The Ethanol Metabolite Acetaldehyde Increases Paracellular Drug Permeability In Vitro and Oral Bioavailability In Vivo

Scott J. Fisher, Peter W. Swaan, and Natalie D. Eddington

Department of Pharmaceutical Sciences, University of Maryland, Baltimore, MD 21201
Running Title Page

a) Running Title: Acetaldehyde increases drug permeability in vivo

b) Corresponding Author:

Peter W. Swaan, Ph.D., Department of Pharmaceutical Sciences, University of Maryland, 20 Penn Street, Baltimore, MD 21201 USA
Tel: (410) 706-0103
Fax: (410) 706-5017
Email: pswaan@rx.umaryland.edu

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ABSTRACT

Alcohol consumption leads to the production of the highly reactive ethanol metabolite, acetaldehyde which may affect intestinal tight junctions and increase paracellular permeability. We examined the effects of elevated acetaldehyde within the gastrointestinal tract, on the permeability and bioavailability of hydrophilic markers and drug molecules of variable molecular weight and geometry. *In vitro* permeability was measured unidirectionally in Caco-2 and MDCKII cell models in the presence of acetaldehyde, ethanol or disulfiram, an aldehyde dehydrogenase inhibitor, which causes acetaldehyde formation when co-administered with ethanol *in vivo*. Acetaldehyde significantly lowered transepithelial resistance in cell monolayers and increased permeability of the low molecular weight markers, mannitol and sucrose; however, permeability of high molecular weight markers, polyethylene glycol and inulin, was not affected. *In vivo* permeability was assessed in male Sprague-Dawley rats treated for six days with ethanol, disulfiram, or saline alone or in combination. Bioavailability of naproxen was not affected by any treatment, while that of paclitaxel was increased upon acetaldehyde exposure. While disulfiram has been shown to inhibit MDR1 P-glycoprotein *in vitro*, our data demonstrate that the known P-gp substrate paclitaxel is not affected by co-administration of disulfiram. In conclusion, we demonstrate that acetaldehyde significantly modulates tight junctions and paracellular permeability *in vitro* as well as the oral bioavailability of low molecular weight hydrophilic probes and therapeutic molecules *in vivo* even when these molecules are substrates for efflux transporters. These studies emphasize the significance of ethanol metabolism and drug interactions outside of the liver.
INTRODUCTION

The metabolism of ethanol leads to the production of acetaldehyde, a highly promiscuous intermediate, that is able to react with various proteins and results in the formation of adducts at concentrations as low as 5 µM (Salmela et al., 1997). Lysine is the major amino acid residue that is associated with adduct formation (Tuma et al., 1987) via an intermediary Schiff’s base (Braun et al., 1995). Proteins that preferentially form adducts with acetaldehyde include hemoglobin, albumin, tubulin, lipoproteins, collagen, and CYP2E1 (Niemela, 1999). While the rate of protein adduct formation remains unclear, the number of nucleophilic amino acid residues (e.g Lys, Cys) within the protein apparently increases total adduct formation (Mauch et al., 1986).

Upon the administration of ethanol, high concentrations of acetaldehyde can be measured in blood. In rats, peak blood acetaldehyde concentrations are in the millimolar range (Kinoshita et al., 1996); in human blood, after ethanol consumption, concentrations have been measured in the micromolar range (Nuutinen et al., 1983), and it should be noted that this range is considered pathophysiologically relevant (Atkinson and Rao, 2001). It has also been estimated that 30 µM free, unreacted, non-adduct acetaldehyde circulates in the blood of heavy drinkers (Brecher et al., 1997).

Emerging evidence suggests that pathophysiologically relevant concentrations of acetaldehyde are also found in the stomach and upper gastrointestinal (GI) tract. Various alcohol dehydrogenase (ADH) isoenzymes (I - IV) occur at different levels along the GI tract of the rat (Julia et al., 1987; Vaglenova et al., 2003). While ADH IV predominates the epithelial surface of the esophagus and stomach, only ADH I is found in the duodenum (Vaglenova et al., 2003). Upon increasing levels of ethanol consumption, ADH I in the gut becomes saturated and it has been observed that CYP2E1 is induced in both stomach and small intestine to levels 25% above controls (Pronko et al., 2002). While ADH metabolism of ethanol in the GI tract is comparatively
lower than that in the liver, it may still contribute significantly to local concentrations of acetaldehyde in the upper GI tract (Pronko et al., 2002).

In concert with adherens junctions, tight junctions and their associated actin filaments compose the apical junctional complex between epithelial cells of the small intestine. The loosening of this complex and the formation of the “leaky gut” in chronic alcoholics has received substantial research interest (Atkinson and Rao, 2001; Keshavarzian et al., 2001; Basuroy et al., 2005). Studies demonstrate that acetaldehyde increases tyrosine phosphorylation via modulation of tyrosine kinases or PTPases in three tight junctional proteins: ZO-1, E-cadherin, and β-catenin, which may be responsible for increased membrane permeability (Atkinson and Rao, 2001). In the lower GI tract, anaerobic bacteria express ADH and have the ability to metabolize ethanol and produce acetaldehyde (Rao, 1998). In both humans and rats, colon acetaldehyde levels may achieve mM concentrations (Koivisto and Salaspuro, 1997; Rao, 1998; Atkinson and Rao, 2001). This is an important fact, as endotoxin is suspected to enter the bloodstream via “leaky” tight junctions and play a role in alcohol-induced liver damage (Keshavarzian et al., 1999).

The concentration of acetaldehyde in the upper GI tract becomes significant as this area represents the site of absorption for most clinically therapeutic drugs. Higher paracellular permeability due to disrupted tight junctions may result in highly variable oral bioavailability; this is especially problematic for drugs with narrow therapeutic windows. It is reported that acetaldehyde formation in the intestine is proportional to the ethanol concentration delivered (Koivisto and Salaspuro, 1997). We hypothesize that the levels of acetaldehyde in the upper small intestine are sufficient to disturb tight junctional organization thereby affecting drug absorption. This study examines changes in paracellular permeability in epithelial cell culture models and in a rat model of acetaldehyde formation. Using hydrophilic markers and drug molecules of variable
molecular weight and geometry we demonstrate that ethanol metabolism can significantly affect drug absorption and disposition.

METHODS

Chemicals and Reagents

Dulbecco’s modified Eagles medium (DMEM), phosphate buffered saline (PBS), non-essential amino acids (NEAA), sodium pyruvate, penicillin, streptomycin, L-glutamine, and trypsin/EDTA were purchased from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS, 0.1 µM filtered) was purchased from Gemini Bio-Products (Woodland, CA). Transwells® 12mm diameter (surface area 1.13 cm²) 0.4 µM pore size were purchased from Corning Costar (Cambridge, MA). Acetaldehyde (AcH), tetraethylthiuram disulfide (disulfiram), D-Mannitol-[1-3H(N)] (specific activity 18 Ci/mmol; radiochemical purity>97%), [3H]-inulin (212 mCi/g; purity ≥95%) and ethanol (EtOH) were purchased from Sigma Chemical Company (St. Louis, MO). [U-14C]-Sucrose (498 mCi/mm; purity >97%) was purchased from Perkin Elmer (Boston, MA). [14C]-PEG 4000 (0.87 mCi/g; purity >95%) and [14C]-naproxen sodium (50 mCi/mm; purity >98%) were purchased from American Radiolabeled Chemicals (St. Louis, MO). [14C]-paclitaxel (68 mCi/mm; purity >97%) was purchased from Moravek Radiochemicals (Brea, CA). Universol® scintillation cocktail was purchased from MP Biomedical (Irvine, CA).

Cell Culture

Human colon adenocarcinoma (Caco-2) cells and Madin-Darby canine kidney (MDCK II) cells were purchased from the American Type Culture Collection (Manassas, VA). Caco-2 cells were used from passage 25 – 45 and MDCKII cells were used from passage 25 – 35 in an effort to derive monolayers with consistent morphological and biological features. All cells were maintained in 75 cm² tissue culture flasks at 37 °C, in a
humidified atmosphere of 5% CO$_2$ and were cultured in high glucose DMEM, supplemented with 10% FBS, 0.1 mM NEAA, 2 mM L-glutamine 1 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were passaged to 80% confluence and were released by trypsinization (0.25% trypsin and 0.25% EDTA). MDCKII and Caco-2 cells for use in study were plated at a seeding density of 50,000 and 80,000 cells/cm$^2$, respectively; in 12-well tissue culture treated Transwells®. Cell media was changed every other day and experiments were performed upon confluence in MDCKII cells (5 days) and upon differentiation in Caco-2 cells (25 days). At this time each monolayer is expected to have polarized, with well developed apical brush-borders and tight junctions. Monolayer integrity was assessed by TEER value as described below.

**Measurement of Transepithelial Resistance (TEER)**

TEER of confluent monolayers was measured as an indicator of monolayer integrity as previously described (Hidalgo et al., 1989), using a Millicell-ERS ohmmeter (Millipore, Bedford, MA). TEER was calculated as Ω·cm$^2$ by multiplying it by the surface area of the insert (1.13 cm$^2$) and subtracting the internal resistance of the insert before calculations (25 Ω·cm$^2$). TEER was measured at 0, 60, 120, 180, 240 and 300 minutes. Baseline TEER values for Caco-2 and MDCKII monolayers were between 450-600 and 200-250 Ω·cm$^2$.

**In Vitro Paracellular Permeability Measurements**

The transport of radiolabeled paracellular markers was measured in cells seeded onto Transwell® plates in the presence or absence of ethanol (20% v/v), acetaldehyde (1 mM) or disulfiram (25 mg/ml). The molecular weights of the markers ranged from mannitol (182.2 Da), sucrose (342.3 Da), PEG (4000 Da) and inulin (5000 Da). Caco-2
cells were used at day 25 and MDCKII cells at day 5 and upon confluence, washed twice in PBS. To assess effects on permeability, transport of markers was measured from apical to basolateral chambers at 60, 120, 180, and 240 minutes (n = 6). Cell treatments were agitated orbitally at no greater than 50 rpm and were performed in sealed air tight containers at 37 °C, to prevent evaporation, and in the study with acetaldehyde, it was replaced each hour. Radioactivity was measured using scintillation counting. Apparent permeability (P_{app}) was calculated as:

\[ P_{app} = \frac{\Delta Q}{\Delta t} \cdot \frac{1}{A \cdot C_{(0)}} \]  

(Eq. 1)

Where \( \Delta Q/\Delta t \) is the linear appearance rate of mass in receiver solution in basolateral chamber, \( A \) is the cross-sectional area of the Transwell®, and \( C_{(0)} \) is the initial concentration of the marker in the apical chamber.

**Animals**

Adult male Sprague-Dawley rats (250 – 280 g) were used in the pharmacokinetic measurement of permeability markers. They were purchased from Harlan (Indianapolis, IN). The study protocols were approved by the Institutional Animal Care and Use Committee of the School of Pharmacy (University of Maryland, Baltimore). Rats were housed in the animal facility at a room temperature of 23 ± 1 °C. Food was withheld 4 hour prior to oral dosing, and rats were allowed free access food post dosing (Purina 5001 Rodent Chow; Purina, St. Louis, MO). Water was given *ad libitum*, and they were maintained on a 12-h light/dark cycle (light on from 7:00 AM to 7:00 PM).

**Ethanol Dosing and In Vivo Permeability Measurements**

Rats in each group (n=5) were treated with saline, disulfiram (100 mg/kg), ethanol 5 g/kg body weight (given as 33% v/v solution in saline, approximately 7M) or
ethanol and disulfiram (5 g/kg body weight (7M) and 100 mg/kg (84M), respectively). Treatments were administered twice daily via oral gavage for a period of six days. On day five, all animals were anesthetized and underwent jugular vein cannulation. On day six, one of four hydrophilic markers ([3H]-mannitol, [14C]-sucrose, [3H]-inulin, or [14C]-PEG 4000) or [14C]-naproxen or [14C]-paclitaxel were given via oral gavage to each group. Serial blood samples were taken at 0, 20, 40, 60, 90, 120, 240, 480, 720, and 1440 minutes post dose. Blood was centrifuged (10,000 x g for 10 min) immediately and plasma was obtained. Scintillation cocktail was added and plasma samples were analyzed for radioactivity by Beckman Coulter LS 6500 multi-purpose Scintillation counter. Non-compartmental pharmacokinetic analysis was performed to determine the radio-marker absorption in each treatment group using WinNonlin version 3.1 (Pharsight, Cary, NC).

Statistical Analysis

All samples in all experiments were analyzed for statistically significant differences between groups using single-factor ANOVA with the α value set a priori at p < 0.05. In vitro and in vivo values are expressed as mean ± standard error of mean (S.E.M) obtained from 6 experiments (in vitro) or 5 samples (in vivo).

RESULTS

Reversibility of Transepithelial Electrical Resistance upon Ethanol and Acetaldehyde Treatment

TEER measurement is a frequent method used to assess the integrity of Caco-2 cell monolayers (Hidalgo et al., 1989; Swaan et al., 1997; Cox et al., 2002; Salama et al., 2003; Salama et al., 2004). TEER was measured each hour over the course of the
experiment (5 hrs). Treatment of Caco-2 cell monolayers with both ethanol (20% v/v) and acetaldehyde (1 mM) significantly lowered TEER values from 1 to 4 hours (Fig. 1A); however, the effect of acetaldehyde was more pronounced. The decrease in TEER values for ethanol and acetaldehyde treatment in MDCKII cell lines paralleled those observed in Caco-2 cells (Fig. 1B). Maximal decrease in TEER was observed upon acetaldehyde treatment at 240 minutes and was 73.5 ± 2.5% in Caco-2 cells and 49.6 ± 3.0% in MDCKII cells (Figure 1), returning to starting TEER values 60 minutes after withdrawal of treatment. This reversibility suggests that the opening of tight junctions is not due to cellular cytotoxicity. These results are consistent with our previous data showing that doses of ethanol and acetaldehyde used here are not cytotoxic (Fisher et al., 2008).

**Ethanol and Acetaldehyde Increase Permeability of Paracellular Markers in Caco-2 and MDCK Cell Models**

To assess the degree of tight junctional opening, various hydrophilic markers of paracellular permeability of increasing molecular weight were employed in the Caco-2 cell monolayer. [3H]-mannitol (182.2 Da), [14C]-sucrose (342.3 Da), [14C]-PEG 4000, (4000 Da), and [3H]-inulin (5000 Da) were evaluated in the presence and absence of disulfiram, ethanol and acetaldehyde. Disulfiram treatment did not result in any significant effect on the permeability of any marker (Fig. 2A). Ethanol treatment significantly increased permeability of both [3H]-mannitol and [3H]-inulin (Fig. 2B). Compared to ethanol and disulfiram, acetaldehyde treatment resulted in the most pronounced increase in P_app for 3 out of the 4 permeability markers as well as higher overall transport of [3H]-mannitol in both cell lines (Figs. 2C,F). Mannitol flux values are generally higher and more variable (0.1-2.0·10^{-6} cm²/s) in the present study compared to other reports (Volpe, 2008) and cannot be explained by low radiopurity of tritiated
mannitol probe. A variety of issues (see: (Volpe, 2008)) may have contributed to this anomaly, but these factors do not necessarily disqualify the significance of our data when comparing control and treated cell monolayers (Fig. 2). Interestingly, apparent permeability for the high molecular weight marker inulin was significantly increased upon alcohol treatment in Caco-2 cells (Fig. 2B) and acetaldehyde treatment in both cell lines (Figs. 2C,F), while the $P_{app}$ of PEG-4000 was unaffected by any treatment modality. It should be noted that permeability values of the sucrose are somewhat higher in Caco-2 cells when compared to MDCK cells (Figs 2A-C vs. 2D-F, respectively). This result could be attributed to the presence, albeit at low expression levels, of sucrase in Caco-2 but not MDCK cell lines (Beaulieu and Quaroni, 1991). Transport of [U-$^{14}$C]-sucrose hydrolysis products [$^{14}$C]-glucose and [$^{14}$C]-fructose would be mediated by solute carrier proteins from the SGLT and GLUT families (Joost and Thorens, 2001; Wood and Trayhurn, 2003; Cheeseman, 2008), potentially resulting in higher apparent permeability measurements for sucrose.

**Acetaldehyde Effects on Paracellular Permeability Markers In Vivo**

*In vitro* studies in both Caco-2 and MDCKII cells suggest that low molecular weight markers have significantly increased permeability upon treatment with acetaldehyde. Based on these results, the bioavailability upon oral administration of various molecular weight markers were examined in the rat. The four markers were used, as described above, in rats treated with ethanol, disulfiram or a combination of the two to generate *in vivo* acetaldehyde. Our laboratory has previously examined the generation of acetaldehyde in the rat *in vivo* and found that the dosing scheme described in the Methods section yields approximately 80 μM acetaldehyde in plasma (Fisher et al., 2008). The percentage [$^{3}$H]-mannitol detected in plasma of the overall dose was significantly higher in rats treated with ethanol/disulfiram, as compared to rats
treated with ethanol alone, disulfiram alone, or control treated. We found similar results with rats who received $[^{14}C]$-sucrose and detected a significant increase in systemic sucrose availability only in rats generating in vivo acetaldehyde (Fig. 3B). The systemic availability of the high molecular weight markers, $[^{14}C]$-PEG and $[^{3}H]$-inulin, was unchanged as compared to control rats for any treatment group (Figs. 3C,D).

Pharmacokinetic analysis for the $[^{3}H]$-mannitol group demonstrates that the area under the curve (AUC) profile of the acetaldehyde generating group was approximately 15-fold greater than that of control rats (45,434 $\pm$ 5,823 $\mu$Ci*min/ml vs. 3,134 $\pm$ 642 $\mu$Ci*min/ml, respectively). Analysis of the $[^{14}C]$-sucrose group showed an approximate 7-fold increase in the AUC of the marker with values of 290.2 $\pm$ 74.7 $\mu$Ci*min/ml for the treatment group vs. 41.4 $\pm$ 7.3 $\mu$Ci*min/ml for the control. These data suggest that in vivo bioavailability is increased in the acetaldehyde-generating rat model in a size-dependent fashion, likely due to increased intestinal permeability via pharmacologically induced opening of the tight junctions.

**Acetaldehyde Affects on Paclitaxel but not Naproxen Bioavailability**

To further define the potential in vivo relevance of ethanol metabolism in the GI tract of the rat, we studied the effect of acetaldehyde production on the systemic availability of a highly bioavailable drug, naproxen, and a drug with limited bioavailability, paclitaxel. Upon oral dosing with $[^{14}C]$-naproxen, there was no significant difference in detected plasma levels in any treatment group (Fig. 5A). However, there was a significant effect ($p < 0.05$) of acetaldehyde production on the bioavailability of paclitaxel (Fig. 5B), with an approximate 4-fold increased in AUC (7051 vs 1875 mg/ml-min vs. control rats). While the overall bioavailability of paclitaxel remained very low (less than 6% of the total dose administered) in every treatment group, this result is significant. All other treatment groups showed no significant influence on paclitaxel bioavailability.
DISCUSSION

The effect of acetaldehyde, which is generated during the course of alcohol consumption, on paracellular permeability and loosening of tight junctions has received increasing interest in recent years (Rao, 1998; Atkinson and Rao, 2001; Seth et al., 2004; Sheth et al., 2004; Basuroy et al., 2005). These studies have demonstrated a pronounced effect of acetaldehyde on the structure of the tight junction, but the extent of tight junctional modulation and its consequent impact on drug permeability has not been investigated. Therefore, the aim of the present study was to examine the effects of acetaldehyde produced from ethanol metabolism in vivo on the degree of tight junctional disruption by assessing the permeability of various marker molecules of increasing molecular weight and geometry. Additionally, we established the effects of ethanol, its major metabolite and drugs used to generate in vivo acetaldehyde on the permeability of marker molecules in cell culture models in vitro. To validate the clinical significance of these findings, we determined the effects of ethanol metabolism in the GI tract on the bioavailability of two drug molecules representing different biopharmaceutics classification system (BCS) classes. Naproxen, an over-the-counter NSAID is a BCS Class I/II compound, displaying high permeability and high/low solubility dependent on pH (Wu and Benet, 2005); paclitaxel, on the other hand, is a representative of compounds in the relatively rare BCS Class IV, which feature poor permeability and solubility characteristics.

Cell lines such as Caco-2 and MDCK form tight junctions upon differentiation, with apical and basolateral membrane, and are generally considered good models for the in vitro study of epithelial permeability. This study examined the effects of ethanol (20% v/v), disulfiram (25 mg/ml) and acetaldehyde (1 mM) on the reversibility of TEER in both Caco-2 (Fig. 1A) and MDCKII cells (Fig. 1B). TEER is an established measure of the integrity of cellular monolayers and represents a qualitative assessment of
paracellular pathway aperture. As anticipated, disulfiram administration did not affect TEER values as this compound is not known to affect tight junction or function as a membrane permeabilizer (Balakirev and Zimmer, 2001). Our data show a significant decrease in cell monolayer TEER values upon treatment of a “sub-lethal” dose of ethanol (20% v/v). These data are consistent with studies by Ma and colleagues (Ma et al., 1995; Ma et al., 1999) who showed that ethanol permeabilizes the membrane at concentrations less than 10% (v/v); further, they speculated that ethanol reversibly affects tight junctional function via disassembly of perijunctional actin and myosin filaments. However, the definitive mechanism(s) behind these observed effects remain to be studied. Under the experimental conditions applied here, acetaldehyde reversibly and significantly decreases TEER values, which is comparable to studies by our laboratory (Fisher et al., 2008) as well as other groups (Atkinson and Rao, 2001; Basuroy et al., 2005). Our previous studies show that acetaldehyde in concentrations up to 2 mM are not significantly cytotoxic (Fisher et al., 2008). The effect of acetaldehyde on monolayer resistance was more pronounced in MDCKII cell monolayers compared to Caco-2 cells (75% vs. 50% of their starting values, respectively). Since MDCKII cell and Caco-2 cells originate from the kidney and the intestine, respectively, these data may highlight the differential effect of ethanol and its metabolites on paracellular integrity in different organs.

To further assess the effect of acetaldehyde on membrane permeability, our study employed several molecular markers of various sizes that cross membranes exclusively via a paracellular route. The permeability of these probes were tested on cell monolayers with fully differentiated cells and junctional complexes. Studies by Knipp and co-workers (Knipp et al., 1997), using a series of structurally unrelated compounds with variable hydrodynamic radii \( r_h \) estimated the pore radius of Caco-2 cells to be approximately 5.2Å, whereas Watson and colleagues accurately established this pore
radius at 4.5 Å using permeability assessment of PEG probes with radii 3.5-7.4 Å (Watson et al., 2001). Parallel to TEER data (Fig. 1), disulfiram had no effect on paracellular permeability of any marker molecules across Caco-2 or MDCKII cell monolayers (Fig. 2A,D), while ethanol increased mannitol ($r_h$ 4.1 Å), but not sucrose ($r_h$ 5.2 Å) permeability (Fig. 2B,E). Acetaldehyde treatment (Fig. 2C,F) effected the greatest permeability increase for both mannitol and sucrose. MDCKII cells only showed a significant increase in marker permeability upon acetaldehyde treatment, which was somewhat unexpected based on the significant decrease in TEER values observed above (Fig. 1). This suggests that ethanol and acetaldehyde have different mechanisms of paracellular permeability enhancement in MDCK cells. Interestingly, Caco-2 cell monolayers displayed a significant permeability increase of the high molecular weight marker, inulin (5kDa), but not PEG (4kDa). This result may be explained by the compact spherical shape of inulin ($r_h$ 10 Å vs. 15.9 Å for PEG4000), which makes it more amenable to paracellular transport. This effect was observed previously by Ghandehari and colleagues (Ghandehari et al., 1997) who demonstrated that the permeability of probes via tight junctions is based on molecular weight, along with properties such as geometry, flexibility, hydrophobicity and charge.

To validate our in vitro results and assess a correlation with in vivo models, we monitored the oral pharmacokinetics and bioavailability of each molecular marker in a rat model exposed to various treatments associated with alcohol metabolism. To simulate the production of acetaldehyde in vivo, rats were treated with a combination of ethanol and the aldehyde dehydrogenase inhibitor, disulfiram. Under these conditions we observed a marked increase in the systemic availability upon oral administration of low, but not high molecular weight probes (Fig. 3). It should be noted that control experiments with ethanol and disulfiram alone did not have an effect on probe availability (Fig. 4). Mannitol has been used as a standard probe used in the assessment of junctional
integrity (Swaan et al., 1994). Thus, the animal data presented here correlate well with our in vitro results indicating an acetaldehyde-induced permeability enhancement for low molecular weight molecules, most likely due to its disruptive effect on intestinal tight junctions. It has been suggested that acetaldehyde disrupts tight junctions via a tyrosine kinase-dependent mechanism (Atkinson and Rao, 2001). Evidence exists for a dramatic reduction of the zonula occludens protein ZO-1 within tight junctions upon application of pathophysiologically relevant levels of acetaldehyde (~650 µM) (Rao, 1998; Atkinson and Rao, 2001). Extending these in vitro observation to our studies, we conclude that acetaldehyde generated in vivo directly affects the intestinal tight junctional complex, thereby causing a temporary, size-and geometry-dependent increase in permeability. Overall, these results indicate that acetaldehyde, not ethanol, is responsible for the observed modulation of tight junctions both in vitro and in vivo.

To further assess the clinical relevance of the above observations, we determined the oral bioavailability of two drug molecules upon administration of saline (control), ethanol, disulfiram, or acetaldehyde (i.e. co-administered disulfiram/ethanol). We selected the NSAID naproxen as a representative control drug with a low molecular weight (252 Da), high solubility at neutral to basic pH and reportedly high permeability and almost complete (>95%) oral bioavailability. These features place naproxen in BCS Class I (since pH influences naproxen solubility, low pH conditions would constitute a Class II categorization). As expected, the addition of ethanol or production of acetaldehyde in the animal during drug administration had no significant effect on naproxen bioavailability (Fig. 5A). The chemotherapeutic paclitaxel, on the other hand, is a relatively high molecular weight drug (854 Da) that is insoluble in aqueous solution. It displays poor permeability characteristics partly due to its affinity for the efflux transporter P-glycoprotein, abundantly expressed along the GI tract. As a result, paclitaxel has low and variable bioavailability and is not traditionally given via oral routes.
of delivery. Owing to its poor permeability and solubility, it is a member of BCS Class IV, which comprises drug molecules that are typically difficult to formulate. Thus, we employed paclitaxel as a representative Class IV drug (Varma and Panchagnula, 2005) to observe the effects of alcohol and its metabolites on the opening of tight junctions. After acetaldehyde production, plasma paclitaxel levels were significantly higher upon oral administration compared to controls (saline, ethanol, disulfiram) (Fig. 5B), even though overall bioavailability remained low (<6%). It should be noted that disulfiram has been shown to be an inhibitor of P-glycoprotein in vitro (Loo et al., 2004), likely by covalent modification of specific cysteine residues in both substrate and ATP binding domains (Loo and Clarke, 2000). However, metabolism of disulfiram, which is rapidly converted upon ingestion in the stomach (Johansson, 1992), would make this result difficult to observe in vivo, especially since the studies were performed at disulfiram concentrations much higher than those achieved clinically. Importantly, co-administration of disulfiram or ethanol with paclitaxel does not lead to increased paclitaxel plasma levels (Fig. 5B), thereby excluding the possibility of an indirect effect of disulfiram or ethanol on P-glycoprotein activity that would influence intestinal paclitaxel permeability. Therefore, the increase in paclitaxel bioavailability observed here upon administration of disulfiram and ethanol cannot be attributed to inhibition of P-glycoprotein; however, an effect of in vivo generated acetaldehyde on P-glycoprotein activity cannot be excluded. These results highlight that perturbation of tight junctions can enhance the permeability of drug molecules and even overcome an opposing force created by efflux transporters.

These studies further demonstrate the pronounced effects of acetaldehyde on intestinal permeability and its impact on drug absorption in chronic alcoholics. Furthermore, data presented here add to mounting evidence that acetaldehyde is responsible for the loosening of tight junctions rather than ethanol. While this series of experiments displays changes in functional assays, it is clear that continued examination
into the mechanism by which acetaldehyde affects tight junctions, adherens junctions, proteins within these junctional complexes or some combination thereof, is necessary. Overall, our data illustrate that modulation of the paracellular permeation pathway by acetaldehyde affects drug absorption and bioavailability depending on drug size, geometry, and BCS classification.
REFERENCES


Footnotes

a) Unnumbered footnotes

b)

c) Send reprint requests to:

Peter W. Swaan, Ph.D., Department of Pharmaceutical Sciences, University of
Maryland, 20 Penn Street, Baltimore, MD 21201 USA; E-mail:
pswaan@rx.umaryland.edu

c) Numbered Footnotes
Legends for Figures

Figure 1. Effect of control (PBS, open diamonds ◊), disulfiram (25 mg/ml, closed circles ●), ethanol (20 %, closed squares ■), and acetaldehyde (1 mM, closed triangles ▲) on TEER measured in A) Caco-2 and B) MDCKII cell monolayers. TEER measured from 0 to 300 minutes. Treatments were removed at 240 minutes and cells were allowed to recover in media for 60 minutes. Each data point represents mean ± SEM of at least 8 experiments. Samples were analyzed for any statistically significant differences between groups using single-factor ANOVA. * indicates statistically significant difference between treatment and control $p < 0.05$ or ** $p < 0.001$.

Figure 2. Effect of disulfiram (25 mg/ml) (A,D), ethanol (20 %) (B,E) and acetaldehyde (1 mM) (C,F) treatment (++) on permeability of [³H]-mannitol, [¹⁴C]-sucrose, [³H]-inulin, and [¹⁴C]-PEG4000 vs. untreated controls (−) in Caco-2 (A-C) and MDCKII (D-F) cells. Permeability was assessed each hour for a period of 4 hours. Each data point represents mean ± SEM of at least 6 experiments. Samples were analyzed for any statistically significant differences between groups using single-factor ANOVA. * indicates statistically significant difference between treatment and control $p < 0.05$ or ** $p < 0.001$.

Figure 3. In vivo bioavailability of low weight molecular markers [³H]-mannitol (182.2 Da) (A) and [¹⁴C]-sucrose (342.3 Da) (B), and high weight molecular markers [¹⁴C]-PEG (4,000 Da) (C) and [³H]-inulin (5,000 Da) (D). Rats treated for six days via oral bolus dose with saline control (open diamonds ◊), 5 g/kg ethanol (open squares □), 100 mg/kg disulfiram (open circles ○) or 5 g/kg ethanol and 100 mg/kg disulfiram (closed triangles ▲). Uptake of marker measured using liquid scintillation as percent of total starting dose.
Each data point represents mean ± SEM of at least 6 experiments from time 0 to 1440 minutes. Samples were analyzed for any statistically significant differences between groups using single-factor ANOVA. * indicates statistically significant difference between treatment group $V_{max}$ and control $V_{max}$ ($p < 0.05$).

**Figure 4** *In vivo* area under the curve (AUC) determinations in the *in vivo* rat model as analyzed by WinNonlin. Male Sprague-Dawley rats ($n = 6$) exposed to saline control (C), 33% ethanol (E), 100 mg/kg disulfiram (D) or ethanol/disulfiram (A) and given 1 of 4 molecular probes, $[^{3}H]$-mannitol, $[^{14}C]$-sucrose, $[^{14}C]$/PEG, and $[^{3}H]$-inulin, respectively. Each data point represents mean ± SEM of at least 6 experiments from time 0 to 1440 minutes. Samples were analyzed for any statistically significant differences between groups using single-factor ANOVA.

**Figure 5.** *In vivo* bioavailability of low molecular weight drug $[^{14}C]$-Naproxen (252.2 Da) (A) and medium molecular weight drug $[^{14}C]$-paclitaxel (853.9 Da) (B). Rats treated for six days via oral bolus dose with saline control (open squares □), 5 g/kg Ethanol (closed circles ●), 100 mg/kg Disulfiram (closed diamonds ♦) or 5 g/kg Ethanol and 100 mg/kg Disulfiram (closed squares ■). Uptake of drug measured using liquid scintillation as percent of total starting dose. Each data point represents mean ± SEM of at least 6 experiments from time 0 to 1440 minutes. Samples were analyzed for any statistically significant differences between groups using single-factor ANOVA.
Figure 1

A

B

Percent TEER from Baseline (%)

Time (min)

Treatment Removed

Treatment Removed