Anti-epileptic drugs and bone loss: phenytoin reduces pro-collagen I and alters the electrophoretic mobility of osteonectin in cultured bone cells.

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Grant supporters: RJAH charitable funds.

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Supplementary Information Available.

Supplementary File.
**Highlights**

- Phenytoin, like valproate, reduces collagen I protein production by osteoblast-like cells.
- Unlike valproate, phenytoin does not reduce osteonectin protein production but instead, alters the electrophoretic mobility of osteonectin.
- Perturbation of these important bone proteins is a possible mechanism to explain bone loss following long-term treatment with phenytoin.
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Disclosures

All authors state that they have no conflicts of interest.

Abstract

Phenytoin is an antiepileptic drug used in the management of partial and tonic-clonic seizures. In previous studies we have shown that valproate, another antiepileptic drug, reduced the amount of two key bone proteins, pro-collagen I and osteonectin (SPARC, BM-40), in both skin fibroblasts and cultured osteoblast-like cells. Here we show that phenytoin also reduces pro-collagen I production in osteoblast-like cells, but does not appear to cause a decrease in osteonectin message or protein production. Instead, a 24h exposure to a clinically relevant concentration of phenytoin resulted in a dose-dependent change in electrophoretic mobility of osteonectin, which was suggestive of a change in post-translational modification status. The perturbation of these important bone proteins could be one of the mechanisms to explain the bone loss that has been reported following long-term treatment with phenytoin.

Keywords

Phenytoin, valproate, collagen, osteonectin, SPARC, AEDs, anti-epileptic drugs, bone.

1. Introduction
Many of the most commonly used anti-epileptic drugs (AEDs) are associated with bone disease, as evidenced by biochemical abnormalities, increased fracture risk and decreased bone mineral density (reviewed by Nakken and Taubøll (2010) and Lee et al., 2010). AEDs that are implicated in hepatic cytochrome p450 dysregulation leading to vitamin D deficiency with subsequent bone loss appear to have the strongest association with bone abnormalities (Välimäki et al., 1994 and Pack 2001). This association does not fully explain the mechanism(s) of AED-induced bone loss however, since an increase in bone turnover with AEDs can occur independently of vitamin D deficiency (Valimaki et al., 1994, and Weinstein 1984).

Despite the clear body of evidence that describes the effects of AEDs on fracture risk and bone mass, few studies have investigated the direct effect of AEDs on bone cells. In a previous study, we examined the effect of the AED, valproate, on an established cell-based model of long bone-derived osteoblasts (hFOB1.19) and found for the first time that valproate reduced the amount of two key bone proteins, collagen I and osteonectin (Humphrey et al., 2013). Collagen I is the main protein component of bone matrix and osteonectin has a major role in bone development and mineralisation (Delany et al., 2003), so reduced levels may contribute to bone loss following long-term treatment with valproate. The aim of this study was to determine whether other commonly used AEDs also reduce levels of these important bone proteins in osteoblast-like cells.

2. Materials and Methods

2.1 AED compounds
AEDs were tested at a range of concentrations that were as close as possible to clinically relevant serum concentration (i.e. phenytoin (5-40 µg/mL, Gallagher and Sheehy, 2000); topiramate (5-40 µg/mL, Hu et al., 2013); levetiracetam (5-40 µg/mL, Bobustuc et al., 2010), lamotrigine (2.5-20µg/mL, Johannessen and Tomson, 2006) and carbamazepine (5-40 µg/mL, Gao and Chuang, 1991) (all from Sigma-Aldrich, UK). AEDs were solubilized in DMSO and stored as 2000-fold stock solutions.

2.2 Western blotting

Human foetal hFOB1.19 osteoprogenitor cells (hFOBs) were cultured as described previously (Humphrey et al., 2013). After establishing that parallel differentiated cultures were producing and mineralising a matrix in culture (Supplementary Figure 1), the hFOBs were treated with vehicle control (i.e. DMSO) or AEDs. Triplicate cultures of control and AED-treated hFOBs were harvested by trypsination after 24 hours of treatment and analysed by western blotting using an antibody against osteonectin (Santa Cruz Biotechnology), as described previously (Fuller et al., 2010).

2.3 Immunofluorescence

hFOBs were grown on coverslips, as described previously (Humphrey et al., 2013). For detection of collagen I, the coverslips were incubated with a pro-collagen I antibody (developed by McDonald, JA and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, The University of Iowa, Department of Biology, Iowa City, IA 52242), as described previously (Fuller et al., 2010). Sequential scans were performed with a Leica TCS SP5 confocal microscope with a 40× objective. To reduce operator bias a fixed laser intensity was used for all image acquisition and images were only acquired from fields with even DAPI staining. To reduce edge effects only the inner two-
thirds of the coverslip were analysed. Immunofluorescence intensity was quantified using Image J software and normalised to DAPI intensity to account for variations in cell number. Statistical analysis was performed using a one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test.

2.5 Gene expression

hFOBs were cultured and treated with AEDs, as described for western blot analysis. RNA was extracted from triplicate pellets of control, valproate-treated and phenytoin-treated cells after 8 and 24 hours of treatment, using an RNAeasy kit (Qiagen). RNA (0.5 μg) was reverse transcribed using the high capacity cDNA archive kit (Applied Biosystems) according to the manufacturer’s instructions. Amplification of the osteonectin, collagen I, ACTB and GAPDH genes was performed using previously validated primers (Supplementary Table 1) and SYBR green master mix with the ABI 7500 real time PCR machine. Quantification of data was performed using the comparative CT (ΔΔCT) method (Livak and Schmittgen, 2001), using the mean from the two endogenous reference genes, GAPDH and ACTB.

3. Results

3.1 Phenytoin treatment of hFOB cells results in a decrease in pro-collagen I immunoreactivity.

Pre-differentiation hFOB cells were grown with or without AEDs at clinically relevant doses. After 24 hours treatment with phenytoin, pro-collagen I immunoreactivity was significantly decreased in a dose-dependent manner, with a maximum of 48% reduction.
with a 20 µg/mL dose (p=0.037) (Figure 1a and 1b). This statistically significant decrease is identical to the decrease observed after a 24 hour treatment of hFOBs with a clinically-relevant concentration of valproate (Humphrey et al., 2013). Collagen I gene expression levels were unaffected, suggesting that phenytoin alters pro-collagen I protein production or turnover (Figure 1c). We were unable to detect a statistically significant decrease in collagen I following treatment with topiramate, levetiracetam, lamotrigine or carbamazepine (data not shown). It was not possible to reliably quantify the effects of AEDs on pro-collagen I levels in post-differentiation hFOB cultures because the high density of cells obtained after differentiation prevents reliable quantification by immunofluorescence. However, a similar trend was evident by qualitative assessment of phenytoin-treated post-differentiation cells (Supplementary Figure 2).

3.2 Phenytoin treatment of hFOB cells causes a dose-dependent change in the electrophoretic mobility of osteonectin.

Unlike previous observations with valproate (Humphrey et al., 2013), treatment of hFOB cells with other commonly used AEDs did not appear to reduce the levels of osteonectin protein following treatment for 8 or 24 hours (as determined by western blot analysis) (Figure 2a). After 24 hours of incubation with phenytoin, however, a small but clear shift in apparent molecular weight of osteonectin was detectable by western blot in treated hFOB cell extracts (Figure 2b). The approximate 2-3kDa difference in electrophoretic mobility was evident following treatment with as little as 5 µg/mL of phenytoin and this change was not apparent following treatment with the other AEDs (Figure 2a). Neither protein (Figure 2b) nor gene expression levels of osteonectin were altered following treatment with phenytoin (Figure 2c).
4. Discussion

In this study we have demonstrated that, like valproate (Humphrey et al., 2013), phenytoin treatment of osteoblast-like cells with a clinically relevant dose results in a reduction of pro-collagen I protein. Despite the profound effect of valproate and phenytoin on pro-collagen I protein production, neither drug appeared to alter the expression levels of the collagen I gene (Figure 1c). Taken together, these results suggest that phenytoin, like valproate, appears to have a direct-effect on osteoblast-like cells by causing them to produce lower amounts of collagen I protein. It seems highly probable that this would have a detrimental effect on the bone forming ability of these cells since osteogenesis imperfecta, the “brittle bone disease”, is caused, in most cases, by mutations in collagen I chains (Rauch and Glorieux, 2004).

None of the other AED compounds tested appeared to alter pro-collagen I protein levels. It is important to acknowledge, however, that some AEDs may require conversion to active metabolites in vivo, and it is not known whether osteoblast-like cells have the capability to do this.

Unlike valproate (Humphrey et al., 2013), none of the other AEDs tested in this study caused a reduction in osteonectin protein levels in osteoblast-like cells, suggesting that the mechanism by which this occurs is not common to all AEDs. Interestingly though, treatment with phenytoin did alter the electrophoretic mobility of osteonectin in SDS-PAGE gels when compared to control-treated cells. Since protein levels were unaltered, the most likely explanation for the shift in electrophoretic mobility is a change in the glycosylation of osteonectin. Osteonectin exists as isoforms of various molecular weights, attributed to differences in glycosylation patterns (Kelm and Mann, 1991), each with different functions and varying affinities for collagen binding (Kaufmann et al., 2004). It seems highly likely, therefore, that alterations in the glycosylation pattern of osteonectin would affect its ability to...
bind to collagen; thus influencing cellular ability to produce normal bone. This may explain a
mechanism by which long-term phenytoin treatment can lead to bone weakness (reviewed in
detail by Nakken and Taubøll (2010) and Lee et al., 2010), especially since two unrelated
cases of osteogenesis imperfecta with a severe bone fragility were caused by rare
homozgyous mutations affecting the collagen I binding region of osteonectin (Mendoza-
Londono et al., 2015).

In summary, the findings in this study suggest that, as with valproate, perturbation of
the important bone proteins, collagen I and osteonectin, could be one of the mechanisms that
lead to bone loss following long-term treatment with phenytoin. The findings presented here
provide a possible future direction for research focusing on the effects of AEDs on bone
metabolism in-vivo.

Acknowledgements

This work was funded by the RJAH charitable funds. Authors’ roles: Study design:
ELH, MG and HRF; conduct of experiments: ELH; drafting manuscript: ELH, MG and HRF.
HRF takes responsibility for the integrity of the data analysis. The authors are grateful to
Professor Glenn Morris for providing access to laboratory facilities.

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Figure Legends

**Figure 1.** Pro-collagen I protein, but not gene expression levels, is reduced in osteoblast-like cells after treatment with phenytoin. Acetone/methanol fixed cells were incubated with the anti-pro-collagen I monoclonal antibody (M-38) and visualised using a goat anti-mouse ALEXA 488. Bar = 100µm. Ten fields of view were chosen at random from each slide using DAPI view to avoid bias and include at least 200 cells over 10 images. The integrated density for each confocal microscope image was measured using ImageJ software and normalised to DAPI staining for each image. A representative image (A) and quantitative measurements from the dose response of phenytoin (PHT) on collagen protein (B) are shown. Collagen I gene expression was measured in cells treated with valproate (VPA) or PHT for 8 or 24hrs. No significant change in gene expression could be detected with either AED (C).

**Figure 2.** Of the AEDs tested, only valproate reduced the levels of osteonectin protein in osteoblast-like cells. Protein extracts from differentiated osteoblast-like cells treated with carbamazepine (CBX), lamotrigine (LAM), with levetiracetam (LEV), topiramate (TOP) and valproate (VPA) were subjected to SDS-PAGE and transferred to nitrocellulose by electroblotting. The blots were probed with antibodies against osteonectin (SPARC, BM-40). Total protein staining with Ponceau S was used as a loading control. After visualization using a chemiluminescent system, the integrated density of the bands for osteonectin and ponceau S were measured using ImageJ software (A). A dose-dependent change in the electrophoretic
mobility of osteonectin was evident, following treatment when treated with PHT (B). Neither VPA nor PHT had any effect on the gene expression levels of osteonectin (C).
Figure 1
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