2007, Verdu et al., 2008). Each of these

tricyclics was studied both *in silico*, in order to model the docking of these molecules to the 3-dimensional structure of the TLR co-receptor molecule MD-2 that is involved in TLR2 and TLR4 signaling activation (Dziarski and Gupta, 2000, Dziarski et al., 2001), and *in vitro* for their potential for activating or inhibiting TLR2 and TLR4 signaling in cell lines over-expressing human TLR2 or TLR4 and its accessory signaling molecules. Select tricyclics were also tested *in vitro* for effects on 1) TLR signaling in a macrophage cell line; 2) their effect on TLR4-dependent proinflammatory cytokine mRNA expression in primary neonatal rat microglia; and 3) interleukin-1 protein output from a mouse microglial BV-2 cell line. Tricyclics were also tested *in vivo* to compare their predicted efficacies in altering the magnitude and duration of intrathecal morphine analgesia in rats and to assess the requirement of TLR4 and MyD88-dependent signaling for potentiating morphine analgesia using TLR4 knockout, MyD88 knockout and wildtype mice. A sampling of other clinically relevant drugs, chosen based on various chemical structural or pharmacological similarities to tricyclics, were also examined *in silico* and *in vitro* for potential TLR effects as an initial exploration of the breadth of TLR actions.

Methods and Materials

Subjects

Pathogen-free adult male Sprague-Dawley rats (n = 6 rats/group for each experiment; 300–375 g; Harlan Labs, Madison, WI, USA) were used. Pathogen-free male Balb/c wild-type, MyD88 knockout and TLR4 knockout mice were used for the knockout studies (knockout lines back crossed onto Balb/c 10 times, n = 6 mice/group for each experiment; 24–32 g; kindly supplied by Dr. Simon Phipps and sourced from Prof. Akira). Mice and rats were housed in temperature (23 ± 3 °C) and light (12 hr:12 hr light:dark cycle; lights on at 0700) controlled rooms with standard rodent chow and water available *ad libitum*. All procedures were performed during the light phase of the light cycle. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Colorado at Boulder (rat) and the Animal Ethics Committee of the University of Adelaide (mouse).

Drugs

The tricyclics amitriptyline, cyclobenzaprine, carbamazepine, oxcarbazepine, imipramine, desipramine and mianserin were used (Sigma, St. Louis, MO, USA). Based in part on structural or pharmacological similarities to tricyclics, representative selective serotonin and/or norepinephrine reuptake inhibitor (venlafaxine, fluoxetine), antipsychotic (haloperidol, rimcazone, chlorpromazine), neuroleptic (mesoridazine, beta-carboline (harmine, norharmine, anti-cholinesterase (tacrine), anti-cholinergic (orphenadrine) and anti-histamine (ketotifen, dephenhydramine) drugs were used as well (Sigma). Morphine sulfate was kindly gifted by Mallinckrodt, Inc. (St. Louis, MO, USA). (+)-Naloxone, (+)-naltrexone and (+)-nalmefene were kindly gifted by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD, USA). (–)-Naloxone and (–)-naltrexone were obtained from Sigma. Where applicable, endotoxin-free status of compounds was confirmed by LAL assay. Drugs were dissolved in endotoxin-free physiological saline (Abbott Laboratories, North Chicago, IL, USA) and handled using aseptic procedures. The lipopolysaccharide (LPS; *Escherichia Coli*) used was serotype 0111:B4 (Sigma). The Pam3CSK4 is a synthetic tripalmitoylated lipopeptide (LP) that mimics the acylated amino terminus of bacterial LPS, which selectively activates TLR2 was purchased from Invivogen (San Diego, CA USA). Where applicable, drugs were prepared and are reported as free base concentrations. Vehicle (saline) was administered equivolume to the drugs under test.

Experiment 1. Tricyclic activation and inhibition of *in vitro* TLR2 and TLR4 signaling: HEK-TLR2 and HEK-TLR4 cell line and reporter protein assay

As we have previously described (Hutchinson et al., 2008c) a human embryonic kidney-293 (HEK293) cell line stably transfected to express human TLR4 was used to assess TLR4 activity. This HEK293 cell line expresses high levels of TLR4, the required TLR4 co-signaling molecules (MD-2 and CD14) and an optimized alkaline phosphatase reporter gene under the control of a promoter inducible by several transcription factors such as NF- κ B and AP-1 (Invivogen, San Diego, CA USA; 293-htlr4a-md2cd14). A parallel HEK-TLR2 (Invivogen, San Diego, CA USA) cell line was also employed here to examine the TLR2 activity of select tricyclics. TLR2 and TLR4 activity in the cells was assessed by measuring the expression of secreted alkaline phosphatase (SEAP) protein that is produced as a consequence of TLR2 or TLR4 activation. As a specificity control, all drugs were also tested on a HEK293 cell line that expressed the SEAP reporter gene but neither TLR.

Experiment 2. Tricyclic docking simulations *in silico* to MD-2

In silico docking simulations were conducted as we have previously described (Hutchinson et al., 2010). The complexed human TLR4 and MD-2 pdb file was obtained from RCSB Protein Data Bank database (PDBID: 3fxi). All ligands were extracted via Molegro Molecular Viewer to eliminate exogenous water molecules and artifacts from crystallization. Modified pdb files were inputted into AutoDock 4.0 (<http://autodock.scripps.edu>), hydrogens added, and resaved in pdbqt format. Drug ligands for docking were gathered using PubChem isomeric SMILES then converted to .pdb using a structure file generator (<http://cactus.nci.nih.gov/services/translate/>). Initially, the *in silico* docking of ligands to the entire TLR4/MD-2 dimer complex was conducted (AutoGrid center set 3.438, -7.805, 2.034; 126 grid points expanding in all directions; GA running number of 100, Max Evals 5×10^6 and 1.0 Å spacing). These data demonstrated that the great majority of the ligands docked with human MD-2 independent of human TLR4 interactions. Therefore, all the ligands were docked to MD-2 alone with greater resolution (AutoGrid center set 27.991, 0.851, 19.625; 126 grid points expanding in all directions; GA running number of 100, Max Evals 5×10^6 and 0.375 Å spacing). All dockings were executed with Lamarckian genetic algorithms. The lowest energy and highest interaction docking conformation was visualized and Binding Energy, Ligand Efficacy, Inhibitory Constant, Intermolecular Energy, van der Waals + Hydrogen Bonds + Disolvation Energy, Electrostatic Energy, Total Internal Energy, Torsional Energy, Unbound Energy, Lowest Energy, Docking Frequency, Rank and the amino acid residues of MD-2 that the ligand conformation interacted with were collected (coded +1 for interaction and -1 for no interaction). These parameters constitute the *in silico* portion of the data. The two sets of *in vitro* data (described below) were then integrated to produce the sum of the percent above control (set to 0%) of the activity of individual ligands in the *in vitro* agonist assay (when tested alone at 10 μ M); and the maximal inhibition of LPS signaling (when tested at 10 μ M) expressed as a percent of the LPS control. Therefore, positive values equaled TLR4 signaling activators and negative values represented TLR4 signaling inhibitors. All the data was then standardized (mean = 0 and standard deviation = 1) to remove the inherent variability between the scores reported thereby removing numerical magnitude bias from correlations. A linear regression was then applied to the sum of all of the *in silico* parameters plotted against the integrated *in vitro* data, with variable multiplication values applied to each *in silico* parameter which were then varied by an iteration process to maximize the correlation between the sum of the *in silico* parameters and the integrated *in vitro* data. This process also provided the opportunity to ascertain which parameters contributed most to the *in vitro* prediction as their multiplication factors are inherently larger. The starting values for the multiplication values were trained based on the individual correlations of each *in silico* parameter to the *in vitro* data, such that the multiplication factor was set to -1 for negative correlations and +1 for positive correlations. This process was conducted using the *in silico* and *in vitro* data from 4 tricyclics

(oxcarbazepine, carbamazepine, amitriptyline and mianserin) enabling a model of *in silico* to *in vitro* prediction to be built that was subsequently tested using 3 different tricyclics (desipramine, imipramine, cyclobenzaprine). The final model was then refined further by the inclusion of the data from all of the tricyclics and related molecules, and tested again on the several of the previously published opioid MD-2 *in silico* models that shared similar MD-2 docking residues; (+)-nalmefene, (+)-naloxone, (-)-naloxone, (+)-naltrexone, (-)-naltrexone, and (-)-morphine.

Experiment 3. Real time microscopy of TLR4 signaling in a stably transfected RAW264.7 mouse macrophage cell line

TLR4 signaling leads to the simultaneous activation of three parallel intra-cellular signaling pathways. Two of these (through NF- κ B and MAPK) are principally responsible for the proinflammatory responses induced by TLR4 activation, while the third parallel pathway (PI3K/Akt1) is more related to cell survival, apoptosis, and cell motility (Dauphinee and Karsan, 2006, Laird et al., 2009). Regardless of the differing downstream effects created by each of these pathways, all three are activated by agonism at TLR4, any can be used as a reflection of TLR4 activation. This does not negate potential interaction of the tested compounds with downstream sites, but rather addresses whether the compounds have a site of action at or near TLR4. As we previously described (Hutchinson et al., 2008c, Hutchinson et al., 2010) a RAW264.7 mouse macrophage cell line stably transfected to express green fluorescent protein (GFP)-tagged Akt1 (Evans and Falke, 2007), with mobilization and cytosolic clearance of GFP-Akt1 was used as an indicator of TLR4 activation.

Experiment 4. *In vitro* TLR4-induced Interleukin-1 release in murine microglial BV-2 cell line

A murine microglial cell line, BV-2, was grown in macrophage serum free media (Invitrogen, Carlsbad, CA, USA) in 75 cm² Primaria-treated flasks (Falcon, BD Biosciences, San Jose, CA, USA) with no supplementation at 5% CO₂ and 37°C. 200,000 cells/well were plated in 6-well Primaria treated plates (Falcon, BD Biosciences, San Jose, CA, USA) for 48 h, in 1.6 ml of the same media, before drug administration. After 48 h, fresh media and either LPS (100 ng/ml) or vehicle were added (n=6 per condition). In addition, doses of amitriptyline, carbamazepine or oxcarbamine were coadministered with the LPS or vehicle (100 μ M, 10 μ M and 1 μ M). Twenty-four hours after drug administration, the supernatants were removed and the cells lysed in 300 μ l of sonication buffer (cold Iscove's culture medium containing 5% fetal calf serum and a cocktail enzyme inhibitor: 100 mM amino-n-caproic acid, 10 mM EDTA, 5 mM benzamidine-HCl, and 0.2 mM phenylmethylsulfonyl fluoride). Sonicated samples were centrifuged at 14,000 rpm at 4°C for 10 min. The cell culture supernatants were immediately measured for interleukin-1 β (IL-1) protein using a commercially available mouse ELISA kit (OptEIA, BD Biosciences, San Jose, CA, USA). Procedures were conducted according to manufacturer's directions. The minimum detection threshold of each of the assays is 6 pg/ml. Protein concentrations were normalized to total protein content as measured by a Bradford protein assay.

Experiment 5. *In vitro* TLR4-induced Interleukin-1 release in neonatal primary rat microglia

Brains from P0/1 neonatal Sprague-Dawley rat pups were collected and the cortices carefully dissected and the overlying meninges removed. The tissue was then minced with a scalpel blade and digested for 30 min in Liberase and DNase (0.1 U per brain) at 37°C with agitation. The cells were triturated with a 27 Gauge hypodermic needle. MEM (100 U/ml penicillin, 100 μ g/ml streptomycin, 0.6% glucose and 2 mM l-glutamine) was added and the cells centrifuged at 250 x g for 5 min at RT. The supernatant was discarded and the cells resuspended in 1 ml of MEM media per brain. The cells were filtered through a 70 μ m and then 40 μ m filters. The cells were plated in 75 cm² tissue culture flasks at 4 $\times$ 10⁶ cells per flask. Cells were incubated

at 37°C and 5% CO₂ until confluence was reached (about 10 days). Media was changed every 3-4 days, with the first change a complete media change and subsequent changes being a 50% media change. Once confluence was reached, microglial cells were shaken from the remaining astrocytes for 90 min at 160 rpm. The media containing the microglia was removed and centrifuged at 300 x g for 5 min at RT. The supernatant discarded and the pellet resuspended in 1 ml fresh MEM media. A small aliquot of cells was counted with trypan exclusion and plated in 96-well v-bottom tissue culture plates at 40,000-50,000 cells per well in 100 µl. 24 h after plating, the drugs were added. Cell cultures were incubated for 4 h with LPS (100 ng/ml, vehicle) and drug (100 µM, 10 µM, 1 µM or vehicle) at 37°C. The cells were centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was discarded and cells processed for mRNA gene expression as previously reported (Hutchinson et al., 2008b) using IL-1 β and TNF- α mRNA primers as previously reported (Hutchinson et al., 2008a).

Experiment 6. *In vivo* effects of intrathecally administered tricyclics on basal pain responsivity and intrathecal morphine analgesia

Catheter implantation—The method of constructing and implanting the indwelling intrathecal catheters in rats was based on that described previously (Hutchinson et al., 2008a). Briefly, intrathecal catheters were implanted under anesthesia (isoflurane; Phoenix Pharmaceuticals, St. Joseph, MO, USA) by threading sterile polyethylene-10 tubing (PE-10 Intramedic Tubing; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA) guided by an 18-gauge needle between the L5 and L6 vertebrae. The catheter was threaded rostrally such that the proximal catheter tip lay over the lumbosacral enlargement. The needle was removed and the catheter was sutured to the superficial musculature of the lower back and the exterior end led subcutaneously to exit through a small incision at the nape of the neck. The catheters were preloaded with drugs at the distal end in a total volume of no greater than 25 µl. Specifically, morphine (15 µg in 1 µl) vs. equivolume saline vehicle was injected with either amitriptyline (64 nmol), or equimolar cyclobenzaprine, carbamazepine or equivolume (1 µl) saline vehicle. The catheters were 90 cm in length, allowing remote drug delivery without touching or otherwise disturbing the rats during the testing. Acute intrathecal drug administration began 2 hr after surgery.

Pain threshold—Rats received at least three 60 min habituations over successive days to the test environment prior to behavioral testing. Latencies for behavioral response to radiant heat stimuli applied to the plantar surface of each hind-paw and tail were assessed using a modified Hargreaves test (Hargreaves et al., 1988). All testing was conducted blind with respect to group assignment. Intrathecal catheter surgery does not affect baseline responses after 2 h recovery from surgery, compared to latencies recorded prior to surgery (Hutchinson et al., 2008a). Briefly, baseline withdrawal values were calculated from an average of 2 consecutive withdrawal latencies of the tail and the left and the right hind-paws, measured at 15 min intervals. Latencies for the baseline responses ranged from 2 to 3 s, and a cut-off time of 10 s was imposed to avoid tissue damage. Nociceptive assessments were then made at 0 (immediately following remote drug delivery), 5 min, 15 min and every 10 min thereafter until completion of the experiment. All testing was conducted blind with respect to group assignment.

Experiment 7: TLR4 knockout, MyD88 knockout and wildtype mice opioid behavioral assessment

Hotplate analgesia assessment—Mice received at least three 5 min habituations to the test environment prior to behavioral testing. Latencies for behavioral responses to the 50°C hotplate were assessed. All testing was conducted blind to group assignment. A cut-off time of 60 s was imposed to avoid tissue damage. Latencies for the hotplate response ranged from 17 to 31 s. Baseline response latencies were recorded prior to drug administration. Examination

of the effect of amitriptyline on morphine analgesia in wildtype and knockout mice was conducted by giving 51 mg/kg amitriptyline (an equimolar dose to previous novel TLR4 targeted therapies published previously (Hutchinson et al., 2010)) i.p. 10 min prior to 2.5 mg/kg morphine i.p. with hotplate latencies recorded prior to the amitriptyline and morphine doses and 20 min after the morphine dose.

Statistics

Graphpad Prism 5.0 was used for all statistical analysis. A two-way ANOVA with Bonferroni post-hoc was used to test the activation and inhibition results obtained from the GFP-Akt RAW264.7 cells and *in vivo* Hargreaves and hotplate analgesia data. A one-way ANOVA with Bonferroni post-hoc was used to assess TLR4 activity in the HEK293-hTLR4 cell line. Error bars on graphs represent standard error of the mean. Data from the Hargreaves test were calculated as the % of maximal possible effect (%MPE) using the following equation:

$$\%MPE = \frac{\text{test latency} - \text{baseline latency}}{\text{cut off} - \text{baseline latency}} \times 100 \quad (\text{Carmody, 1995}).$$

Statistical comparisons are indicated on the figures for clarity and significance was set at $p < 0.05$.

Results

Experiment 1. Activation and inhibitory effects of tricyclics *in vitro* on TLR2 and TLR4 signaling: HEK293-hTLR4 and HEK293-hTLR2 cell line and reporter protein assays

Given that we have previously demonstrated that some xenobiotics, including opioids, can have TLR4 activity (Hutchinson et al., 2010), it is important to define whether other clinically relevant therapeutic drugs may also possess TLR actions, either inducing inhibition or activation. In order to explore this issue, tricyclics were tested *in vitro* either alone to determine their ability to activate either TLR4 or TLR2 signaling, or in the presence of the classical TLR2 or TLR4 agonists to determine their ability to inhibit TLR signaling. As can be seen in Figure 1, amitriptyline (Figure 1A; $n = 3$ for each condition) showed significant ($P < 0.01$) dose dependent inhibition of TLR4 signaling, followed in order of inhibitory potency by imipramine (Figure 1B; $P < 0.05$), mianserin, cyclobenzaprine and desipramine (summarized below). In contrast, carbamazepine (Figure 1C) and oxcarbazepine (Figure 1D) exerted no inhibitory effects. Instead, mild and moderate ($P < 0.05$) agonist effects on TLR4 signaling were observed, respectively. Similar results were also obtained when the ligands were examined in the absence of LPS, with the TLR4 signaling inhibitors having no significant effect on basal TLR4 signaling, but carbamazepine ($126 \pm 2.2\%$) and oxcarbazepine ($170.8 \pm 4.6\%$) displaying significant TLR4 signaling activation ($P < 0.05$). Select tricyclics were also assessed on the human TLR2 cell line (Figure 1E-G) and demonstrated to have similar actions as observed for TLR4, with amitriptyline inhibiting TLR2 (Figure 1E), carbamazepine having no effect (Figure 1F), and oxcarbazepine potentiating TLR2 signaling (Figure 1G). Additional tricyclic and related ligands were also examined for their TLR4 signaling inhibitory and activation properties. Harmine, ketotifen, rimcazone, norharmane, chlorpromazine, tacrine, mesoridazine, haloperidole, fluoxetine, venlafaxine, diphenhydramine and orphenadrine were tested, with their response data summarized in Figure 2. Importantly, to ensure the responses were TLR2/TLR4 specific, each compound was tested on a cell line only expressing the reporter gene and not TLR2 or TLR4. No significant non-TLR2/TLR4 signaling was observed in these cells ($P > 0.05$).

Experiment 2. Tricyclics dock *in silico* to MD-2

To gain additional insight into the interaction of tricyclics with TLRs, which may explain the resulting modifications of TLR signaling, an *in silico* docking analysis was conducted using the recently published high-resolution crystalline structure of the protein complex of human

TLR4 and MD-2 (Park et al., 2009) and the *in silico* docking software suite AutoDock 4. Using AutoDock 4, 100 independent docking simulations were run for each ligand with the entire dimer of human TLR4 and MD-2. It was observed that the majority of the ligands docked with greater preference to the LPS binding cleft of MD-2, independent of any interactions with TLR4, as we observed previously for opioid *in silico* docking. Current docking simulations have higher confidence in predicting the binding site than calculating the binding energies. Therefore, the *in silico* docking was repeated with MD-2 alone with a more thorough search on the protein surface (grid size = 0.375 Å), with the resulting ligand docking conformations, frequency of each ligand docking conformation and the estimated energy for each docking conformation being generated. In each case, the optimal conformation with the lowest energy and greatest frequency was selected as the docking conformation of preference for each ligand and the protein residues this conformation interacted with were collected.

All of the ligands docked with varying success to the LPS binding pocket of MD-2 with an average of 49 out of 100 dockings to this location with as many as 98 (mainserin) and as few as 13 (venlafaxine) dockings for this choice conformation. Importantly, in each case, despite the quality of the docking the best conformation for each ligand docked to the same MD-2 pocket. Activators and inhibitors are predicted to dock similarly, as the software does not allow for global conformation change resulting from the docking of a ligand. Conformational change upon agonist binding is necessary to facilitate signal transduction versus inhibition. Notably, a recently solved TLR4/ligand complex crystal structure showed that activators and inhibitors occupy the same binding site in the TLR4/MD-2 association (Park et al., 2009). There were specific residues that ligands interacted that were shared with the majority of the opioids screened. Specifically phenylalanine 147 and 76, and isoleucine 63 were critical in the ligand recognition.

In order for these *in silico* data to have validity, they should be relatable to *in vitro* results. Given the MD-2/TLR4 interaction is pivotal to TLR4 signaling, alterations in MD-2's binding to TLR4 would be expected to impact TLR4 signaling, possibly by changing TLR4 activation or inhibition (Slivka et al., 2009). Therefore, it is feasible for the *in silico* predictions to have *in vitro* consequences. As such, an *in silico* to *in vitro* prediction model was built using 4 tricyclic ligands (oxcarbazepine, carbamazepine, amitriptyline and mianserin; see grayed circles for “integrated *in vitro* score” in Figure 2A) and subsequently tested on 3 different tricyclic ligands spanning both TLR4 activators and inhibitors (desipramine, imipramine, cyclobenzaprine; see black squares for “actual *in vitro* score” in Figure 2A). The *in silico* to *in vitro* prediction model successfully determined with high specificity (Figure 2A: predicted open squares points are obscured by and actual score filled squares) the stimulation and inhibition categorical assignment of all 3 the tricyclics tested on TLR4 signaling. The ability to predict the extent of activation and inhibition of TLR4 signaling was very promising with 0.02%, 8.7% and 0.7% error associated with the predictions of desipramine, imipramine and cyclobenzaprine, respectively. The model was successfully built when all nineteen ligands were included in the additional adaptation (Figure 2B). This complete model was then used to test the previously characterized opioids that shared similar MD-2 docking residues; (+)-nalmeferene, (+)-naloxone, (-)-naloxone, (+)-naltrexone, (-)-naltrexone, and (-)-morphine. There is a robust correlation between the *in silico* prediction and the *in vitro* results, establishing that based on the MD-2 *in silico* docking, the ligands were all assigned correctly as either TLR4 signaling activators or inhibitors with some variability in the degree of ligand activity.

Experiment 3. *In vitro* effects of amitriptyline on TLR4 signaling: Real time microscopy of intracellular TLR4 signaling in RAW264.7 cells

Experiment 1 and 2 provide evidence that amitriptyline can inhibit TLR2- and TLR4-mediated signaling *in vitro*, as previously shown for other novel TLR signaling inhibitors (Hutchinson et al., 2008c, Hutchinson et al., 2010). However, these cell line data alone provide no indication as to where in the signaling cascade this blockade of signaling may be occurring, especially given the high lipophilicity of the ligands granting them ready access to intracellular compartments. Certainly there is precedence for other drugs interfering with TLR signaling through non-receptor mediated mechanisms (Noman et al., 2009). To begin to approach this issue, we utilized a RAW264.7 mouse macrophage cell line that stably expresses green fluorescent protein (GFP)-tagged Akt1. As noted above, the PI3K/Akt1 pathway is one of 3 parallel intracellular signaling pathways which are all activated upon TLR4 signaling. The other 2 (NF- κ B and MAPK) are the predominant pathways leading to proinflammatory responses resulting from TLR4 activation, while the PI3K pathway is predominantly responsible for cell survival, apoptosis, and cell motility effects (Dauphinee and Karsan, 2006, Laird et al., 2009). The advantages of this cell line are that: (a) TLR4 signaling activates Akt1, as well as NF- κ B and MAPK, and (b) TLR4-induced activation of Akt1 by PI3 kinase is a very early step in the TLR4 signaling cascade, quite close to TLR4 (Dauphinee and Karsan, 2006, Laird et al., 2009). Thus, if TLR4-induced Akt1 activation is affected by the drugs under test then an interaction is most likely to be occurring at or close to the TLR4 complex. Notably, this does not negate potential interaction of the tested compounds with downstream sites, but rather addresses simply whether the compounds have a site of action at or near TLR4. Under basal conditions, Akt1 is diffusely distributed in the cytosol (Figure 3A) but, upon activation, rapidly moves to the membrane site where an Akt1 activating event is occurring (Figure 3B). In the case of LPS-induced TLR4 signaling, activated Akt1 moves out of the cytosol to the TLR4 lipid raft (Ojaniemi et al., 2003). As expected, the classical TLR4 agonist LPS (200 ng/ml in 1.2 ml total dish volume) reliably induced robust, rapid clearance of GFP-Akt1 from the cytosol to the cell membrane (Figure 3A; n = 10 cells/group from a minimum of 4 separate plates). In contrast, preincubation with the TLR4 signaling inhibitor amitriptyline (200 μ M; Figure 3C) significantly attenuated the LPS-induced Akt1 response. We have previously documented that morphine activates Akt1 in this cell line, an effect that appears to be mediated via TLR4 since Akt1 activation is blocked by the TLR4 competitive inhibitor LPS-R/S (Hutchinson et al., 2008c, Hutchinson et al., 2010). The ability of amitriptyline to inhibit morphine-induced TLR4 signaling was also examined here as the *in silico* docking results of Experiment 2 suggest the two disparate ligands may be exerting their effects in a similar location via interactions with MD-2. Amitriptyline was able to significantly inhibit morphine induced Akt1 membrane localization (Figure 3D), thereby supporting the *in silico* evidence from Experiment 2.

Since TLR4-induced Akt1 activation is an early event in its intracellular signaling cascade (Dauphinee and Karsan, 2006, Laird et al., 2009), this suggests that amitriptyline may be blocking either LPS activation of TLR4, TLR4 interaction/activation of the TRIF/TRAM complex, the subsequent activation of PI3K, or PI3K phosphorylation of Akt1. To clarify whether the blockade of the LPS and (–)-morphine Akt1 response by amitriptyline reflects a general suppression of Akt1 itself, rather than an effect on a specific signaling pathway, complement 5a (C5a; 25 ng/ml) was applied to the amitriptyline blocked cells as C5a activates PI3K/Akt1 via a TLR4-independent pathway. C5a immediately precipitated significant Akt1 membrane localization quantified by cytosolic clearance (Figure 3). Thus, amitriptyline appears to be, at minimum, disrupting the LPS and (–)-morphine induced TLR4 signaling cascade, either at the level of LPS/morphine binding or TLR4 interaction/activation of the TRIF/TRAM complex or via this method of activation of PI3K, but not via an alteration of the action of PI3K itself. Whether this or other tricyclics may also directly modulate downstream

components of the signaling cascades activated by TLR4 signaling, other than Akt1, cannot be known from these data.

Experiment 4. *In vitro* effects of tricyclics on TLR-induced Interleukin-1 release in murine microglial BV-2 cell line

The impact tricyclics have on the proinflammatory consequences of LPS (TLR4) and PAM3CSK4 (TLR2, 100 ng/ml) signaling in the murine microglial BV-2 cell line was assessed. Amitriptyline and carbamazepine (Figure 4A) dose dependently inhibited TLR4 signaling induced increases in IL-1 protein ($P < 0.001$). In contrast, oxcarbazepine caused a significant potentiation of LPS induced-expression of IL-1 (Figure 4A; $P < 0.0001$). Interestingly, amitriptyline was also found to significantly inhibit PAM3CSK4-induced IL-1 expression (Figure 4B).

Experiment 5. *In vitro* effects of tricyclics on TLR-induced Interleukin-1 β and Tumor necrosis factor- α mRNA expression in neonatal primary rat microglia

The impact tricyclics have on the proinflammatory consequences of TLR4 (LPS) signaling in primary rat microglia was assessed. Amitriptyline and carbamazepine dose dependently inhibited TLR4-induced elevations in IL-1 β (Figure 5A) and TNF- α (Figure 5B) mRNA ($P < 0.001$).

Experiment 6. *In vivo* effects of intrathecally administered tricyclic antidepressants on basal pain responsivity and intrathecal morphine analgesia

Opioid-induced, TLR4/MD-2-mediated, glial activation has been proposed to oppose acute opioid analgesia (Hutchinson et al., 2007, Hutchinson et al., 2008a, Hutchinson et al., 2008b, Hutchinson et al., 2009, Hutchinson et al., 2010). Therefore, inhibition of TLR4 signaling by tricyclics is hypothesized to block this glial opposition of acute morphine analgesia. Moreover, the degree of potentiation of analgesia is hypothesized to be related to the extent of TLR4 signaling blockade. To test these hypotheses, the potentiation of acute intrathecal morphine analgesia by tricyclics was assessed. Equimolar doses of three tricyclics with differing *in vitro* and *in silico* TLR4/MD-2 activity were co-administered intrathecally with morphine and the resulting analgesia was quantified. Specifically, amitriptyline, cyclobenzaprine and carbamazepine, with high to low predicted TLR4 signaling inhibitory capacity. Morphine alone produced profound and rapid analgesia that lasted for 100 min (Figure 6; $n = 6$ per condition; $P < 0.001$). Co-administration of amitriptyline with morphine produced a profound potentiation of morphine analgesia ($P < 0.01$), with no change in withdrawal latency when tested alone (Figure 6A; $P > 0.05$). An equimolar dose of cyclobenzaprine also produced profound potentiation of morphine analgesia ($P < 0.05$), albeit less than that produced by amitriptyline (Figure 6B). Finally, carbamazepine produced no change in morphine analgesia (Figure 6C; $P > 0.05$). The extent of potentiation of morphine analgesia *in vivo* has the same rank order as was observed for these three tricyclics *in vitro* in the HEK293-hTLR4 cell results and the *in silico* docking results.

Experiment 7: Genetic knockout of TLR4 or MyD88 signaling blocks amitriptyline potentiation of morphine analgesia

In order to extend the results of Experiment 6, and clarify the involvement of TLR4 and its MyD88-dependent signaling cascade in amitriptyline's potentiation of opioid analgesia, two different genetic knockout strains were examined and compared to wild-type mice. Low dose morphine (2.5 mg/kg) caused limited increases in the hotplate latency (analgesia) in wildtype mice, but caused significant analgesia in TLR4 knockout and MyD88 knockout animals (Figure 7, $P < 0.001$) as we have reported previously (Hutchinson et al., 2010). Amitriptyline

coadministration significantly potentiated morphine analgesia in wild-type mice ($P < 0.001$) without any additional effect in TLR4 or MyD88 knockout animals (Figure 7; $P > 0.05$).

Discussion

Using *in vitro*, *in vivo* and *in silico* techniques, we demonstrate that clinically employed tricyclics of diverse pharmacologies possess previously unrecognized TLR2 and TLR4 activity ranging from signaling inhibition to signaling activation. Based on *in silico* docking simulations, it appears that this action may potentially be mediated via interaction with the TLR accessory protein, MD-2. Since morphine has recently been documented to: (a) dock to the same MD-2 binding pocket as shown here for tricyclics, (b) activate TLR4 signaling, shown here to be blocked by amitriptyline, and (c) morphine analgesia is compromised by co-occurring morphine-induced TLR4 activation, the consequence of tricyclic inhibition of morphine-induced TLR4 signaling was assessed *in vivo*. This demonstrated that intrathecal co-administration of morphine with tricyclics of varying TLR4 putative activity potentiated morphine analgesia in the same rank order as their *in vitro* and *in silico* MD-2 activity data predicted. Moreover, genetic knockout mouse studies revealed that amitriptyline potentiation of morphine analgesia is TLR4 and MyD88 dependent. Hence, combined with *in vitro* and *in vivo* results, *in silico* docking simulations of tricyclic interaction with MD-2 predicted the observed *in vivo* activity. Together, these data represent a novel addition to an understanding of the pharmacologies of these tricyclics and have implications for their primary indications and the treatment of pain.

Tricyclics, such as tricyclic antidepressants, are commonly used “off label” for chronic pain states such as neuropathic and inflammatory pain (Eisenach and Gebhart, 1995a, b, McQuay et al., 1996, Abdel-Salam et al., 2003, Hauser et al., 2009). Indeed, antidepressants, especially tricyclic antidepressants, are widely used as first-line drugs for the treatment of neuropathic pain (Ikeda et al., 2009). However, while diverse actions have been proposed to contribute to their anti-allodynic properties, the complete mechanisms of actions of such drugs remain unknown (Mico et al., 2006).

As the present data support that tricyclics can either not alter (chlorpromazine), potentiate (oxcarbazepine), or inhibit (amitriptyline) TLR2 and TLR4 signaling, as measured by the altered expression of a reporter protein downstream of TLR2 or TLR4 activation, these data suggest a possible non-neuronal site(s) of action of these compounds, in addition to their previously characterized neuronal sites. Given that amitriptyline blocked *in vitro* TLR4-mediated Akt1 activation in response to either LPS or (–)-morphine, and the action of amitriptyline was MyD88-dependent, these data support that a site of action may be either at, or very close to, TLR4 itself. This conclusion is based on the fact that TLR4 activation leads, in turn, to MyD88 and Akt1 activation as two of the very early intracellular events (Dauphinee and Karsan, 2006, Laird et al., 2009). *In silico* MD-2 docking simulations support an action of the tricyclics at or above at minimum TLR4 and likely TLR2, hence lending computer modeling support to this hypothesis. Moreover, the ability of the full tricyclic *in silico* model to predict the TLR4 activity of several structurally disparate opioids previously established to have TLR4 activity also adds credence to the MD-2 site of action hypothesis.

The variable response of carbamazepine in the TLR4 and TLR2 HEK293 cell lines where no activity was observed, versus that of the BV-2 microglial cell line and primary neonatal rat microglial cultures where inhibition was quantified, cannot be explained at this stage. *In vivo* analgesia potentiation data indicates that carbamazepine has no effect on morphine-induced analgesia, agreeing with the hypotheses generated by the HEK293 data. However, the ability of carbamazepine to inhibit LPS induced IL-1 protein and mRNA expression in the BV-2 microglial cell line and primary rat microglia culture does not agree with this hypothesis.

Bioactivation of carbamazepine to a TLR2 or TLR4 active metabolite, such as carbamazepine-10,11-epoxide is plausible, but would require additional studies to be proven. As such, the cell type specific cell cultures appear to be more sensitive to displaying inhibitory results, rather than a negative result.

A potential, alternative explanation for the modulation of TLR4 (and possibly TLR2) signaling by tricyclics may be that some, but not all, tricyclics alter TLR2 and/or TLR4 signaling by disrupting lipid raft formation via their actions as inhibitors of acid sphingomyelinase. Acid sphingomyelinase is required for formation of the functional TLR4 receptor complex in lipid rafts (Cuschieri et al., 2007); whether this is also true for TLR2 is at present unknown. Assembly of, at minimum, the TLR4 receptor complex upon LPS stimulation is dependent upon ceramide, a sphingolipid metabolite produced by acid sphingomyelinase following LPS binding to CD14 (Cuschieri et al., 2007). One constraint on assuming that inhibition of acid sphingomyelinase accounts for the effects reported here lies in the fact that desipramine, imipramine, and amitriptyline have been reported to inhibit acid sphingomyelinase with the rank ordering of desipramine > imipramine > amitriptyline (Albouz et al., 1986), which is the opposite rank order documented here for these compounds in inhibiting TLR4 signaling (Figure 2A).

While the present data suggest at least an interaction of tricyclics at or close to TLR4 and likely TLR2, that does not imply whether or not the compounds may also have additional direct actions on downstream components of the TLR2 and TLR4 signaling cascades. A major consequence of blocking the downstream effects of TLR2 and/or TLR4 activation, and the one most relevant for pain control via TLR2 and/or TLR4 blockade, is the inhibition of production of proinflammatory cytokines. Two signaling molecules that have major control over the production of proinflammatory cytokines in the TLR2 and TLR4 signaling cascades are p38 MAPK and NF- κ B. Thus it is relevant to ask whether current literature predicts that tricyclics directly inhibit either of these. Regarding NF- κ B, treatment of immortalized neurons and PC12 pheochromocytoma (that is, cells unlikely to express TLR4) with amitriptyline or desipramine led to an increase in NF- κ B activation (Post et al., 2000, Bartholoma et al., 2002), rather than a decrease that would have been predicted if the suppression of NF- κ B-mediated SEAP expression by tricyclics in the present study were to be explained by a direct inhibition of NF- κ B by these drugs independent of TLR2 or TLR4. A similar picture arises upon an examination of the prior literature on p38 MAP kinase effects reported for tricyclics. Of the existing literature, only one prior paper that we are aware of examined the effects of tricyclics on LPS (Hwang et al., 2008), which would signal through TLR4. Here, clomipramine and imipramine were reported to decrease production of nitric oxide and tumor necrosis factor in microglia and astrocyte cultures as well as inhibit the expression of inducible nitric oxide synthase and proinflammatory cytokines such as interleukin-1 and tumor necrosis factor at mRNA levels. Associated with these changes was an inhibition of I κ B degradation, NF- κ B nuclear translocation and inhibition of phosphorylation of p38 MAP kinase, as would be predicted by the current study indicating an inhibition of TLR4 signaling by these tricyclics. In contrast, when tricyclics were studied in cells unlikely to express TLR4 or in cells stimulated by drugs acting through non-TLR4 pathways, tricyclics were reported to either have no effect on p38 MAP kinase (Chang et al., 2008) or to activate it (Otczyk et al., 2008, Lu et al., 2009), rather than inhibit it. These data are again not consistent with a direct inhibitory effect of tricyclics on p38 MAP or NF- κ B independent of the TLR4 signaling pathway. Clearly, directed research will be needed to clarify where along the TLR2 and TLR4 signaling pathway the tricyclics are having their inhibitor effects, whether this is limited to just TLR2 and TLR4, and whether any cofactors are necessary for the effects observed.

Interestingly, intrathecally administered tricyclics such as amitriptyline attenuate inflammatory pain and neuropathic pain, especially when combined with opioids (Eisenach and Gebhart, 1995a, Gray et al., 1998, Esser and Sawynok, 1999). In this regard, amitriptyline

has been reported to inhibit the development of morphine tolerance and to suppress the elevation of microglial proinflammatory cytokines that occurs as a consequence of chronic morphine (Tai et al., 2006), actions again consistent with a mechanism of action involving TLR4. A role for TLR2 in this mechanism has yet to be proven, but is possible given the similar proinflammatory downstream signaling consequences. The evidence of *in vivo* potentiation of intrathecal morphine analgesia by three tricyclics presented here extends these chronic morphine behavioral data. In addition, the failure of amitriptyline to further potentiate morphine analgesia in TLR4 or MyD88 knockout mice demonstrates the pivotal role these targets play in amitriptyline's pharmacological action. Moreover, given the potentiation of intrathecal morphine analgesia shares the same rank order as predicted from the *in silico* docking and *in vitro* TLR4 signaling inhibition data, this also provides *in vivo* validation of the *in silico* and *in vitro* data, TLR4/MD-2 hypothesis and a potential strategy for future drug development.

Finally, oxcarbazepine is currently used successfully in the treatment of orofacial pain (Reisner and Pettengill, 2001) but showed TLR4 activation in the present study, which is opposite to the result predicted from successful treatment of pain demonstrated in the Reisner et al. data. However, it is notable that oxcarbazepine is a prodrug for its active metabolite licarbazepine. Using the full tricyclic MD-2 *in silico* docking model presented here, MD-2 docking results indicate that licarbazepine is predicted to inhibit TLR4 signaling approximately 30%, in agreement with the clinical data demonstrating the prodrug nature of oxcarbazepine (Rogawski, 2006). Therefore, the potential TLR4/MD-2 actions of such drug metabolites should also be considered in addition to the parent compounds, just as we have previously found specific morphine metabolites to be important for understanding the effects of morphine via TLR4/MD-2 (Hutchinson et al., 2010, Lewis et al., 2010).

The present results support and extend prior studies reporting that at least some tricyclics prevent effects induced by LPS and PAM, and hence TLR4 and TLR2, respectively. Imipramine has been reported to inhibit the induction of iNOS and proinflammatory cytokine mRNAs, inhibit the activation of p38 MAPK, and inhibit the activation and nuclear translocation of NF κ B in LPS-stimulated microglial cultures (Hwang et al., 2004). Imipramine and desipramine have each been reported to suppress LPS-induced apoptosis, proposed to be via inhibition of Bcl-2 (Huang et al., 2007). In addition, imipramine has been observed to inhibit proinflammatory cytokine production by LPS-stimulated monocytes (Xia et al., 1996) and inhibit blood levels of these cytokines following systemic LPS administration (Dredge et al., 1999). Lastly, amitriptyline has been found to suppress proinflammatory cytokine release in LPS-stimulated mixed glia and pure microglial cultures (Obuchowicz et al., 2006). While such observations occur in the literature, the present study is the first to provide data suggesting suppression of TLR4 signaling as a potential underlying mechanism.

Given the multiple lines of evidence supporting anti-inflammatory and glial suppressive effects of tricyclics, one can naturally ask why they are not singularly effective in controlling chronic pain if such pain has a major glial component. One answer may lie in the fact that these compounds are complex chemical structures that clearly affect multiple sites of action such that their net effects reflect the summation of multiple individual ones. For example, tricyclic antidepressants induce significant adverse effects that limit their clinical use. Many antidepressants have side effects, including anticholinergic actions affecting ~60% of patients (Verdu et al., 2008) producing blurred vision, dry mouth, constipation, and urinary retention (Dredge et al., 1999, Saarto and Wiffen, 2007). In addition, they can cause postural hypotension, heart block and arrhythmias (Saarto and Wiffen, 2007). Other adverse effects including sedation, drowsiness or orthostatic hypotension are due to the antihistaminic and α 2 adrenergic actions of tricyclics (Saarto and Wiffen, 2007). Antidepressants can also induce physical dependence and withdrawal symptoms, adding to their adverse effects. Alternatively,

the doses required to achieve steady-state central nervous system concentrations capable of TLR4 signaling inhibition may be above those currently clinically prescribed owing to the side effect limitations outlined above. Whilst glia appear a possible cellular candidate of the cellular source of pronociceptive proinflammation that tricyclics counteract, the contributory and/or permissive role of other cell types within the CNS remains to be elucidated.

In summary, these data provide new insights into novel sites of action of tricyclics. Additional research is required to compare the rank order of anti-allodynic potency of tricyclics in human populations and animal models of neuropathic pain to confirm the current TLR2/TLR4 hypothesis. Additionally, the role of TLR2 and TLR4 in the primary indication (TLR2/TLR4 signaling inhibition) or off target side effects (TLR2/TLR4 signaling activation) of each of the tricyclics examined here may warrant further investigation given the expanding role of both glia and TLR2/TLR4 in a variety of pathologies. In addition, the roles of active metabolites and the potential for co-factors to modulate TLR2 and TLR4 activation should also be examined. In conclusion, these data demonstrate the TLR2 and TLR4 action of another structurally disparate class of small molecule xenobiotics highlighting the influential nature of at minimum TLR4 and likely TLR2 and their potential role in the pathologies where these clinically employed TLR2 and TLR4 signaling inhibitors are indicated.

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Abbreviations

cAMP	Cyclic Adenosine Monophosphate
EAAT	Excitatory Amino Acid Transporters
HEK293	Human Embryonic Kidney 293 cells
i.t.	intrathecal
MD-2	Myeloid Differentiation protein 2
MyD88	Myeloid differentiation primary response gene (88)
PDE	Phosphodiesterase
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4

Literature Cited

- Abdel-Salam OM, Nofal SM, El-Shenawy SM. Evaluation of the anti-inflammatory and anti-nociceptive effects of different antidepressants in the rat. *Pharmacol Res* 2003;48:157–165. [PubMed: 12798668]
- Albouz S, Le Saux F, Wenger D, Hauw JJ, Baumann N. Modifications of sphingomyelin and phosphatidylcholine metabolism by tricyclic antidepressants and phenothiazines. *Life Sci* 1986;38:357–363. [PubMed: 3945166]
- Bartholoma P, Erlandsson N, Kaufmann K, Rossler OG, Baumann B, Wirth T, Giehl KM, Thiel G. Neuronal cell death induced by antidepressants: lack of correlation with Egr-1, NF-kappa B and

- extracellular signal-regulated protein kinase activation. *Biochem Pharmacol* 2002;63:1507–1516. [PubMed: 11996893]
- Carmody J. Avoiding fallacies in nociceptive measurements. *Pain* 1995;63:136. [PubMed: 8577485]
- Chang HC, Huang CC, Huang CJ, Cheng JS, Liu SI, Tsai JY, Chang HT, Huang JK, Chou CT, Jan CR. Desipramine-induced apoptosis in human PC3 prostate cancer cells: activation of JNK kinase and capase-3 pathways and a protective role of $[Ca^{2+}]_i$ elevation. *Toxicology* 2008;250:9–14. [PubMed: 18606486]
- Cuschieri J, Bulger E, Billgrin J, Garcia I, Maier RV. Acid sphingomyelinase is required for lipid Raft TLR4 complex formation. *Surgical infections* 2007;8:91–106. [PubMed: 17381401]
- Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. *Lab Invest* 2006;86:9–22. [PubMed: 16357866]
- Dredge K, Connor TJ, Kelly JP, Leonard BE. Differential effect of a single high dose of the tricyclic antidepressant imipramine on interleukin-1 β and tumor necrosis factor- α secretion following an in vivo lipopolysaccharide challenge in rats. *Int J Immunopharmacol* 1999;21:663–673. [PubMed: 12609461]
- Dziarski R, Gupta D. Role of MD-2 in TLR2- and TLR4-mediated recognition of Gram-negative and Gram-positive bacteria and activation of chemokine genes. *J Endotoxin Res* 2000;6:401–405. [PubMed: 11521063]
- Dziarski R, Wang Q, Miyake K, Kirschning CJ, Gupta D. MD-2 enables Toll-like receptor 2 (TLR2)-mediated responses to lipopolysaccharide and enhances TLR2-mediated responses to Gram-positive and Gram-negative bacteria and their cell wall components. *J Immunol* 2001;166:1938–1944. [PubMed: 11160242]
- Eisenach JC, Gebhart GF. Intrathecal amitriptyline acts as an N-methyl-D-aspartate receptor antagonist in the presence of inflammatory hyperalgesia in rats. *Anesthesiology* 1995a;83:1046–1054. [PubMed: 7486155]
- Eisenach JC, Gebhart GF. Intrathecal amitriptyline. Antinociceptive interactions with intravenous morphine and intrathecal clonidine, neostigmine, and carbamylcholine in rats. *Anesthesiology* 1995b;83:1036–1045. [PubMed: 7486154]
- Esser MJ, Sawynok J. Acute amitriptyline in a rat model of neuropathic pain: differential symptom and route effects. *Pain* 1999;80:643–653. [PubMed: 10342426]
- Evans JH, Falke JJ. Ca^{2+} influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. *Proc Natl Acad Sci USA* 2007;104:16176–16181. [PubMed: 17911247]
- Gray AM, Spencer PS, Sewell RD. The involvement of the opioidergic system in the antinociceptive mechanism of action of antidepressant compounds. *Br J Pharmacol* 1998;124:669–674. [PubMed: 9690858]
- Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 1988;32:77–88. [PubMed: 3340425]
- Hauser W, Bernardy K, Uceyler N, Sommer C. Treatment of fibromyalgia syndrome with antidepressants: a meta-analysis. *JAMA* 2009;301:198–209. [PubMed: 19141768]
- Huang YY, Peng CH, Yang YP, Wu CC, Hsu WM, Wang HJ, Chan KH, Chou YP, Chen SJ, Chang YL. Desipramine activated Bcl-2 expression and inhibited lipopolysaccharide-induced apoptosis in hippocampus-derived adult neural stem cells. *J Pharmacol Sci* 2007;104:61–72. [PubMed: 17510525]
- Hutchinson MR, Bland ST, Johnson KW, Rice KC, Maier SF, Watkins LR. Opioid-induced glial activation: mechanisms of activation and implications for opioid analgesia, dependence and reward. *ScientificWorldJournal* 2007;7:98–111. [PubMed: 17982582]
- Hutchinson MR, Coats BD, Lewis SS, Zhang Y, Sprunger DB, Rezvani N, Baker EM, Jekich BM, Wieseler JL, Somogyi AA, Martin D, Poole S, Judd CM, Maier SF, Watkins LR. Proinflammatory cytokines oppose opioid-induced acute and chronic analgesia. *Brain Behav Immun* 2008a;22:1178–1189. [PubMed: 18599265]
- Hutchinson MR, Lewis SS, Coats BD, Skyba DA, Crysdale NY, Berkelhammer DL, Brzeski A, Northcutt A, Vietz CM, Judd CM, Maier SF, Watkins LR, Johnson KW. Reduction of opioid withdrawal and

- potentiation of acute opioid analgesia by systemic AV411 (ibudilast). *Brain Behav Immun* 2009;23:240–250. [PubMed: 18938237]
- Hutchinson MR, Northcutt AL, Chao LW, Kearney JJ, Zhang Y, Berkelhammer DL, Loram LC, Rozeske RR, Bland ST, Maier SF, Gleeson TT, Watkins LR. Minocycline suppresses morphine-induced respiratory depression, suppresses morphine-induced reward, and enhances systemic morphine-induced analgesia. *Brain Behav Immun* 2008b;22:1248–1256. [PubMed: 18706994]
- Hutchinson MR, Zhang Y, Brown K, Coats BD, Shridhar M, Sholar PW, Patel SJ, Crysdale NY, Harrison JA, Maier SF, Rice KC, Watkins LR. Non-stereoselective reversal of neuropathic pain by naloxone and naltrexone: involvement of toll-like receptor 4 (TLR4). *Eur J Neurosci* 2008c;28:20–29. [PubMed: 18662331]
- Hutchinson MR, Zhang Y, Shridhar M, Evans JH, Buchanan MM, Zhao TX, Slivka PF, Coats BD, Rezvani N, Wieseler J, Hughes TS, Landgraf KE, Chan S, Fong S, Phipps S, Falke JJ, Leinwand LA, Maier SF, Yin H, Rice KC, Watkins LR. Evidence that opioids may have toll like receptor 4 and MD-2 effects. *Brain Behav Immun* 2010;24:83–95. [PubMed: 19679181]
- Hwang CK, Kim CS, Choi HS, McKercher SR, Loh HH. Transcriptional regulation of mouse mu opioid receptor gene by PU.1. *J Biol Chem* 2004;279:19764–19774. [PubMed: 14998994]
- Hwang J, Zheng LT, Ock J, Lee MG, Kim SH, Lee HW, Lee WH, Park HC, Suk K. Inhibition of glial inflammatory activation and neurotoxicity by tricyclic antidepressants. *Neuropharmacology* 2008;55:826–834. [PubMed: 18639562]
- Ikeda T, Ishida Y, Naono R, Takeda R, Abe H, Nakamura T, Nishimori T. Effects of intrathecal administration of newer antidepressants on mechanical allodynia in rat models of neuropathic pain. *Neurosci Res* 2009;63:42–46. [PubMed: 18992286]
- Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB, Lee SJ. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. *J Biol Chem* 2007;282:14975–14983. [PubMed: 17355971]
- Laird MH, Rhee SH, Perkins DJ, Medvedev AE, Piao W, Fenton MJ, Vogel SN. TLR4/MyD88/PI3K interactions regulate TLR4 signaling. *J Leukoc Biol*. 2009
- Lewis SS, Hutchinson MR, Rezvani N, Loram LC, Zhang Y, Maier SF, Rice KC, Watkins LR. Evidence that intrathecal morphine-3-glucuronide may cause pain enhancement via toll-like receptor 4/MD-2 and interleukin-1beta. *Neuroscience* 2010;165:569–583. [PubMed: 19833175]
- Li Y, Li H, Zhang Y, Sun X, Hanley GA, LeSage G, Sun S, Peng Y, Yin D. Toll-like receptor 2 is required for opioids-induced neuronal apoptosis. *Biochem Biophys Res Commun* 2010;391:426–430. [PubMed: 19914204]
- Li Y, Sun X, Zhang Y, Huang J, Hanley G, Ferslew KE, Peng Y, Yin D. Morphine promotes apoptosis via TLR2, and this is negatively regulated by beta-arrestin 2. *Biochem Biophys Res Commun* 2009;378:857–861. [PubMed: 19071087]
- Lu T, Huang CC, Lu YC, Lin KL, Liu SI, Wang BW, Chang PM, Chen IS, Chen SS, Tsai JY, Chou CT, Jan CR. Desipramine-induced Ca-independent apoptosis in Mg63 human osteosarcoma cells: dependence on P38 mitogen-activated protein kinase-regulated activation of caspase 3. *Clin Exp Pharmacol Physiol* 2009;36:297–303. [PubMed: 18986328]
- McQuay HJ, Tramer M, Nye BA, Carroll D, Wiffen PJ, Moore RA. A systematic review of antidepressants in neuropathic pain. *Pain* 1996;68:217–227. [PubMed: 9121808]
- Mico JA, Ardid D, Berrocoso E, Eschaler A. Antidepressants and pain. *Trends Pharmacol Sci* 2006;27:348–354. [PubMed: 16762426]
- Milligan ED, Watkins LR. Pathological and protective roles of glia in chronic pain. *Nat Rev Neurosci* 2009;10:23–36. [PubMed: 19096368]
- Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 2007;19:3–10. [PubMed: 17275324]
- Noman AS, Koide N, Hassan F, IIE-K, Dagvadorj J, Tumurkhuu G, Islam S, Naiki Y, Yoshida T, Yokochi T. Thalidomide inhibits lipopolysaccharide-induced tumor necrosis factor-alpha production via down-regulation of MyD88 expression. *Innate Immun* 2009;15:33–41. [PubMed: 19201823]
- Obuchowicz E, Kowalski J, Labuzek K, Krysiak R, Pendzich J, Herman ZS. Amitriptyline and nortriptyline inhibit interleukin-1 release by rat mixed glial and microglial cell cultures. *Int J Neuropsychopharmacol* 2006;9:27–35. [PubMed: 15963243]

- Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. *Eur J Immunol* 2003;33:597–605. [PubMed: 12616480]
- Otczyk M, Mulik K, Budziszewska B, Jaworska-Feil L, Basta-Kaim A, Kubera M, Jagla G, Nowak W, Lason W. Effect of some antidepressants on the low corticosterone concentration-induced gene transcription in LMCAT fibroblast cells. *J Physiol Pharmacol* 2008;59:153–162. [PubMed: 18441395]
- Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 2009;458:1191–1195. [PubMed: 19252480]
- Post A, Crochemore C, Uhr M, Holsboer F, Behl C. Differential induction of NF-kappaB activity and neural cell death by antidepressants in vitro. *Eur J Neurosci* 2000;12:4331–4337. [PubMed: 11122343]
- Reisner L, Pettengill CA. The use of anticonvulsants in orofacial pain. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001;91:2–7. [PubMed: 11174562]
- Rogawski MA. Diverse mechanisms of antiepileptic drugs in the development pipeline. *Epilepsy Res* 2006;69:273–294. [PubMed: 16621450]
- Saarto T, Wiffen PJ. Antidepressants for neuropathic pain. *Cochrane Database Syst Rev*. 2007 CD005454.
- Slivka PF, Shridhar M, Lee GI, Sammond DW, Hutchinson MR, Martinko AJ, Buchanan MM, Sholar PW, Kearney JJ, Harrison JA, Watkins LR, Yin H. A peptide antagonist of the TLR4-MD2 interaction. *Chembiochem* 2009;10:645–649. [PubMed: 19184989]
- Song P, Zhao ZQ. The involvement of glial cells in the development of morphine tolerance. *Neurosci Res* 2001;39:281–286. [PubMed: 11248367]
- Tai YH, Wang YH, Wang JJ, Tao PL, Tung CS, Wong CS. Amitriptyline suppresses neuroinflammation and up-regulates glutamate transporters in morphine-tolerant rats. *Pain* 2006;124:77–86. [PubMed: 16697108]
- Tanga FY, Natile-McMenemy N, DeLeo JA. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* 2005;102:5856–5861. [PubMed: 15809417]
- Verdu B, Decosterd I, Buclin T, Stiefel F, Berney A. Antidepressants for the treatment of chronic pain. *Drugs* 2008;68:2611–2632. [PubMed: 19093703]
- Watkins LR, Hutchinson MR, Johnston IN, Maier SF. Glia: novel counter-regulators of opioid analgesia. *Trends Neurosci* 2005;28:661–669. [PubMed: 16246435]
- Watkins LR, Hutchinson MR, Rice KC, Maier SF. The “toll” of opioid-induced glial activation: improving the clinical efficacy of opioids by targeting glia. *Trends Pharmacol Sci* 2009;30:581–591. [PubMed: 19762094]
- Xia Z, DePierre JW, Nassberger L. Tricyclic antidepressants inhibit IL-6, IL-1 beta and TNF-alpha release in human blood monocytes and IL-2 and interferongamma in T cells. *Immunopharmacology* 1996;34:27–37. [PubMed: 8880223]

Toll Like Receptor 4

Figure 1. Tricyclics modify human TLR4 and TLR2 signaling *in vitro*

Tricyclic compounds of diverse pharmacologies modify lipopolysaccharide (LPS)-induced toll-like receptor 4 (TLR4) signaling (A-D). HEK293-hTLR4 cells were incubated with LPS (log doses from 0 to 100 ng/ml) with vehicle (media control) 1, 10 or 100 μ M, amitriptyline (A), imipramine (B), carbamazepine (C), oxcarbazepine (D). TLR4 signaling inhibition and activation could be observed as reflected by secreted alkaline phosphatase (SEAP) levels. Corresponding changes caused by tricyclics on TLR2 signaling were also quantified using HEK293-hTLR2 cells incubated with PAM3CSK4 (log doses from 0 to 100 ng/ml) with vehicle (media control) 1, 10 or 100 μ M, amitriptyline (E), carbamazepine (F), oxcarbazepine (G). $n = 3$ /group.

Figure 2. *in silico* MD-2 docking to *in vitro* TLR4 signaling prediction model

A: 4 tricyclics were used to build the *in silico* to *in vitro* prediction model which was subsequently tested on 3 different tricyclics, with the predicted and actual *in vitro* scores displayed. Superimposed actual and predicted values are indicated by the white box within the black box symbol. B: The modified complete 19 ligand *in silico* to *in vitro* model was retested on the structurally disparate 4,5-epoxymorphians examined previously (Hutchinson et al., 2010), with the predicted and actual *in vitro* scores displayed.

Figure 3. Amitriptyline inhibits LPS and (-)-morphine induced TLR4 Akt1 signaling

Basally, GFP-Akt1 is distributed evenly throughout the cytosol (A), but after Akt1 activation via signaling cascades such as those recruited by TLR4, rapid localization to the membrane occurs (B). LPS plus vehicle (200 ng/ml; ■; panel C) or (-)-morphine (200 μM; ♦, panel D) cause this significant membrane localization of GFP-Akt1 quantified by cytosolic clearance of GFP-Akt1 in a stably expressing RAW264.7 cell line. Pretreatment with amitriptyline (200 μM) significantly attenuates subsequent LPS and (-)-morphine-induced GFP-Akt1 membrane localization. To ensure the TLR4-selectivity of the inhibition the Akt1 blockade, cells were then stimulated with C5a (25 ng/ml), which utilizes a non-TLR4 pathway to activate Akt1.

Notably, C5a triggers significant GFP-Akt1 membrane localization. n = 10 cells/group from a minimum of 4 separate plates.

Figure 4. Modulation of LPS and PAM induced-Interleukin-1 response in Microglial BV-2 cell line LPS (100ng/ml) induced IL-1 expression is significantly inhibited by amitriptyline and carbamazepine *in vitro*, whilst oxcarbazepine significantly augments IL-1 expression (A; $P < 0.001$). Amitriptyline also significantly reduced PAM3CSK4 (100 ng/ml) induced IL-1 expression (B; $P < 0.001$). $n = 6$ /group.

Figure 5. Amitriptyline inhibits LPS induced-Interleukin-1 mRNA expression in primary neonatal rat microglia

LPS (100ng/ml) induced IL-1 β (A; $P < 0.0001$) and TNF- α (B; $P < 0.0001$) mRNA expression is significantly attenuated by amitriptyline *in vitro*. n = 4-6/group.

Figure 6. Differing potentiation of morphine analgesia by tricyclics

Intrathecal (i.t.) co-administration of morphine (15 μ g) with amitriptyline (A; 64 nM), cyclobenzaprine (B; 64 nM) or carbamazepine (C; 64 nM) led to differing potentiation of morphine tailflick analgesia (morphine + vehicle grey filled squares; morphine in combination with tricyclics black squares). Tricyclics and vehicles produced no change in withdrawal latencies (open diamonds). $n = 6/\text{group}$

Figure 7. Genetic knockout of TLR4 or MyD88 results in the loss of further potentiation of morphine analgesia by amitriptyline *in vivo*

Morphine analgesia (2.5 mg/kg) is significantly potentiated by genetic knockout of TLR4 or MyD88 compared to wild-type mice. Amitriptyline (51 mg/kg) significantly potentiated morphine analgesia in wild-type mice ($P < 0.001$) but has no additional effect in TLR4 or MyD88 knockout mice. $n = 6/\text{group}$