

## Effects of phenytoin and carbamazepine on calcium transport in Caco-2 cells

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### Abstract

Adverse effects of anti-seizure/anti-epileptic medications on bone density have been observed and reported since the early 1960s. Phenytoin and carbamazepine are two commonly prescribed anti-epileptic drugs most frequently associated with osteomalacia including fractures, bone demineralization, and reduced bone formation. The mechanism by which anti-epileptic drugs induce bone loss is not fully explained. We hypothesized that anti-epileptic drugs may impair dietary calcium absorption in the intestine. Using Caco-2 cells, a model transport system for study of the function of the intestinal epithelium, we determined the effects of several anti-epileptic drugs on intestinal epithelial calcium transport. In our system, phenytoin and carbamazepine dose-dependently inhibit active calcium transport from the apical to basolateral side of Caco-2 cells under physiologic calcium conditions. Vitamin D ameliorates the anti-epileptic drug-induced decrease in calcium permeability.

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**Keywords:** Anti-epileptic drugs; Osteomalacia; Permeability; Vitamin D

### 1. Introduction

Adverse effects of anti-seizure/anti-epileptic medications on bone density have been observed and reported since the early 1960s. A study evaluating hip fractures in women over 65 years of age found that women taking anti-epileptic drugs had twice the risk of developing a hip fracture (Cummings et al., 1995). With anti-epileptic drug therapy, osteomalacia, or a decrease in bone density, is observed (Stephen et al., 1999). Phenytoin and carbamazepine are two commonly prescribed anti-epileptic drugs frequently associated with osteomalacia including fractures, bone demineralization, and reduced bone formation (Pack, 2003). Newer

agents (e.g. topiramate, lamotrigine, gabapentin) appear to be less causative of osteomalacia but long-term studies are not as complete as for the older agents (Stephen et al., 1999).

Long-term treatment with anti-epileptic drugs is associated with greater adverse effects on bone density; this is especially problematic as anti-epileptic drugs are commonly prescribed for chronic anti-seizure effects and patients are often treated for multiple decades with an effective agent. Children are especially sensitive to the bone density-depleting effects of anti-epileptic drugs (Dent et al., 1970; Stephen et al., 1999).

The mechanism by which anti-epileptic drugs induce bone loss is not fully explained. Hypocalcemia is associated with anti-epileptic drug treatment and with osteomalacia. Levels of hypocalcemia with chronic anti-epileptic treatment vary between 3% and 30%, and higher incidences are associated with poly-therapy (Gough et al., 1986). Increases in serum markers of bone resorption including increased osteocalcin and increased ICTP (cross-linked carboxy

*Abbreviations:*  $P_e$ , permeability; TEER, transepithelial electrical resistance;  $EC_{50}$ , 50% effective concentration.

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terminal telopeptide I of type I collagen) levels are positively correlated with anti-epileptic drug therapy (Valimaki et al., 1994; Verrotti et al., 2000). Plausible mechanisms for anti-epileptic drug-induced hypocalcemia include induction of vitamin D catabolism, inhibition of parathyroid hormone-induced calcium mobilization, or decreased dietary calcium absorption. Several studies have failed to find a consistent correlation between altered vitamin D levels and anti-epileptic drug treatment; but induction of vitamin D catabolism may be involved in combination with other mechanisms (Gough et al., 1986; Verrotti et al., 2000). Studies in rats have suggested that both impaired intestinal calcium absorption and inhibition of PTH response are associated with phenytoin treatment (Pack and Morrell, 2001).

Caco-2 cells are cultured human colon adenocarcinoma cells and a very well-characterized model system for study of the intestinal epithelium. At critical density, Caco-2 cells form a polar monolayer with apical (normally adjacent to the intestinal lumina) and basolateral (normally adjacent to the blood supply) sides, tight junctions, brush border membranes and the selective permeability of intact intestinal epithelium. Quantitation of drug transport across Caco-2 cells and calculation of drug permeability is commonly used to estimate oral bioavailability of pharmaceutical agents (Mandagere et al., 2002). Caco-2 cells have also been used to study intestinal calcium transport, including the effect of vitamin D to enhance intestinal calcium absorption (Giuliano and Wood, 1991; Surendran et al., 1995; Jovani et al., 2001). To test our hypothesis that anti-epileptic drugs which affect bone health inhibit intestinal calcium permeability, we assessed the effects of phenytoin and carbamazepine, which are known to affect bone health, and gabapentin and topiramate, which are not associated with bone disease, on active calcium transport in Caco-2 cells.

## 2. Materials and methods

Caco-2 cells, a human colon epithelial carcinoma cell line, were received from ATCC (Bethesda, MD). Penicillin/Streptomycin antibiotic mixture (10,000 IU penicillin, 10,000 µg/mL streptomycin) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Cellgro/Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Nonessential amino acids (10 mM at 100×) were obtained from Gibco-BRL (Gaithersburg, MD). Transwell plates (3.0 µm pore size) were purchased from Corning-Costar (Corning, NY). Phenytoin (5,5-diphenylhydantoin), carbamazepine (5H-dibenzazepine-5-carboxamide) and Scintisafe® gel were obtained from Fisher Scientific (Pittsburgh, PA). Topiramate (2,3:4,5-Di-O-isopropylidene-β-D-fructopyranose sulfamate) was purchased from Toronto Research Chemicals (North York, Ont.); gabapentin (1-(aminomethyl)cyclohexanecetic acid) was from USP (Rockville, MD). Active vitamin D (1,25-dihydroxycholecalciferol) was purchased from Sigma-Aldrich (St. Louis, MO).

### 2.1. Caco-2 cell culture

Frozen Caco-2 cells were routinely revived, cultured and used between the 26th and 30th passages. Cells were grown in 150 cm<sup>2</sup> tissue culture flasks with 30 mL DMEM supplemented with 10% FBS, 4 mL/L antibiotic mixture, 25 mg/L amphotericin B, and 100 µM nonessential amino acids. Medium was replaced every 2–3 days and cells grew to approximately 80% confluency before subculturing. Cells used in calcium transport experiments were seeded on Transwell® (Corning) inserts at a density of  $2.5 \times 10^5$  cells per insert (4.71 cm<sup>2</sup>) and grown for 21–22 days in supplemented DMEM, described above, to allow for development of tight junctions. Medium (1.5 mL apical, 2.5 mL basolateral) was changed every other day for 14 days and then daily for another 7 days prior to use for transport studies on day 21. All cells were maintained in an atmosphere of 5% CO<sub>2</sub>–95% air at 37 °C.

### 2.2. Transepithelial calcium transport studies

Caco-2 cells were grown to confluency and maintained in Transwell® culture for 21–22 days until tight junctions formed as described previously (Rodriguez-Proteau et al., 2006). Cells in Transwell® at 21–22 days post-confluency were then incubated with the indicated concentrations of vehicle or anti-epileptic drug in culture medium (apical and basolateral sides) for 24 h. Anti-epileptic drugs phenytoin, carbamazepine, or topiramate were dissolved in 100% DMSO (vehicle) at 100× final concentration before application to cells. Gabapentin was dissolved at 1000× final concentration in dH<sub>2</sub>O.

After 24 h drug pretreatment, monolayer integrity and maintenance of tight junctions in the Caco-2 cells were confirmed by transepithelial electrical resistance (TEER) readings using a World Precision Instrument (EVOM, Sarasota, FL). Transepithelial resistance was calculated as follows: (resistance of Transwell® containing cells – resistance of Transwell® without cells)  $\Omega \times 4.71 \text{ cm}^2$ . Only cultures meeting the criteria of resistance greater than 500  $\Omega \text{ cm}^2$ , indicating formation of tight junctions, were used for transport studies.

After TEER readings were completed, cells were rinsed with Hanks Balanced Salt Solution (HBSS; 0.4 g/L KCl, 0.06 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.1 g/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.1 g/L MgSO<sub>4</sub>·7H<sub>2</sub>O, 8 g/L NaCl, 0.35 g/L NaHCO<sub>3</sub>, 0.09 g/L Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 4.5 g/L D-glucose, and 2.383 g/L Hepes). HBSS was made to pH 6.8 for apical application and to pH 7.4 for basolateral application to mimic the *in vivo* intestinal cell environments. Apical and basolateral HBSS media with 7.5 mM calcium containing fresh drug solutions were added to each transwell and allowed to equilibrate at 37 °C in a 5% CO<sub>2</sub> incubator for 30 min. Calcium transport from apical to basolateral sides of the polar cell monolayer was monitored by spiking the apical HBSS medium with 5 µCi/mL radioactive <sup>45</sup>CaCl<sub>2</sub> at time zero and sampling the basolateral medium at various times. Basolateral to apical

transport was monitored by spiking the basolateral medium at time zero. Calcium transport was quantified by sampling the apical or basolateral medium at 20 min intervals over a 3 h time period following addition of radioactive calcium. Samples (50  $\mu$ L) in duplicate were withdrawn and replaced with 100  $\mu$ L of fresh drug-containing HBSS with calcium after each collection. Samples were transferred to 7 mL scintillation vials, 5 mL of scintillant was added, and a liquid scintillation counter was used to quantitate the amount of  $^{45}\text{CaCl}_2$  radioactivity present in the basolateral samples. For apical to basolateral transport (and vice versa for basolateral to apical transport), the starting specific activity of  $^{45}\text{CaCl}_2$  in the spiked apical medium at time zero ( $[\text{CaCl}_2]_{\text{total}}$ ), and the background transport in the basolateral medium ( $[\text{CaCl}_2]_{\text{zero}}$ ) were calculated using the known molar concentration of  $\text{CaCl}_2$  in the media and by sampling both sides of the transwell at time zero. Percent cumulative  $\text{CaCl}_2$  transported at each time point (% transport) was then calculated as  $([\text{CaCl}_2] \text{ in basolateral medium at time } t - [\text{CaCl}_2]_{\text{zero}})/([\text{CaCl}_2]_{\text{total}} + [\text{CaCl}_2]_{\text{zero}}) \times 100$ . Effective permeability ( $P_e$ ) of calcium as a function of calcium transport over time was calculated under conditions of calcium homeostasis (equimolar calcium in apical and basolateral buffers) according to the formula:  $P_e \text{ (cm/s)} = \% \text{ transport} \times V/(A \times t)$  where  $V$  = volume of apical medium (1.5 mL),  $A$  = surface area (4.71  $\text{cm}^2$ ) and  $t$  = time (s) post  $^{45}\text{CaCl}_2$  addition.

Maximal effects and  $\text{EC}_{50}$  values for the dose response relationship between drug concentration and inhibition of calcium permeability were calculated by non-linear regression analysis for one-site inhibition using GraphPad<sup>®</sup> Prism 4 software. For all figures, significance is indicated and corresponds to  $p < 0.05$  by non-paired  $t$ -test compared to control (vehicle-treated) samples or by ANOVA for multiple sample comparisons with a Neuman–Keuls post-test.

### 2.3. Determination of optimum calcium concentrations for maximum calcium transport

Caco-2 cells were grown and incubated on Transwell<sup>®</sup> inserts and treated 24 h prior to experiment with vehicle or 5  $\mu\text{g/mL}$  phenytoin in cell culture medium as described above. Thirty minutes prior to addition of radioactive  $^{45}\text{CaCl}_2$ , HBSS (apical or basolateral) including three different  $\text{CaCl}_2$  concentrations (1 mM, 7.5 mM or 15 mM) was

added with vehicle or 5  $\mu\text{g/mL}$  phenytoin. Calcium transport apical to basolateral was quantitated and  $P_e$  calculated as described above.

### 2.4. Transepithelium calcium transport studies with vitamin D and modified calcium concentration

Calcium transport studies were conducted as described above, with the inclusion of a 48 h pretreatment with the active form of vitamin D, 1,25-dihydroxy vitamin D. Vitamin D (100 nM), ethanol vehicle (0.04% v/v) or no vehicle was added to both the apical and basolateral cell culture media 48 h prior to initiation of transport studies. Twenty-four hours prior to experiment, fresh medium containing vitamin D, ethanol vehicle or no vehicle was replenished in the transwells with the addition of either phenytoin (6.6  $\mu\text{M}$ ), carbamazepine (10  $\mu\text{M}$ ) or DMSO vehicle (1% v/v). Apical and basolateral HBSS media with 2.5 mM calcium containing fresh drug solutions was added to each transwell and allowed to equilibrate as described above. At experimental time zero, the HBSS medium was spiked with radioactive  $^{45}\text{CaCl}_2$  on the apical side only in the context of equilateral media concentrations of 2.5 mM  $\text{CaCl}_2$ . Basolateral samples were taken at varying times after  $^{45}\text{CaCl}_2$  addition; % calcium transport was quantitated and  $P_e$  calculated as described above.

## 3. Results

### 3.1. Calcium transport and permeability ( $P_e$ ) following antiepileptic drug treatment

To assess the effects of anti-epileptic drugs on transport and  $P_e$  of calcium, Caco-2 cells were treated for 24 h with varying concentrations of phenytoin, carbamazepine, gabapentin or topiramate. TEER readings of Caco-2 cells were taken after 24 h exposure to all concentrations of all drugs tested. We observed no statistical difference from vehicle-treated control cells for any concentration of any anti-epileptic drug tested, suggesting that tight junction integrity was not affected by selected drug treatments. Table 1 shows the average TEER readings for the most effective concentrations of phenytoin and carbamazepine and the highest concentrations of gabapentin and topiramate. All other concentrations tested also did not affect TEER readings (data not shown).

Table 1  
TEER Readings

	DMSO	Gabapentin	Topiramate	Phenytoin	Carbamazepine	Ethanol	Vitamin D
Mean	639	673	710	654	589	534	590
SE	48	45	46	39	72	75	96
<i>n</i>	15	4	4	7	4	3	3

TEER readings ( $\Omega \text{cm}^2$ ) were calculated as described in materials and methods following 24 h treatment with DMSO (1% v/v), gabapentin (480  $\mu\text{M}$ ), Topiramate (60  $\mu\text{M}$ ), phenytoin (6.6  $\mu\text{M}$ ) or carbamazepine (10 mM). TEER readings were calculated following 48 h treatment with ethanol (0.04% v/v) or vitamin D (100 nM). Duplicate readings were averaged for each treatment per experiment and mean TEER readings (Mean)  $\pm$  standard error (SE) were calculated for the indicated number of separate experiments (*n*).

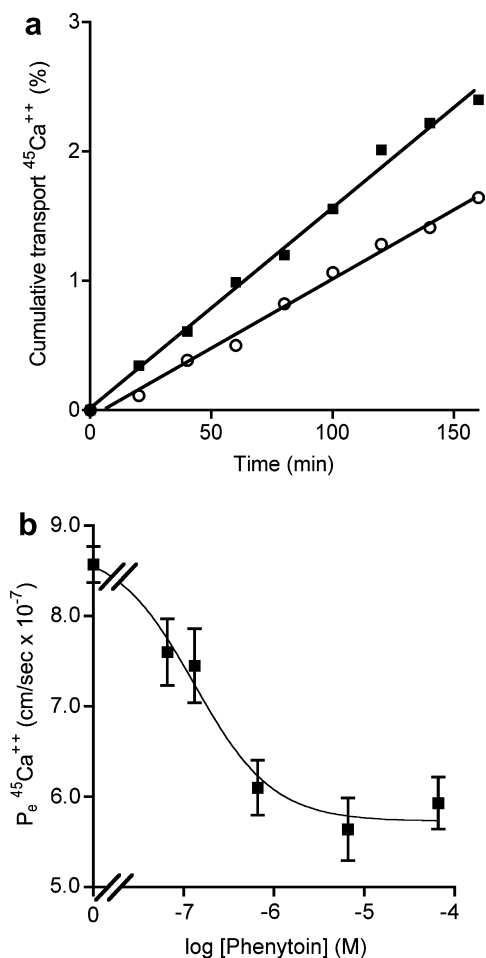


Fig. 1. Concentration-dependent effect of 24 h phenytoin pretreatment on calcium permeability in Caco-2 cells. (a) Effect of 24 h pretreatment with 6.6  $\mu\text{M}$  phenytoin (open circle) or vehicle (filled square) on apical to basolateral calcium transport over 160 min. (b) Effect of varying phenytoin concentration on Caco-2 cell calcium permeability calculated from transport data similar to that shown in part A. Transport of  $\text{CaCl}_2$  was quantitated and  $P_e$  calculated as described in methods. Permeability data shown is  $\pm$  standard error averaged from three independent experiments conducted in duplicate. Data from all samples pretreated with phenytoin were statistically different at  $p < 0.05$  compared to vehicle pretreated controls.

Calcium permeability was assessed after 24 h anti-epileptic drug treatment by measuring the transport of radioactive calcium across Caco-2 cell monolayers in the continuing presence of anti-epileptic drug over 3 h. The effects of the anti-epileptic drug phenytoin on the apical to basolateral transport (influx) of calcium are shown in Fig. 1. Influx calcium transport was linear over 160 min in the absence and presence of all concentrations of phenytoin tested under conditions of equilateral (equimolar apical and basolateral) 7.5 mM calcium concentration (Fig. 1a and data not shown). Concentrations of phenytoin tested (66 nM to 66  $\mu\text{M}$ ) included the therapeutic total plasma concentration range of 12–24  $\mu\text{M}$  and the therapeutic free (unbound) plasma range of 1.2–2.4  $\mu\text{M}$  (McNamara, 2005). Phenytoin caused a significant and concentration-dependent inhibition of apical to basolateral  $P_e$  of calcium with

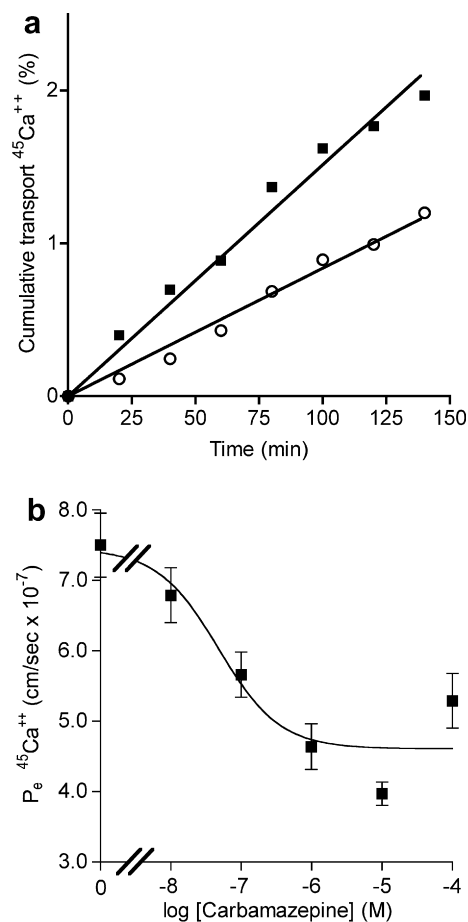


Fig. 2. Concentration-dependent effect of 24 h carbamazepine pretreatment on calcium permeability in Caco-2 cells. (a) Effect of 24 h pretreatment with 10  $\mu\text{M}$  carbamazepine (open circle) or vehicle (filled square) on apical to basolateral calcium transport over 140 min. (b) Effect of varying carbamazepine concentration on Caco-2 cell permeability calculated from data similar to that shown in part A. Transport of  $\text{CaCl}_2$  was quantitated and  $P_e$  calculated as described in methods. Permeability data shown is  $\pm$  standard error averaged from three independent experiments conducted in duplicate. Data from all samples pretreated with carbamazepine at a concentration of 0.1  $\mu\text{M}$  or greater were statistically different at  $p < 0.05$  compared to vehicle pretreated controls.

an  $\text{EC}_{50}$  value of  $157 \pm 5$  nM and a maximal decrease of 41% compared to vehicle (Fig. 1b). Basolateral to apical (efflux)  $P_e$  of calcium in Caco-2 cells was small (less than 5% of calcium influx) and was unaffected by phenytoin at any concentration from 66 nM to 66  $\mu\text{M}$  (data not shown).

The effect of the anti-epileptic drug, carbamazepine, on calcium transport is shown in Fig. 2. Apical to basolateral (influx) transport of calcium was linear over 140 min in the absence and presence of carbamazepine at all concentrations tested (Fig. 2a and data not shown) under conditions of equilateral 7.5 mM calcium as above with phenytoin. Concentrations of carbamazepine tested, 10 nM to 100  $\mu\text{M}$ , included the recommended therapeutic total plasma concentration range of 25–50  $\mu\text{M}$  or therapeutic free (unbound) plasma concentration range of 6–12  $\mu\text{M}$  (McNamara, 2005). In a dose dependent manner, carbamazepine significantly

Table 2

EC<sub>50</sub> values and dose ranges for inhibition of calcium  $P_e$  in Caco-2 cells by various anti-epileptic drugs

	Phenytoin	Carbamazepine	Gabapentin	Topiramate
Experimental CR	66 nM–66 $\mu$ M	10 nM–100 $\mu$ M	120–480 $\mu$ M	6–60 $\mu$ M
Therapeutic PC	1.2–2.4 $\mu$ M	6–12 $\mu$ M	70–120 $\mu$ M	15–60 $\mu$ M
EC <sub>50</sub>	157 $\pm$ 5 nM	51 $\pm$ 3 nM	NE	NE
Maximal inhibition	41%	54%		

Experimental concentration ranges (Experimental CR) tested as described in Figs. 1–3 and 5 are compared to recommended therapeutic plasma concentrations (Therapeutic PC) for the same anti-epileptic drugs. The EC<sub>50</sub> values for inhibition of calcium  $P_e$  (EC<sub>50</sub>) and percent maximal inhibition (maximal inhibition) are also listed where determined. NE = no effect at any tested concentration.

inhibited apical to basolateral (influx)  $P_e$  of calcium by a maximum of 54% with an EC<sub>50</sub> value of 51  $\pm$  3 nM (Fig. 2b). As with phenytoin, basolateral to apical (efflux)  $P_e$  of calcium in Caco-2 cells was unaffected by carbamazepine at any concentration tested to 100  $\mu$ M (data not shown).

To compare the effect of phenytoin on calcium  $P_e$  to the effects of newer anti-epileptic drugs lacking known adverse effects on bone health, gabapentin and topiramate were each tested in the Caco-2 cell calcium transport assay. Gabapentin was tested at concentrations of 120 and 480  $\mu$ M, corresponding to and a bit higher than the target therapeutic plasma range of 70–120  $\mu$ M; topiramate was tested at 6  $\mu$ M and 60  $\mu$ M, corresponding to the target therapeutic plasma range of 15–60  $\mu$ M (Johannessen et al., 2003). Neither topiramate nor gabapentin significantly affected apical to basolateral  $P_e$  of calcium under conditions identical to those described above demonstrating phenytoin inhibitory effects (Fig. 3). In fact, topiramate at the high concentration (60  $\mu$ M) displayed a non-significant tendency to increase calcium  $P_e$ . The resultant effects of phe-

nytoin, carbamazepine, gabapentin and topiramate on the  $P_e$  of calcium in Caco-2 cells, along with corresponding tested and recommended therapeutic concentrations, are summarized in Table 2.

### 3.2. Calcium permeability in Caco-2 cells as a function of equilateral calcium concentration

The effects of various extracellular calcium concentrations on calcium  $P_e$  in Caco-2 cells is shown in Fig. 4. Maximum apical to basolateral  $P_e$  of calcium ( $2.02 \pm 0.19 \times 10^{-6}$  cm/s) occurs at a concentration of 1 mM calcium, twice the  $P_e$  ( $0.99 \pm 0.03 \times 10^{-6}$  cm/s) found at 7.5 mM calcium. At 1 mM calcium, the  $P_e$  of calcium was decreased 44% with the addition of 6.6  $\mu$ M phenytoin. Permeability was decreased by 29% and 22% with the addition of 6.6  $\mu$ M phenytoin at 7.5 and 15 mM calcium concentrations, respectively. Phenytoin has greatest effects on transport of calcium at low calcium concentrations which most stimulate transport.

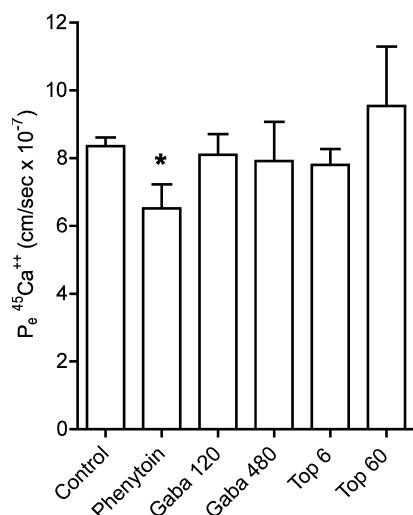


Fig. 3. Effect of 24 h gabapentin and topiramate pretreatment on calcium permeability in Caco-2 cells. Effect of 24 h pretreatment with 6.6  $\mu$ M phenytoin (Phenytoin), 120  $\mu$ M gabapentin (Gaba 120), 480  $\mu$ M gabapentin (Gaba 480), 6  $\mu$ M topiramate (Top 6), and 60  $\mu$ M topiramate (Top 60) on apical to basolateral calcium permeability over 160 min. Transport of calcium was quantitated and permeability calculated as described in methods. Permeability data shown is  $\pm$  standard error averaged from 3 to 6 independent experiments conducted in duplicate. \* $p$  < 0.05 compared to vehicle treated controls.

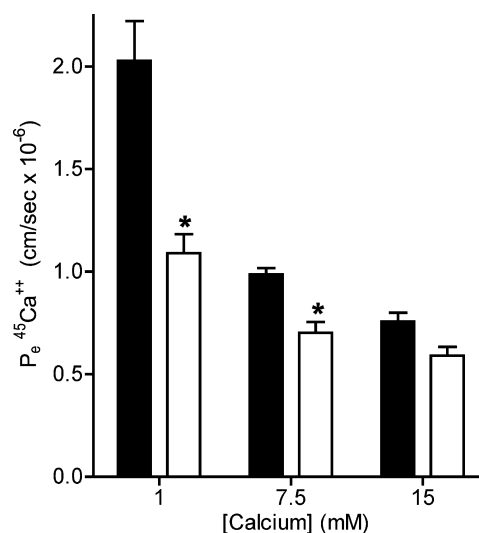


Fig. 4. Effect of extracellular calcium concentration on the permeability of <sup>45</sup>CaCl<sub>2</sub> with or without phenytoin pretreatment. Caco-2 cells were pretreated for 24 h with 6.6  $\mu$ M phenytoin (white bars) or vehicle (black bars). Apical and basolateral media, in the continuing presence of phenytoin or vehicle, were changed 30 min prior to experimentation to include the indicated concentrations of calcium. Transport of calcium was quantitated over 140 min and  $P_e$  calculated as described in methods. Data shown is  $\pm$  range averaged from two independent experiments conducted in duplicate. \* $p$  < 0.05 compared to vehicle treated controls.



### 3.3. Calcium permeability in Caco-2 cells pre-treated with vitamin D and phenytoin or carbamazepine

We sought to quantitate the effects of phenytoin and carbamazepine on calcium transport under conditions where calcium transport was actively stimulated in the Caco-2 cells. We demonstrated in Fig. 4 that decreased calcium concentration stimulates calcium transport. As previously reported, vitamin D increases the  $P_e$  of calcium in Caco-2 cells (Fleet and Wood, 1999). Ethanol (0.04% v/v), used as vehicle for vitamin D, has effects in our assay and doubles the  $P_e$  of calcium in the absence of vitamin D or vehicle (Fig. 4). Ethanol has been suggested to alter membrane fluidity of intestinal epithelial cells to non-selectively affect permeability (Bikle et al., 1986). Although not significantly different from DMSO-treated cells, there was a tendency toward lower TEER readings in ethanol-treated cells (Table 1), suggesting lower tight junction integrity. Nevertheless, after accounting for ethanol vehicle effects, 100 nM vitamin D increases the  $P_e$  of Caco-2 cells to calcium by 20–30%.

We conducted further experiments to quantitate phenytoin and carbamazepine inhibition of calcium  $P_e$  under conditions of stimulated calcium transport, i.e. with reduced (2.5 mM) equilateral calcium following 48 h vitamin D pretreatment. In the presence of ethanol (0.04% v/v), phenytoin (6.6  $\mu$ M) compared to no phenytoin control decreased the  $P_e$  of calcium by  $42 \pm 6\%$ , which is the same as phenytoin's effect in the absence of ethanol (Fig. 1 above). In the presence of vitamin D, phenytoin decreased the  $P_e$  of calcium by  $20 \pm 7\%$  (Fig. 5a). Thus, vitamin D pretreatment attenuated the phenytoin-induced decrease in calcium  $P_e$  by half, suggesting an ameliorative effect of vitamin D to stimulate active calcium transport and blunt the inhibition of calcium transport by phenytoin.

In the presence of ethanol (0.04% v/v), carbamazepine (10  $\mu$ M) compared to no carbamazepine control decreased the  $P_e$  of calcium by  $35 \pm 4\%$  (Fig. 4b), similar to carbamazepine effects in the absence of ethanol (Fig. 1 above). In the presence of vitamin D, carbamazepine decreased the  $P_e$  of calcium by  $8 \pm 4\%$  (Fig. 5b). Although carbamazepine has a statistically significant effect to reduce the  $P_e$  of calcium in Caco-2 cells following vitamin D pretreatment, vitamin D very effectively reverses most of the carbamazepine-induced inhibition of calcium  $P_e$ .

## 4. Discussion

The  $P_e$  of calcium across Caco-2 cells as a function of rate of transport was quantitated in the absence and presence of varying concentrations of anti-epileptic drugs. We determined that carbamazepine and phenytoin at therapeutic drug concentrations significantly inhibit calcium transport in our model system, whereas we did not observe an inhibitory effect of gabapentin or topiramate. The lack of significant effect of the newer anti-epileptic drugs, gabapentin and topiramate, to affect calcium transport in the Caco-

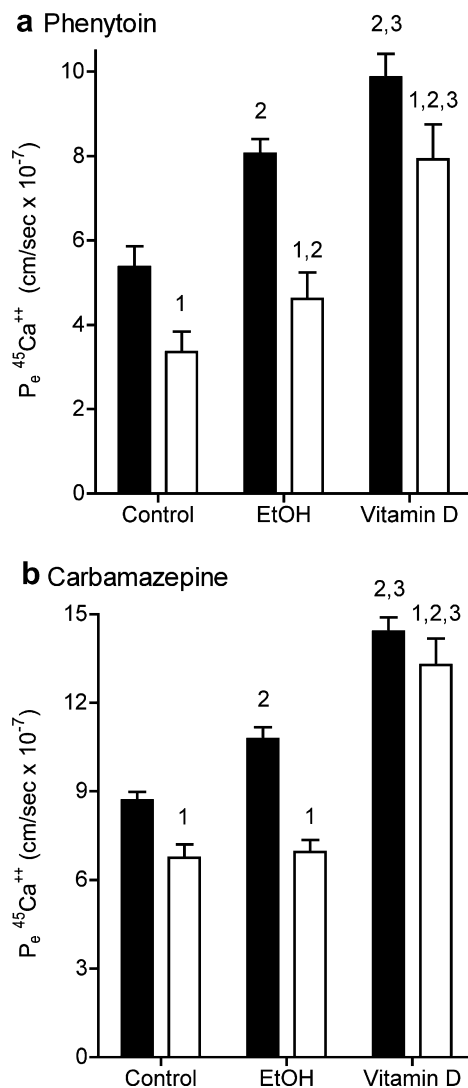


Fig. 5. Effect of vitamin D on Caco-2 cell permeability to calcium in the presence of phenytoin or carbamazepine. (a) Cells were pretreated with 100 nM active vitamin D (Vit D) or vehicle (EtOH) for 24 h followed by addition of 6.6  $\mu$ M phenytoin (white bars) or DMSO vehicle (black bars) for another 24 h in the continuing presence of vitamin D or ethanol vehicle. (b) Cells were pretreated with 100 nM active vitamin D (Vit D) or vehicle (EtOH) for 24 h followed by addition of 10  $\mu$ M carbamazepine (white bars) or DMSO vehicle (black bars) for another 24 h in the continuing presence of vitamin D or ethanol vehicle. Transport of calcium was quantitated over 120 min in 2.5 mM equilateral calcium, and permeability relative to drug treatment was calculated as described in methods. Permeability data shown is  $\pm$  standard error averaged from three independent experiments conducted in duplicate. <sup>1</sup> $p < 0.05$  for phenytoin (a) or carbamazepine (b) treated samples compared to DMSO vehicle treated pairs. <sup>2</sup> $p < 0.05$  for ethanol-pretreated or vitamin D-pretreated samples compared to no ethanol-pretreated control. <sup>3</sup> $p < 0.05$  for vitamin D-pretreated samples compared to ethanol-pretreated samples with or without phenytoin (a) or carbamazepine (b).

2 cells coincides with decreased reports of these newer agents to negatively affect bone health. Table 2 illustrates that the tested anti-epileptic drug concentrations overlapped the range of recommended therapeutic plasma levels for adults.  $EC_{50}$  values for inhibition of calcium  $P_e$  by phenytoin and carbamazepine are below the recommended

therapeutic concentration ranges, providing a rationale for *in vivo* effects of these agents to inhibit intestinal calcium absorption and ultimately adversely affect bone health.

To limit our studies to active calcium transport, permeability was assessed in our system at physiologically relevant, equimolar apical and basolateral calcium concentrations. In this condition of equilateral calcium, contributions from passive paracellular transport are minimized. We also quantitated the effects of phenytoin on transport of calcium under conditions of varying equilateral calcium concentrations. The transport and  $P_e$  of calcium were significantly stimulated at low calcium concentrations, most likely reflecting the importance of the active transport mechanism at lower concentrations of calcium to maintain calcium homeostasis. Phenytoin's greatest inhibitory effect occurs under conditions of low calcium concentration, supporting a hypothesis that phenytoin or carbamazepine blocks active, as opposed to non-regulated passive, calcium transport.

The mechanism by which phenytoin and carbamazepine decrease bone density and lead to increased risk of fracture and osteomalacia has been studied previously, but no definitive mechanism reported. Our results support an effect of anti-epileptic drugs on intestinal epithelial calcium transport with decreased transport presumably leading to decreased serum calcium *in vivo*. Decreased serum calcium initiates a cascade of events to move calcium from bone into plasma that is reversed upon obtainment of sufficient serum calcium. Decreased bone density may then be the result of reduced dietary calcium uptake from the intestine due to anti-epileptic drugs blocking intestinal transport.

Our results demonstrating a 40–50% decrease in intestinal permeability to calcium with therapeutically relevant levels of phenytoin and carbamazepine suggest that chronic treatment with these anti-epileptic agents is sufficient to affect bone health, particularly in children with poor vitamin D intake. A reduction of average daily calcium intake from 700 to 900 mg/day (recommended) to 440 mg/day in children resulted in significantly lower bone mineral densities and smaller skeletons (Black et al., 2002). The increased inhibition of calcium transport by phenytoin at lower calcium concentration highlights the importance of maintaining sufficient dietary calcium intake to ameliorate the drug's effects.

Active vitamin D, as expected, was shown to be an important enhancer of active transport and  $P_e$  of calcium in Caco-2 cells (Fleet and Wood, 1999). Application of vitamin D approximately doubled the  $P_e$  of calcium in the Caco-2 cells. With anti-epileptic drug treatment, calcium permeability was lowered in the presence of vitamin D, but not to as great an extent as in the absence of vitamin D. The importance of maintaining sufficient vitamin D levels either through the diet or exposure to sunlight is highlighted by our study.

Effects of anti-epileptic drugs to reduce vitamin D levels, as suggested by some studies, may compound the anti-epileptic drug effect to reduce calcium absorption (Hahn, 1976; Gough et al., 1986; Verrotti et al., 2000). Cyp3A4 is induced by phenytoin and carbamazepine treatment; vita-

min D is metabolized primarily by Cyp3A4 in the small intestine and liver (Xu et al., 2006). Thus, by reducing vitamin D levels, chronic phenytoin or carbamazepine treatment would be expected to have greater effects to inhibit dietary calcium absorption in the intestine.

Conversely, by inducing Cyp3A4, vitamin D induces its own metabolism while potentially also increasing metabolism and reducing intestinal transport of phenytoin or carbamazepine. Treatment of Caco-2 cells with active vitamin D (100 nM) increased expression of Cyp3A4 mRNA levels by 200–500-fold and induced Cyp3A activity by 200-fold along with modestly increasing *p*-glycoprotein (MDR1 gene product) levels (Schmiedlin-Ren et al., 1997; Aiba et al., 2005). Increased metabolism of carbamazepine by Cyp3A4 (phenytoin is primarily metabolized by Cyp2C) or increased efflux of phenytoin by MDR1/*p*-glycoprotein (carbamazepine is not a *p*-glycoprotein substrate) may constitute mechanisms for vitamin D amelioration of phenytoin's and carbamazepine's effects on calcium transport.

Maintenance of adequate dietary calcium and vitamin D levels is of greater concern in patients on long-term phenytoin or carbamazepine therapy. Drezner (2004) recommends use of calcium supplements and vitamin D for prophylaxis as well as treatment of osteoporosis or osteomalacia. Recommendations include 400–2000 IU/day vitamin D for prophylaxis, 2000–4000 IU/day for osteoporosis, and 5000–15,000 IU/day for 3–4 weeks for osteomalacia. While emphasizing vitamin D intake, Drezner acknowledges the importance of sufficient calcium intake and additionally recommends an intake of 600–1000 mg/day. Our results serve to emphasize the importance of achieving adequate vitamin D intake in patients receiving chronic phenytoin or carbamazepine treatment to promote improved dietary calcium absorption.

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