

**THE HERITABILITY OF ANTINOCICEPTION II:
PHARMACOGENETIC MEDIATION OF THREE OVER-THE-COUNTER
ANALGESICS IN MICE**

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ABSTRACT

Chromosomal loci containing genes affecting antinociceptive sensitivity to morphine have been identified, but virtually nothing is known about the genetic mediation of sensitivity to over-the-counter analgesics. Such knowledge would be of great clinical interest, since prodigious interindividual variability has been noted in the efficacy of these ubiquitously used drugs. In the present study we assessed heritability and genetic correlations among three over-the-counter analgesics in mice of 12 inbred mouse strains on the 0.9% acetic acid (i.p.) writhing test. Analgesics included the centrally acting analgesic, acetaminophen (150 mg/kg, s.c.), and the non-steroidal anti-inflammatory drugs (NSAIDs) indomethacin (40 mg/kg, s.c.) and lysine-aspirin (800 mg/kg, s.c.). Significant strain differences in sensitivity to each of the drugs were observed, with narrow-sense heritability estimates ranging from 23-45%. Similar strains were sensitive and resistant, respectively, to the two NSAIDs ($r_s = 0.64$). In contrast, a completely different pattern of sensitivities was observed for acetaminophen, implying genetic dissociation ($r_s = 0.29$ and 0.02) compared to the NSAIDs. Additional experiments were performed on two strains, C57BL/6 and DBA/2, with extreme sensitivities to acetaminophen. Plasma acetaminophen levels in these strains were not significantly different during the time of antinociception assessment, suggesting the existence of genetic factors affecting acetaminophen pharmacodynamics rather than pharmacokinetics.

Acetaminophen (paracetamol) and nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used medications in the world, both by prescription and over-the-counter. The physiological basis of their antiinflammatory, antinociceptive/antihyperalgesic and antipyretic actions has been debated for some time. The classical explanation that these drugs work via inhibition of peripheral prostaglandin synthesis has been seriously challenged by evidence for central actions (see Yaksh et al., 1998). The discovery of multiple cyclooxygenase enzymes (see Smith and DeWitt, 1996)—the constitutively-expressed cyclooxygenase-1 and the inducible cyclooxygenase-2—represented a great advance in the understanding of NSAID action, and the recent discovery of cyclooxygenase-3, a splice variant of the cyclooxygenase-1 gene (Chandrasekharan et al., 2002), may explain acetaminophen action. However, it remains unknown whether any cyclooxygenase isoform is necessary and/or sufficient for the antinociceptive actions of over-the-counter analgesics. Evidence from transgenic knockout mice lacking these genes has failed to converge (Ballou et al., 2000; Guhring et al., 2002; see Wallace, 1999), and thus a new approach may be of value.

Considerable variability in both the antinociceptive and antiinflammatory responses to individual over-the-counter drugs has been reported (e.g., Huskisson et al., 1976; Scott et al., 1982; Bellamy, 1985; Day et al., 1988; Walker et al., 1994). This variability has rendered it all but impossible to rank such drugs in terms of therapeutic efficacy. In clinical practice, failure to obtain relief with one drug leads to rotation among the common NSAIDs until a satisfactory response is achieved or a switch is made to narcotic analgesics. The cause of this variability is unknown, but is likely unrelated to disease variability as it can be demonstrated in controlled experimental situations (e.g., Walker

et al., 1994). Although pharmacokinetic variations among subjects are a favored explanation of over-the-counter antinociceptive variability, several studies have failed to demonstrate such a relationship (see Walker, 1995). Other proposed explanations of response variability to over-the-counter drugs include sex differences (Walker and Carmody, 1998) and the strength of the placebo effect (Amanzio et al., 2001).

Genetic factors have never been seriously considered as an explanation of variable sensitivity to over-the-counter drugs, although antinociceptive sensitivity to opioids is known to be strongly affected by inherited genetic factors (see Mogil, 1999). The chromosomal locations of genes underlying morphine sensitivity in mice are known (Bergeson et al., 2001; see Mogil, 1999), and pharmacological evidence supports the candidacy of the *Oprm* (μ -opioid receptor) and *Htr1b* (serotonin-1B receptor) genes as contributing to variable morphine antinociception in sensitive (DBA/2J) versus resistant (C57BL/6J) strains (see Mogil, 1999). Very little is known about genetic factors underlying variable responses to any other analgesic drug. In a recent study, we demonstrated a surprisingly high genetic correlation between antinociceptive sensitivity to morphine and four neurochemically distinct analgesics: the κ -opioid agonist, U50,488; the cannabinoid, WIN55-212,2; the nicotinic agonist, epibatidine; and, the α_2 -adrenergic agonist, clonidine (Wilson et al., 2003). In that study, the same 12 strains were tested for their antinociceptive response to the five drugs, and similar strains were found to be sensitive and resistant, respectively, to each one. This finding implicates the same gene(s) in the mediation of sensitivity to *all* of the drugs. In addition, for all drugs a high correlation was obtained between initial nociceptive sensitivity (on the 49°C tail-withdrawal test) and subsequent drug response (Wilson et al., 2003).

Thus, the purposes of the present study were two-fold. First, we wished to investigate whether sensitivity to over-the-counter antinociception is heritable in the mouse, exhibiting inbred strain mean differences. Second, we wished to investigate whether sensitivity to different over-the-counter drugs would correlate genetically, as we had found previously for other centrally acting drugs (Wilson et al., 2003), or would show genetic dissociation, which might be predicted from the clinical reality described above. Acetaminophen, indomethacin and lysine-acetylsalicylic acid (lysine-aspirin) were chosen for this study as they are known to differ greatly in their anti-inflammatory efficacy (McCormack and Brune, 1991), cyclooxygenase enzyme selectivity (e.g., Meade et al., 1993), mode of antagonistic action on cyclooxygenase enzymes (see Smith and DeWitt, 1996) and effects on prostaglandin-independent signaling mechanisms (see Tegeder et al., 2001). To these ends, we determined the antinociceptive sensitivity of 11 inbred mouse strains to acetaminophen (150 mg/kg, s.c.), indomethacin (40 mg/kg, s.c.) and lysine-aspirin (800 mg/kg, s.c.) on the 0.9% acetic acid abdominal constriction (writhing) test. For each drug, full dose-response relationships were then examined in extreme-responding strains. For one drug, acetaminophen, follow-up experiments were performed to establish the generalizability of the strain differences and to investigate whether those differences were due to pharmacodynamic or pharmacokinetic factors.

MATERIALS AND METHODS

Subjects

Male and female breeders of the outbred strains ND4 Swiss Webster (Hsd:ND4) or CD-1 (Hsd:ICR) were obtained from Harlan Sprague Dawley Inc. (Indianapolis, IN). Male and female breeders of eleven inbred strains—129P3, A, BALB, C3H/He, C57BL/6, C57BL/10, CBA, DBA/2, and RIIS, all “J” substrains—were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice used in the studies were bred in our temperature-controlled ($20\pm 2^{\circ}\text{C}$) vivarium under a 12:12 h light/dark cycle (lights on at 07:00 h). Mice were weaned at 18-21 days of age, housed with same-sex littermates in groups of 2-5, and given ad lib access to Purina mouse chow and water. Both sexes were used in these experiments.

All mice were tested in a quiet room just meters away from the vivarium. Every attempt was made to counterbalance strains across testing schedules, but the vagaries of breeding success prevented true counterbalancing. Nonetheless, all mice were handled equivalently, and all testing was performed on at least two strains simultaneously. Also, to control against day-to-day variability exerting an undue influence on these data, we tested saline-treated mice along with drug-treated mice in every session. Each mouse used was only once, and given a single dose of one drug. Sample sizes ranged from $n=5-24$ per dose per strain (depending on strain availability) in all experiments.

Drugs

Acetaminophen and indomethacin (Sigma, St. Louis, MO) were dissolved in a solution of physiological saline and 1,2-propanediol in a ratio of 87.5%:12.5%, and titrated to pH=7 with NaOH. Acetaminophen was injected subcutaneously (s.c.; 10 ml/kg) in doses ranging from 0-400 mg/kg; indomethacin in doses ranging from 0-160 mg/kg. A pilot study performed in both an outbred (ND4 Swiss Webster) and an inbred (DBA/2) strain confirmed that this vehicle did not produce antinociception on the tests used (data not shown). We were unable, however, to solubilize or adequately suspend acetylsalicylic acid (aspirin) in any vehicle that did not itself produce antinociception. For this reason, we used water-soluble lysine-aspirin in these experiments. We are unaware of any published difference in the pharmacodynamic mechanisms underlying the antinociceptive effects of lysine-aspirin relative to the reference compound. Lysine-aspirin (Aspegic; Synthelabo, France) was dissolved in normal saline, and injected s.c. in doses ranging from 0-1600 mg/kg.

Pilot experiments using outbred mice revealed linear and dose-dependent antinociception from each drug (data not shown), with the following half-maximal antinociceptive doses (AD_{50} s; see below) for each drug: acetaminophen (116 mg/kg), indomethacin (41 mg/kg), and lysine-aspirin (460 mg/kg). We chose 150 mg/kg acetaminophen and 40 mg/kg indomethacin as “probe” doses for the inbred strain survey. For lysine-aspirin, a sex difference was observed, such that the AD_{50} for males was 386 mg/kg and for females was 1255 mg/kg. For this reason, we decided on 800 mg/kg as an appropriate probe dose. It should be noted that this sex difference has been

subsequently found to be unreliable, as we were unable to replicate it in ND4 Swiss Webster mice or any other inbred strain.

Nociceptive Assessment

Mice were assessed for nociceptive sensitivity using the 0.9% acetic acid abdominal constriction (writhing) test as described by Koster (1959). A similar model has been shown to be useful in correlating the ED₅₀ values of NSAID antinociception in mice with those in humans (Pong et al., 1985). Although this assay features considerable variability (e.g., a non-negligible percentage of non-responders), which is particularly problematic for the experiments described herein, it is the only common nociceptive assay in the mouse to feature reliable sensitivity to weak over-the-counter analgesics (see Wilson and Mogil, 2001). Although over-the-counter drugs are reported to be effective in the tonic phase of the formalin test (see Yaksh et al., 1998), we did not observe convincing antinociception at non-toxic doses in pilot studies. We have been able to reduce the percentage of non-responding mice in the writhing test by using a slightly higher acetic acid concentration, 0.9%, than is commonly used in this assay (0.6%).

All mice were acclimatized to the procedure room for at least 1 h prior to testing. All testing occurred near midphotoperiod (10:00–16:00 h). Mice were placed on a glass surface within transparent Plexiglas cylinders (29 cm high; 30 cm diameter) and allowed 30 min to habituate to the cylinder. At that point the mice were weighed, injected with drug or vehicle (10 ml/kg, s.c.), and then placed back into the cylinder. Twenty min later, 0.9% acetic acid (in saline) was injected intraperitoneally (i.p.; 10 ml/kg). Mice were placed in the cylinders once again and observed continuously for 30 min. Stereotypical

writhes—lengthwise constrictions of the torso with a concomitant concave arching of the back—were counted over this period in 5-min bins. Four mice were observed and scored at a time. In all cases, the experimenter was kept "blind" to the drug/vehicle solutions.

In some acetaminophen experiments the 50°C hot-plate test was used. This assay is a modification of the classic hot-plate test; the lower stimulus intensity is designed to increase the test's sensitivity to weak analgesics (Ankier, 1974). Mice were placed on a metal surface maintained at 50.0±0.2°C (IITC Model PE34M-HC). Locomotion of the mouse on the plate was constrained by 20-cm high Plexiglas walls to an area 14 x 14 cm. Latency to respond to the heat stimulus was measured to the nearest 0.1 s. Mice remained on the plate until they performed either of two behaviors regarded as indicative of nociception: hindpaw lick or hindpaw shake/flutter. Subjects were tested immediately before, and at various time points after injection of saline or acetaminophen; they remained in their home cages in between tests. A cut-off latency of 150 s was imposed to prevent the possibility of tissue damage.

Data Analysis

In writhing experiments, where within-animal baselines cannot be obtained, antinociception was quantified by reference to the saline-treated control group for each strain. Percent antinociception was calculated as: $[(\text{saline-treated mean writhes} - \text{drug-treated writhes}) / \text{saline-treated mean writhes}] \times 100$, with the saline-treated mean writhes value being calculated separately for each strain in each experiment. In hot-plate experiments, percent antinociception was quantified with respect to the subject's own baseline, as: $[(\text{post-injection latency} - \text{baseline latency}) / (150 - \text{baseline latency})] \times 100$.

Half-maximal antinociceptive doses (AD_{50} s), potency ratios and associated 95% confidence intervals were calculated from percent antinociception data by the method of Tallarida and Murray (1981).

The presence of strain and sex differences was assessed with ANOVA. In all cases, a criterion α level of 0.05 was employed. In a single case, a datum from one mouse receiving acetaminophen was removed from the data set after being identified as a statistical outlier (Studentized residual = -3.7).

Because there are no dominance effects in inbred strains, narrow-sense heritability (h^2) can be estimated from the between-strain variance (V_a) and the within-strain/error variance (V_e) using the formula: $h^2 = V_a/(V_a+V_e)$, which is based on the population intraclass correlation coefficient. The variance components were estimated in our sample using PROC VARCOMP (SAS version 8.2, The SAS Institute, Cary, NC). Type I sums of squares estimation (in which observed mean squares are equated to expected mean squares and solved for the appropriate variance component) was used because this is more robust to potential violation of normality assumptions. A fixed-effect of dose was considered in each ANOVA. Because strains were chosen without respect to the traits under investigation, these values are likely to be reflective of the variability present in the overall mouse population (Hegmann and Possidente, 1981).

Genetic correlations among traits (vehicle-treated sensitivity, acetaminophen antinociception, indomethacin antinociception, ASA antinociception) were assessed using correlation coefficients applied to the relevant strain means. Since the joint distribution of the phenotypes was not normal, Spearman's correlation (r_s) for rank data was employed.

Full dose-response curves were collected in extreme-responding strains and used to calculate the half-maximal antinociceptive dose (AD_{50}) for each inbred mouse strain, using the method of Tallarida and Murray (1981).

Acetaminophen Pharmacokinetics

In order to assess whether strain differences in antinociception from acetaminophen were due to pharmacokinetic or pharmacodynamic factors, we assessed antinociceptive sensitivity and plasma acetaminophen levels in B6 and D2 mice at 30, 60 or 120 min post-injection. Immediately following behavioral assessment of thermal nociceptive sensitivity at one of the three post-injection time points, mice were decapitated and trunk blood was collected and placed on ice. The blood was centrifuged and plasma (about 0.1 ml) was removed and freeze-dried according to VIRTUS Technology lyophilization protocols.

Plasma concentrations of acetaminophen were determined by a modification of published HPLC procedures (Granados-Soto et al., 1993). Briefly, to 100 μ l of freeze-dried mouse plasma was added 50 μ l of 0.5 mg/ml phenacetin (internal standard) and the mixture extracted with 5 ml ethyl acetate. The solvent was then evaporated and the residue redissolved in 50 μ l methanol. Samples (10 μ l) were injected onto a HPLC system, which consisted of a C8 HPLC column (Platinum EPS C8 100A, 5 μ M; 150 x 4.6 mm; Alltech Associates Pty. Ltd., Australia) and eluted with a mixture of 0.05 M sodium acetate buffer (pH=4.0) with acetonitrile (92.5:7.5) at a constant flow of 1 ml/min. The effluent from the column was recorded by UV detection at 254 nm using a Shimadzu UV Detector (SPD10Avp, Shimadzu Corporation, Japan). Retention times for acetaminophen and the internal standard were 3.4 and 11.3 min, respectively. The limit

of determination of acetaminophen was 0.5 $\mu\text{g/ml}$, and the inter-assay coefficient of variation was 6%.

Concentration-time data were analyzed using non-linear mixed effects modeling implemented in P-PHARM Version 1.3 (Gomeni et al., 1994). The population approach examines fixed (e.g., pharmacokinetic model parameters such as clearance and volume of distribution) and random (e.g., inter-animal variance of pharmacokinetic parameters and residual variability) effects (Aarons, 1991).

Preliminary analysis of the pooled plasma concentration-time data for acetaminophen indicated that a one-compartment pharmacokinetic model best described the disposition of this drug in the mouse. The parameters of the combined pharmacokinetic model were the apparent volume of distribution (V/F ; where F is the fraction of the dose that gets absorbed and was assumed to be unity), first order absorption rate constant (K_a) and apparent clearance (CL).

Random effects are considered to consist of inter-individual variability in each pharmacokinetic parameter with the remaining variability being termed the residual or unexplained variability within animals (Gomeni et al., 1994). In this study the inter-animal variability in pharmacokinetic parameters was best described using a normal distribution and the residual variability which encompasses measurement error and model misspecification was assumed to be constant across the study population.

P-PHARM generates the population mean of pharmacokinetic parameters and an estimate of the inter-animal variability in this parameter (expressed as a percent coefficient of variation). Posterior Bayesian parameter estimates for each animal are also

generated. This allows the concentration time profile in plasma to be determined despite only one sample being available in each animal.

RESULTS

Strain differences in baseline sensitivity on the writhing test

As expected, inbred strain differences were observed in the sensitivity to 0.9% acetic acid in vehicle-treated mice. In all three experiments, there was a significant main effect of strain (acetaminophen: $p < 0.05$; indomethacin: $p < 0.001$; lysine-aspirin: $p < 0.001$). Estimates of the heritability of writhing test sensitivity calculated from these three data sets range from $h^2 = 0.22$ -0.61. Although the three experiments were performed separately and by two different experimenters, significant genetic correlations were observed between responses to 0.9% acetic acid in all cases ($r_s = 0.69$ -0.74, $p < 0.001$). Also, significant genetic correlations ($r_s = 0.73$ -0.90, $p < 0.001$) were observed between vehicle-treated strain means in this experiment and strain means of mice given no s.c. injection and receiving 0.6% acetic acid in a previous study (Mogil et al., 1999), suggesting that neither the vehicle injection nor the increased acetic acid concentration markedly affected responses.

Given the stability of strain sensitivities to this nociceptive model over time and experimenters, to increase the accuracy of the antinociceptive sensitivity estimates we pooled all vehicle data (n per strain = 13-25). Once pooled, ANOVA revealed a significant main effect of strain ($F_{10,194} = 7.94$; $p < 0.001$) corresponding to a heritability estimate of $h^2 = 0.34$. Neither the main effect of sex nor the sex x strain interaction was significant, although a trend for higher sensitivity in females was observed ($p = 0.27$).

Strain differences in sensitivity to acetaminophen

Strain means of mice treated with vehicle and 150 mg/kg acetaminophen are shown in Fig. 1a; percent antinociception scores calculated from these data are shown in Fig. 1b. ANOVA revealed a significant main effect of strain on acetaminophen antinociception ($F_{10,100} = 3.02, p < 0.005$), corresponding to a heritability estimate of $h^2 = 0.24$. The main effect of sex and sex x strain interaction were not significant.

A perusal of Fig. 1b suggests that five strains were clear “responders” to acetaminophen (A, AKR, C3H/He, C57BL/6 and C57BL/10), having percent antinociception scores ranging from 40 to 70%, whereas the remaining strains were “weak responders” (and non-responders at this particular dose) with scores ranging from -2.2 to 23%. Thus, to confirm the reliability of these findings, we chose one strain from each category, C57BL/6 (responder) and DBA/2 (weak responder), and compiled full dose-response curves (0-800 mg/kg). As shown in Fig. 1c, the strain difference persists over a wide range of acetaminophen doses, with AD_{50} s differing in these strains by approximately 5-fold.

Strain differences in sensitivity to indomethacin

Strain means of mice treated with vehicle and 40 mg/kg indomethacin are shown in Fig. 2a; percent antinociception scores calculated from these data are shown in Fig. 2b. ANOVA revealed a significant main effect of strain on acetaminophen antinociception ($F_{10,62} = 1.99, p = 0.05$), corresponding to a heritability estimate of $h^2 = 0.23$. The main effect of sex was not significant, but the sex x strain interaction was highly significant ($F_{10,62} = 3.02, p < 0.005$). This interaction manifested itself as significantly greater

indomethacin antinociception in males of two strains relative to females (129P3, AKR), and significantly greater antinociception in females of two strains relative to males (BALB/c, RIIS). Caution should be applied, however, since sex-specific sample sizes were too low to engender confidence in these findings. Overall conclusions regarding genetic correlations were unchanged when male and female indomethacin data were considered separately.

A perusal of Fig. 2b suggests that four strains were clear responders to indomethacin (129P3, C57BL/10, C58 and DBA/2), having percent antinociception scores ranging from 60 to 75%, whereas the remaining strains were weaker responders. Unlike in the acetaminophen experiment, all strains displayed convincing antinociception at the dose of indomethacin used. To confirm the reliability of these findings, we chose one strain from each category, C58 (responder) and CBA (weak responder), and compiled full dose-response curves (0-160 mg/kg). As shown in Fig. 2c, the strain difference persists over a wide range of indomethacin doses, although calculation of precise AD_{50} s was complicated by incomplete efficacy in both strains.

Strain differences in sensitivity to lysine-aspirin

Strain means of mice treated with vehicle and 800 mg/kg lysine-aspirin are shown in Fig. 3a; percent antinociception scores calculated from these data are shown in Fig. 3b. ANOVA revealed a significant main effect of strain on lysine-aspirin antinociception ($F_{10,58} = 5.73$, $p < 0.001$), corresponding to a heritability estimate of $h^2 = 0.45$. The main effect of sex and sex x strain interaction were not significant, although the main effect of sex approached significance ($p=0.11$; males: $59.3 \pm 5.7\%$; females: $50.1 \pm 5.2\%$).

A perusal of Fig. 3b suggests that most strains were clear responders to lysine-aspirin, having percent antinociception scores ranging from 40 to 90%, whereas two strains were weak responders (BALB/c, CBA). To confirm the reliability of these findings, we chose one strain from each category, 129P3 (responder) and BALB/c (weak responder), and compiled full dose-response curves (0-1600 mg/kg). As shown in Fig. 3c, the strain difference persists over a wide range of lysine-aspirin doses, with AD_{50} s differing in these strains by approximately 4-fold.

Genetic correlations

There appeared to be essentially no correlation between strain means of vehicle-treated mice (i.e., baseline sensitivity on the 0.9% writhing test) and antinociceptive sensitivity to acetaminophen, indomethacin or lysine-aspirin ($r_s = -0.12, -0.23, -0.19$, respectively). This lack of genetic correlation was also observed when experiment-specific vehicle means were used instead of combined vehicle means (not shown).

As shown in Figure 4, a significant genetic correlation ($r_s = 0.64$) was obtained between sensitivities to indomethacin and lysine-aspirin, the two NSAIDs tested. However, neither indomethacin nor lysine-aspirin showed a genetic correlation with acetaminophen ($r_s = -0.02, 0.29$, respectively), confirming the impression that different strains were sensitive to acetaminophen than to the two NSAIDs. The same conclusion was arrived at when Pearson correlation coefficients were applied (not shown).

Finally, there appeared to be very little genetic correlation between sensitivity to the three analgesics tested here and five analgesics tested previously (morphine, U50,488,

WIN55,212-2, epibatidine, clonidine) (Wilson et al., 2003), with Spearman correlation coefficients ranging from $r_s = -0.34$ to 0.50, and averaging $r_s = 0.14$.

Sensitivity of C57BL/6 and DBA/2 mice to acetaminophen on the 50°C hot-plate test

To establish the generalizability of these findings to a nociceptive assay other than the writhing test, we tested C57BL/6 and DBA/2 mice for their sensitivity to acetaminophen antinociception on the 50°C hot-plate test. As shown in Figure 5a, a strain difference in the same direction as in the writhing test was observed. Calculation of precise AD_{50} s was complicated by incomplete efficacy in both strains, especially DBA/2, in which 50% antinociception was never achieved at any dose. Doses higher than 800 mg/kg could not be tested because of lethal toxicity.

Acetaminophen pharmacokinetics in C57BL/6 and DBA/2 mice

The mean plasma acetaminophen concentration over time following a 400 mg/kg (s.c.) acetaminophen injection in the same two mouse strains is shown in Figure 5b. The temporal profiles of acetaminophen concentrations in C57BL/6 and DBA/2 are very similar, despite the fact that C57BL/6 mice display robust antinociception on the hot-plate test at this dose and DBA/2 mice display no antinociception whatsoever (see Fig. 5a). This result was confirmed by the pharmacokinetic modeling analysis which indicated that the clearance, volume of distribution and absorption rate constant were not different, with any apparent differences being small (see Table 1).

DISCUSSION

To our knowledge, this study is the first to examine the genetic mediation of over-the-counter antinociceptive efficacy, although a large literature exists concerning genetic factors contributing to the toxicity of these compounds (see Giri, 1993). As expected based on our previous experience with multiple analgesics (Wilson et al., 2003), all three compounds tested here displayed mild-to-moderate heritability. This work represents the first step towards identification of genes whose inheritance may predict drug efficacy. Information about the pharmacogenetics of this drug class may lead to more efficient and successful management of a number of pain conditions. These findings may also have more immediate application to the management of postoperative pain in laboratory animals (see Liles and Flecknell, 1992).

The efficacy of over-the-counter analgesics on the mouse writhing test is well known (see Taber, 1974). Given the variable stimulus intensity of different nociceptive assays and the presently demonstrated strain differences in sensitivity, it is difficult to comment on the relation between antinociceptive magnitudes observed herein and those in the literature. We do note, however, that the ratio of acetaminophen:indomethacin:lysine-aspirin AD_{50} s in ND4 Swiss Webster mice, for which complete dose-response curves were compiled for all three drugs, was 116 mg/kg:41 mg/kg:460 mg/kg. This ratio is comparable to similar ratios collected previously in mice (Pong et al., 1985; Hunskaar and Hole, 1987). We do not believe that our current demonstration of acetaminophen antinociception on the hot-plate test should be interpreted as contradicting the general notion that over-the-counter drugs are ineffective on acute, thermal assays of nociception.

In our hands, only a sensitive strain displayed robust acetaminophen antinociception against a weak thermal stimulus (50°C), at a very high dose (400 mg/kg), and with submaximal efficacy (see Fig. 5).

Genetic Correlations Among Over-the-Counter Analgesics

We observed presently a moderate-to-large genetic correlation between sensitivity to lysine-aspirin and indomethacin ($r_s = 0.64$), but no correlation between either of these drugs and acetaminophen ($r_s = 0.02$ and 0.29 , respectively). Since genetic correlation implies overlapping physiological mediation (see Mogil, 2000), it is worth a brief mention of the known commonalities and dissociations among these drugs.

Acetaminophen has always been the outlier among over-the-counter drugs, displaying very weak anti-inflammatory effects (at least peripherally) (McCormack and Brune, 1991; but see Honore et al., 1995). It has therefore been argued that acetaminophen, unlike NSAIDs, may work in the central nervous system (e.g., Piletta et al., 1991). However, evidence for central actions of NSAIDs too is now overwhelming (see Yaksh et al., 1998), with dose-dependent suppression of pain behavior in animal models produced by intrathecal or intracerebroventricular injections of a number of NSAIDs at doses far lower than those required for peripheral effects. There exist many published dissociations between aspirin and indomethacin action as well. Whereas aspirin inhibits inducible nitric oxide synthase, transcription factor NF- κ B and Erk protein kinase in murine cell lines (independent of cyclooxygenase-2 inhibition), indomethacin (and acetaminophen) do not (see Tegeder et al., 2001). Aspirin can block neurogenic inflammation when injected centrally, indomethacin and steroid anti-inflammatory

drugs can not (Catania et al., 1991). Even the mode of cyclooxygenase antagonism differs between aspirin, a non-competitive antagonist producing irreversible acetylation of the cyclooxygenase site, and indomethacin, which causes a conformational change (see Smith and DeWitt, 1996).

Despite these differences, the current finding of genetic correlation between lysine-aspirin and indomethacin directly predicts the existence of genes commonly (pleiotropically) affecting the two NSAIDs, and the existence of distinct genes affecting acetaminophen antinociception. One obvious possibility is that NSAID antinociception may be affected by allelic variation in *Ptgs2*, the murine gene encoding cyclooxygenase-2, whereas acetaminophen antinociception is affected by allelic variation in *Ptgs1*, the gene encoding cyclooxygenase-1 and, more importantly for acetaminophen, the cyclooxygenase-3 splice variant (Chandrasekharan et al., 2002). This possibility is being investigated at the present time using quantitative trait locus (QTL) analysis (see Mogil, 1999), but our recent experience cautions against the assumption that antinociceptive variation need be explained by allelic variation at the gene coding for the drug's molecular binding site. As mentioned above, surprisingly high genetic correlations were observed for antinociceptive sensitivity to five neurochemically distinct compounds (Wilson et al., 2003). Such a finding all but eliminates the possibility that genetic variability in the level or functionality of each drug's receptor was responsible for variability in antinociceptive response. Indeed, variability in antinociceptive efficacy may derive from allelic variation at any gene producing a protein involved in: a) the neural circuit mediating drug antinociception, from binding site to effector site; b) signal transduction mechanisms within any neuron in that circuit; and/or c) pharmacokinetic

mechanisms impacting drug concentration at relevant binding sites. With regard to the latter, it appears that at least for acetaminophen, the relevant genes are playing a pharmacodynamic role, since strain-dependent hot-plate antinociception was not accompanied by evidence of strain-dependent pharmacokinetic parameters (see Table 1) or plasma acetaminophen concentration (see Figure 5).

Genetic Correlations Between Writhing Test Sensitivity and Over-the-Counter Drug Antinociception

Our previous investigation into genetic mediation of antinociception revealed, in addition to genetic correlation among disparate analgesics, that antinociceptive sensitivity was correlated with initial nociceptive sensitivity (Wilson et al., 2003). This phenomenon was not observed presently, with genetic correlations between writhing test sensitivity and acetaminophen, indomethacin and lysine-aspirin antinociception of $r_s = -0.12, -0.23, -0.19$, respectively. It is difficult to speculate on whether the contradictory findings are secondary to the nociceptive assays being used (tail-withdrawal and formalin tests vs. writhing test) or the different drug classes being tested. It is also the case that genetic correlations between the drugs tested in our previous study (morphine, U50,488, WIN55,212-2, epibatidine, clonidine) and those tested presently were almost all very low ($r_s = -0.34$ – 0.50 ; mean $r_s = 0.12$; all corrected for sign). This low correlation may indicate genetic independence of over-the-counter analgesics from those known to activate descending pain modulatory systems, but the lack of correlation may also be explained by the different nociceptive assays used in each case (e.g., Elmer et al., 1997).

Conclusions

The finding of genotype-dependent sensitivity to over-the-counter drug antinociception represents the first step in the identification of genes associated with such variability. This, in turn, may shed new light on the mechanism of action of these important therapeutics. Given the known spinal and supraspinal actions of over-the-counter drugs (see Yaksh et al., 1998), their myriad cyclooxygenase-independent actions and interactions with central opioidergic and serotonergic circuitry (e.g., Bjorkman, 1995), it is far from clear that the antinociceptive effects of this drug class can be adequately explained by cyclooxygenase inhibition alone. Thus, the fact that the cyclooxygenase genes are now well known does not preclude the important involvement of any number of additional genes. For example, an intriguing new hypothesis (Guhring et al., 2002) is that NSAIDs, by virtue of blocking cyclooxygenase, may liberate arachadonic acid to be transformed by an unknown pathway to anandamide, which has been shown to produce antinociception when released endogenously (Walker et al., 1999).

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FOOTNOTES

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Table 1. Population mean pharmacokinetic parameters for C57BL/6 and DBA/2 mice following 400 mg/kg s.c. acetaminophen, with inter-animal variability expressed as coefficient of variation (CV).

Strain	Parameter ^a	Mean	CV
C57BL/6	CL (ml/min/kg)	12.1	28.1
	V (L/kg)	0.58	38.6
	K _a (min ⁻¹)	0.020	37.3
DBA/2	CL (ml/min/kg)	14.1	18.5
	V (L/kg)	0.87	15.4
	K _a (min ⁻¹)	0.067	36.1

^aCL: clearance; V: volume; K_a: absorption rate constant.

Figure Legends

Fig. 1. Genotype dependence of acetaminophen antinociception in 11 inbred mouse strains. Total number of writhes in a 30-min period following 0.9% acetic acid injection is shown in A; mice were pretreated with vehicle (see text) or 150 mg/kg acetaminophen. Raw data shown in A are converted to percent antinociception scores for acetaminophen-treated mice in B. In C, dose-response relationships are shown for a sensitive (C57BL/6) and resistant (DBA/2) strain. Bars and symbols in all graphs represent mean (\pm S.E.M.). *Significantly different from the other strain, $p < 0.05$.

Fig. 2. Genotype dependence of indomethacin antinociception in 11 inbred mouse strains. Total number of writhes in a 30-min period following 0.9% acetic acid injection is shown in A; mice were pretreated with vehicle (see text) or 40 mg/kg indomethacin. Raw data shown in A are converted to percent antinociception scores for indomethacin-treated mice in B. In C, dose-response relationships are shown for a sensitive (C58) and resistant (CBA) strain. Bars and symbols in all graphs represent mean (\pm S.E.M.). *Significantly different from the other strain, $p < 0.05$.

Fig. 3. Genotype dependence of lysine-aspirin antinociception in 11 inbred mouse strains. Total number of writhes in a 30-min period following 0.9% acetic acid injection is shown in A; mice were pretreated with vehicle (see text) or 800 mg/kg lysine-aspirin. Raw data shown in A are converted to percent antinociception scores for lysine-aspirin-treated mice in B. In C, dose-response relationships are shown for a

sensitive (129P3) and resistant (BALB/c) strain. Bars and symbols in all graphs represent mean (\pm S.E.M.). *Significantly different from the other strain, $p < 0.05$.

Fig. 4. Genetic correlations among over-the-counter analgesics. Symbols represent ranked inbred strain means, from most sensitive (1) to least sensitive (11). Spearman's correlation coefficients are shown in each scatterplot. As can be seen, similar strains are sensitive and resistant, respectively, to lysine-aspirin and indomethacin. By contrast, a different set of strains are sensitive and resistant to acetaminophen.

Fig. 5. Strain-dependent acetaminophen antinociception in C57BL/6 and DBA/2 mice is not accompanied by differential acetaminophen plasma concentrations. In A, dose-response relationships of acetaminophen antinociception on the 50°C hot-plate test are shown. Bars represent mean (\pm S.E.M.) percent antinociception (see text). Both strains exhibit submaximal acetaminophen antinociception on this assay, but at 400 mg/kg C57BL/6 mice display robust antinociception whereas DBA/2 mice display none at all. However, as shown in B, at this same dose, both strains display equivalent concentrations of plasma acetaminophen (symbols represent mean \pm S.E.M. plasma concentrations; see text). *Significantly different from the other strain, $p < 0.05$.

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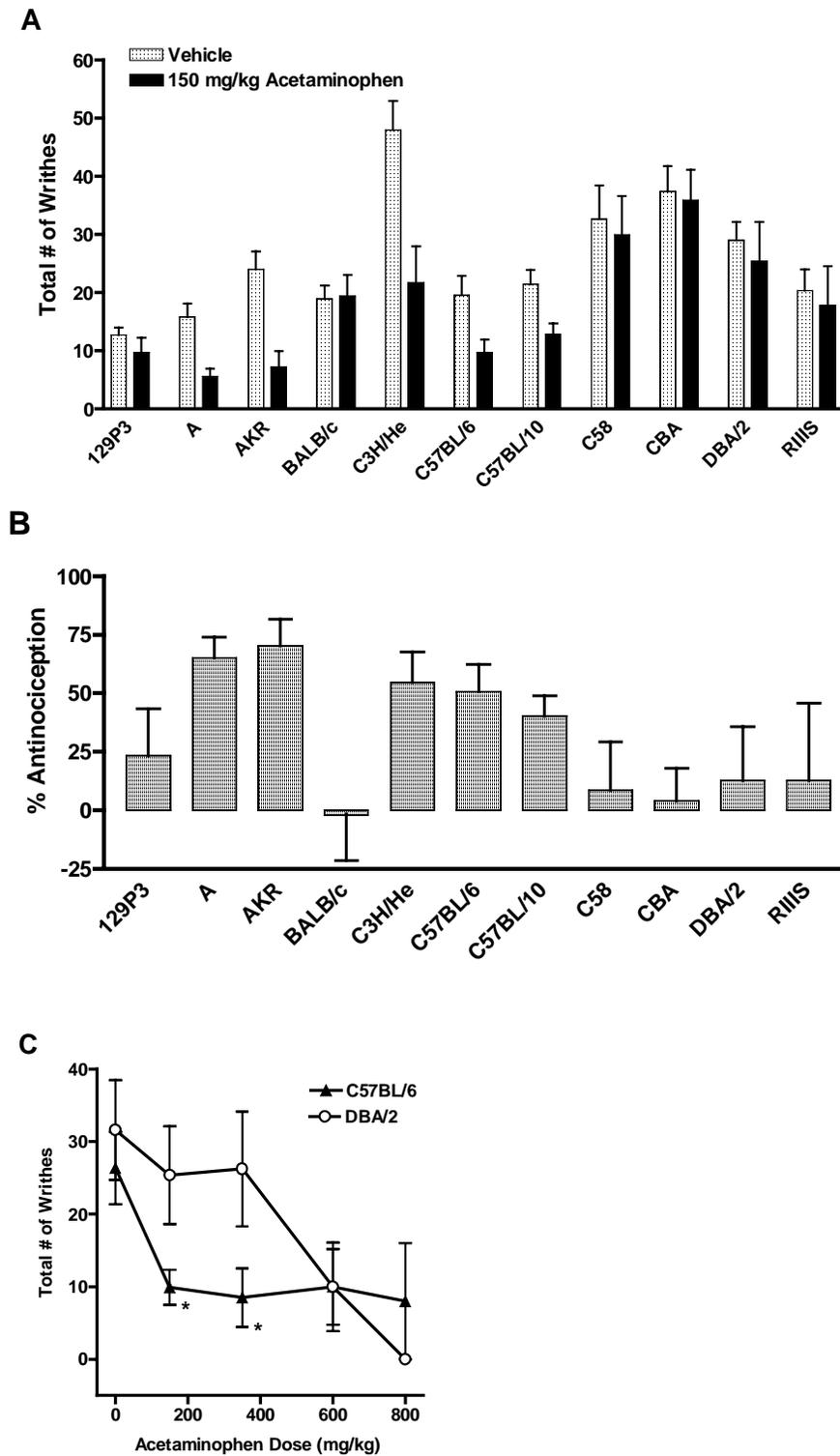


Figure 1

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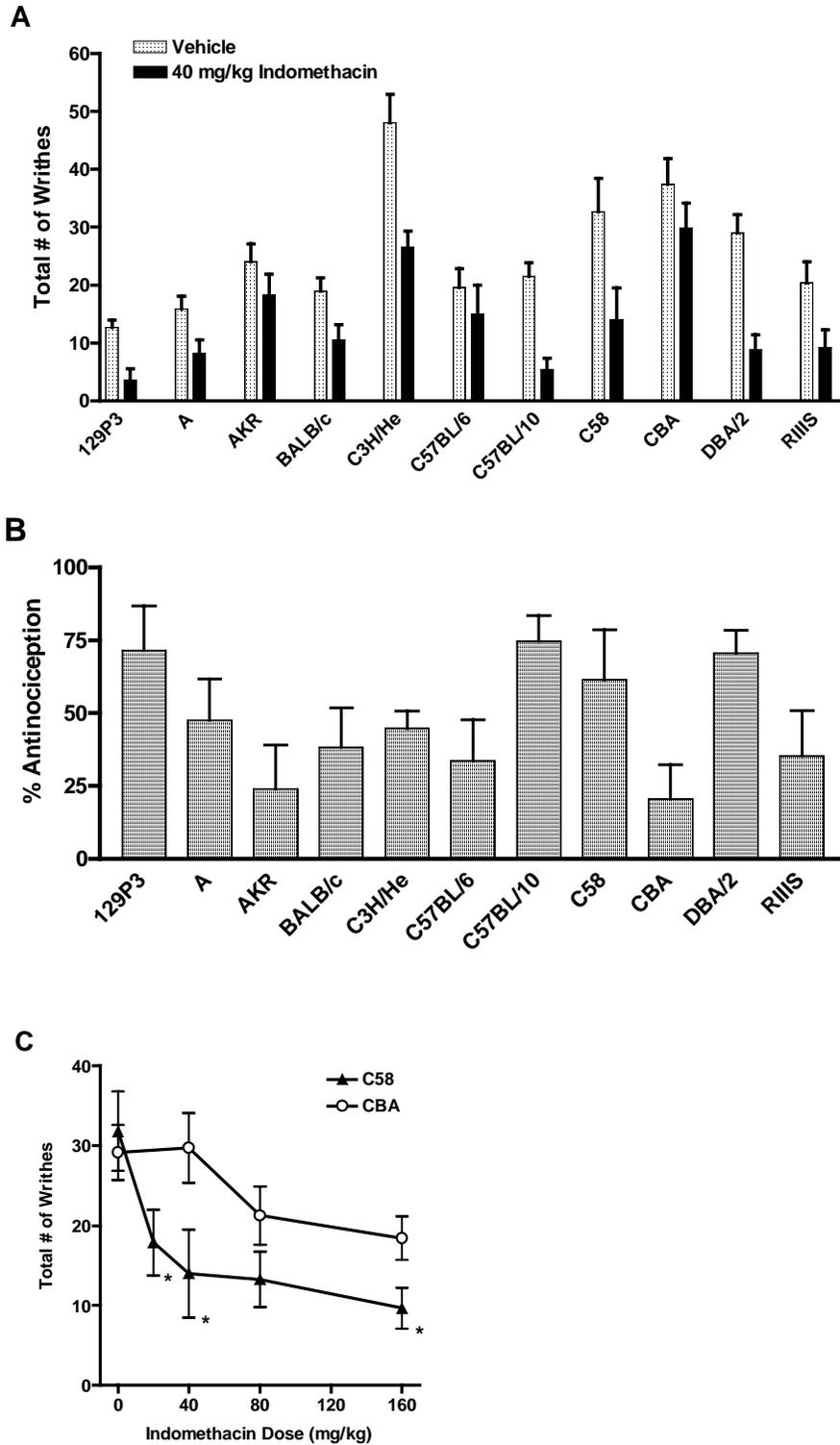


Figure 2

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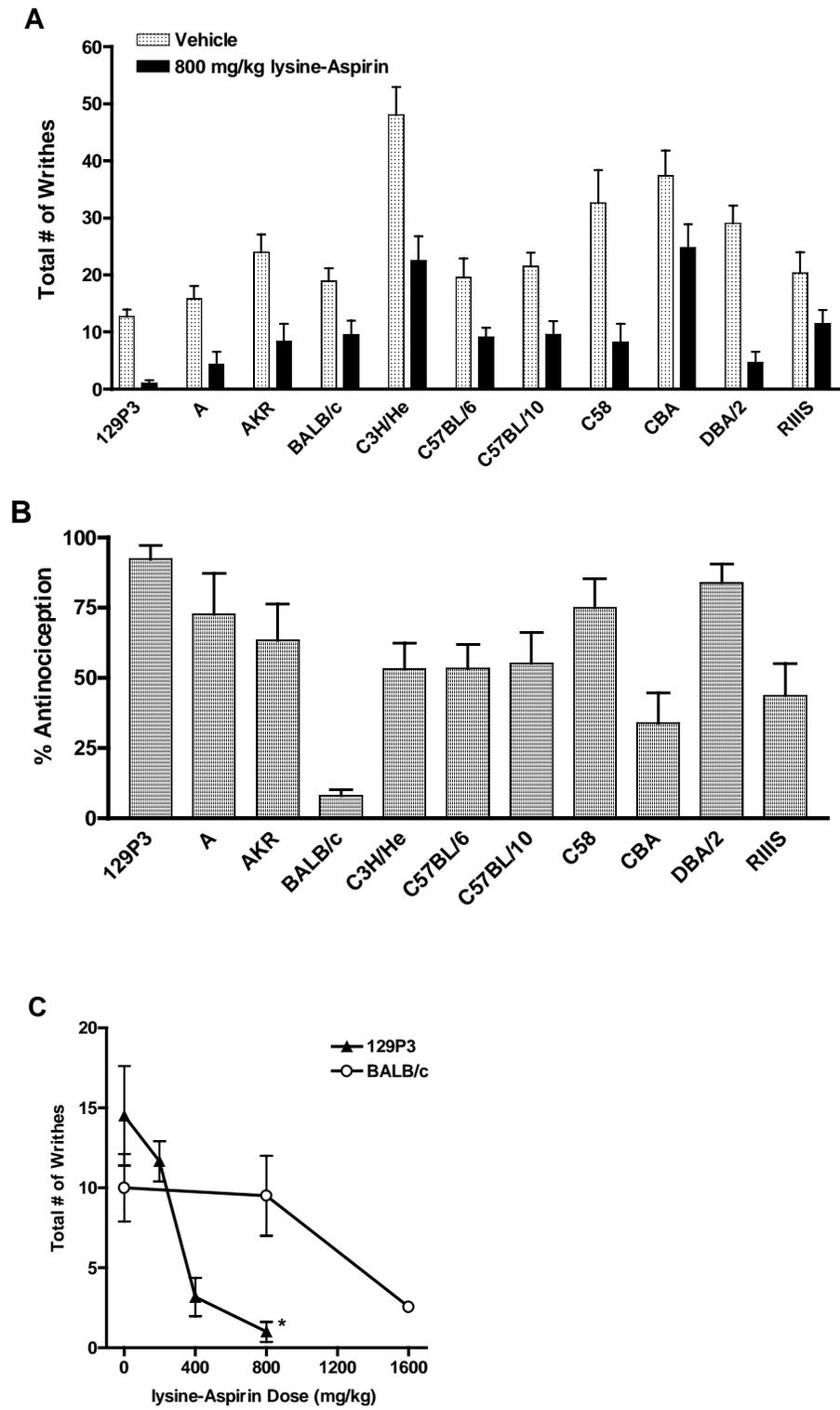


Figure 3

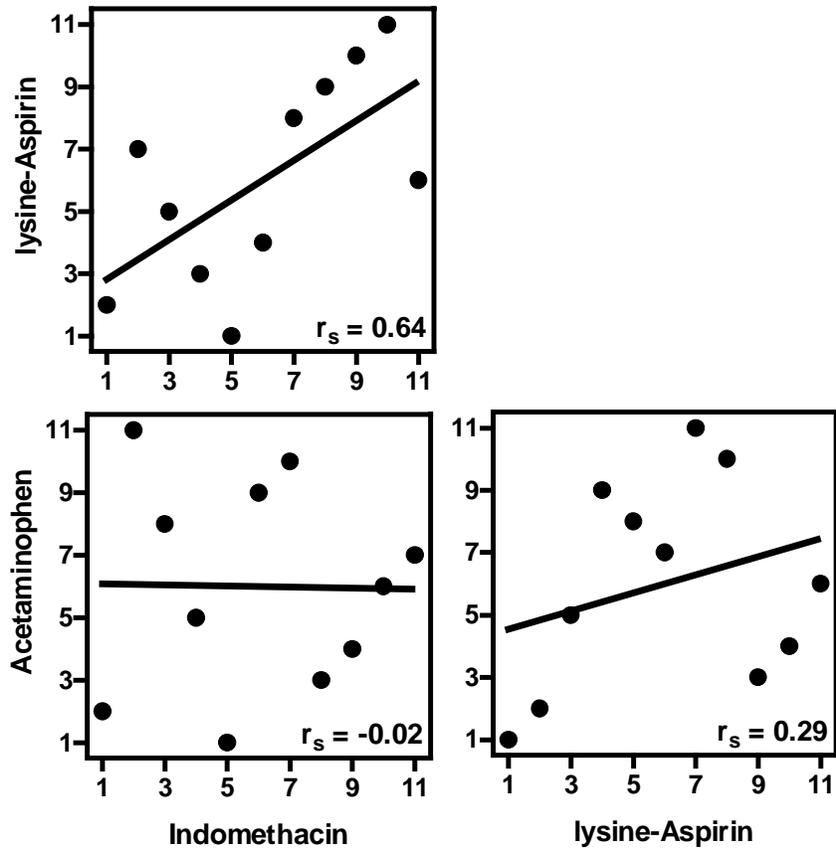


Figure 4

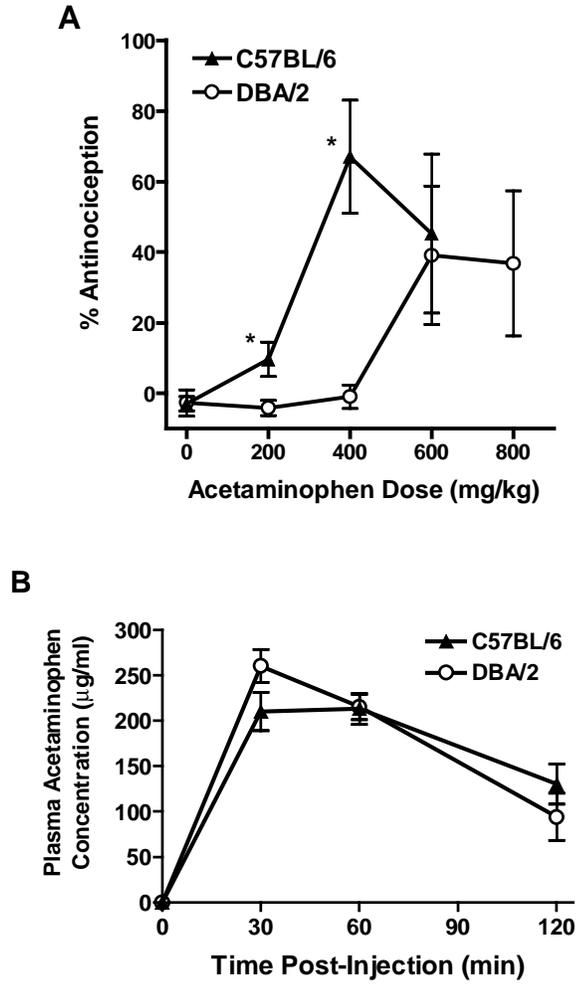


Figure 5