Chapter 5

This study provides the first experimental evidence on the potential role of raloxifene in preventing and ameliorating PHT and SVP-induced bone loss without affecting the antiepileptic efficacy of these AEDs. The study extends previous reports of adverse effect of AEDs on bone and report the same in Swiss strain albino female mice for the first time. Further, the lack of bony effects observed following LTM treatment suggests that the same could be a better alternative to PHT or SVP in female epileptic patients or those having a risk factor for osteoporosis.

5.1 Effect of raloxifene on seizures and on antiepileptic efficacy of PHT, SVP and LTM

There are no consensus guidelines available for addressing the effect of AEDs on bone health even though it is advisable to screen all patients on chronic AED therapy for early diagnosis of possible effects on bone health (Sheth and Harden, 2007) and to prescribe CVD supplements in all patients susceptible to bone loss (though latter has also been questioned in some cases). There are not many studies available on the use of anti-osteoporotic agents that can be prescribed along with AED therapy and thus the physicians are not aware about the possible interaction of anti-osteoporotic agents in modifying the protective efficacy of AEDs. This makes it important to determine the effect of anti-osteoporotic agents on seizures. Such information is also significant from the point of view of prescribing an AED to an epileptic woman suffering from osteoporosis and vice versa.

We investigated the effect of raloxifene alone and in concurrent administration with either PHT or SVP for 4 months in electroshock-induced seizures. We found that chronic treatment with raloxifene (RLX) failed to significantly modify the hind limb extension (HLE) produced by electroshock (ES)-induced seizures. Unlike the findings of Scharfman and co-workers (2009) where RLX treatment improved survival after status epilepticus in rats, we did not find any protective effect of RLX in the ES model though a non-statistical increase in the latency to HLE and reduction in duration of HLE was observed. The effect of RLX on seizures, thus, needs to be investigated in other seizure models including threshold models to confirm its antiepileptic efficacy, if any. When administered with PHT, SVP or LTM, it elicited results similar to that produced by PHT, SVP and LTM per se. This indicates that RLX does not alter the antiepileptic efficacy of PHT, SVP or LTM ruling out any possible pharmacodynamic interaction of RLX with these AEDs on chronic use (Table 37).
5.2 AEDs-induced alterations in bone and effect of raloxifene/CVD/CVDD on the same

We observed bony alterations in Swiss albino female mice following four months treatment with PHT and SVP, hence presenting mouse models of bone loss where potential anti-osteoporotic therapies could be investigated. Since LTM failed to produce any change in either histopathology, BMD or bone turnover markers, it can be deduced that LTM may be a safer alternative in epileptic females who are prone to osteoporosis or having a risk factor for osteoporosis though further clinical investigations are definitely warranted.

Histopathology

In our previous study (Khanna et al. 2011), we administered PHT for 3 months which was found to produce histopathological changes in the femoral bones but not in the lumbar bones. Here, we extended the duration of therapy by one month and observed the changes in lumbar bones too. SVP, at both the doses studied, exhibited an increase number of osteoclasts, and rarefaction of bone matrix though changes were more pronounced at 300 mg/kg. LTM treated mice, however, at both the doses, did not show any histopathological changes in the bony architecture.

Bone Mineral Density

Bone loss, as observed in histopathology, was further confirmed by bone mineral density (BMD) measurements using dual energy X-ray absorptiometry (DEXA) technique, considered as gold standard for diagnosis of osteoporosis. Both PHT and SVP significantly reduced the BMD which is consistent with the earlier preclinical and clinical studies reporting reduced BMD following these AEDs (Boluk et al., 2004; Nissen-Meyer et al., 2007; Pack et al., 2008; Lee et al., 2010). Chronic treatment with PHT reduced BMC but SVP did not affect BMC as much as it affected BMD. The adverse bony effects of SVP as observed in this study were congruent with some previous clinical findings (Boluk et al. 2004; Guo et al. 2001) but inconsistent with others (Triantafyllou et al. 2010). A study by Senn and co-workers (Senn et al. 2010) reported strain-specific effects of valproate in animals. They identified two strains of mice that were sensitive (C3H/HeJ and Balb/c) and one strain (A/J) resistant to valproate-induced bone deficits. In our study, we extend the findings of this study to report an additional strain (Swiss albino mouse) to be sensitive to valproate-induced bony deficits. LTM (100 and 200 mg/kg) did not reduce BMD after 4
months of treatment. This is in agreement with the findings on BMD reported by Nissen-Meyer and co-workers on rats (Nissen-Meyer et al. 2007). The latter study, however, reported that LTM reduced biomechanical strength of the femoral neck. Since the latter is mainly a trabecular bone, we investigated whether LTM produces alterations in the lumbar vertebrae (L2-L4), another trabecular bone. No changes in lumbar vertebrae were, however, evident in our study and hence it may be possible that LTM exerts differential effects on femoral neck and lumbar sites requiring investigation. Clinically too, there have been varied reports. While LTM monotherapy was reported to have no harmful effects on bone strength and metabolism after one year of treatment and no obvious secondary effect on mass, quality (influenced by bone micro-architecture, geometry, and bone matrix composition) and remodelling of bone (Koo et al., 2013), a recent report showed that LTM compromised BMD comparable to oxcarbazepine (OXC) after two years of treatment (Beniczky et al., 2012). Since the previous study (Koo et al., 2013) reported the results obtained from a single center, was conducted in limited number of patients and did not take into account the estimation of fracture risk, it is possible that increasing the duration of treatment or studying in a larger population may result in adverse effects on bone following LTM administration. However, a recent study on young adult epileptic patients also support our findings on LTM that patients who switched to LTM had comparatively higher BMD in both lumbar spine and femur as compared to those on enzyme inducing AEDs such as PHT (Phabphal et al., 2013).

Preventive (RLX & CVD) and therapeutic (RLX & CVDD) treatment significantly restored the reduced BMD following PHT and SVP in both femur and lumbar vertebrae (Table 28, 29). The efficacy of RLX in increasing lumbar BMD has been shown in the multiple outcome of raloxifene evaluation (MORE) trial where an increased BMD in the lumbar spine reduced the risk of vertebral also reported the efficacy of RLX in the prevention of vertebral fractures by increasing bone mass and bone mechanical strength (Seeman et al., 2006). Further, a recent antiepileptic drug and osteoporosis prevention trial (ADOPT) in epileptic males indicated the beneficial effects on BMD at various sites including lumbar spine following CVD supplementation (Lazzari et al., 2013) in agreement with our results. Since raloxifene has been reported to have more effect on trabecular bones, we further examined its effects on biochemical markers of bone turnover in trabecular bone (lumbar vertebrae) only.
Bone Turnover Markers

The findings were further confirmed by measurement of bone turnover markers (BTMs) which provides dynamic information regarding skeletal status and is complementary to BMD analysis (Brown et al., 2009). The BTMs assess either osteoblastic synthetic activity or post release metabolism of pro-collagen, bone resorption markers being considered better indicators than bone formation markers (Vasikaran et al., 2006).

Alkaline phosphatase (ALP) is a glycoprotein found on the surface of osteoblasts and is produced in large amounts during early differentiating osteoblasts (Christenson, 1997). It is, thus, an excellent indicator of osteoblastic bone formation. Both PHT (35 mg/ kg) and SVP (300 mg/kg) reduced ALP in the vertebrae indicating reduced osteoblastic activity and lower bone formation. Preventive as well as therapeutic treatment with RLX, CVD or CVDD reversed the reduced ALP activity in lumbar bones (L2-L4) indicating enhanced bone formation (Table 23). Our study is in line with the previous reports demonstrating positive correlation between ALP and RLX treatment (Johnell et al., 2002). This was related to the fact that RLX increased osteoblast-specific transcription factor Cbfa1/Runx2 and α2 pro-collagen type I chain mRNAs (Taranata et al., 2002). Moreover, the tissue selective estrogenic effect of RLX on bone which promotes estrogen mediated differentiation of osteoblast by RLX cannot be ruled out in explaining its effect on increased ALP activity, a marker of bone formation (Johnell et al., 2002). SVP (100 mg/kg) and LTM (at both 100 and 200 mg/kg) did not affect ALP activity.

Bone resorption markers; tartrate resistant acid phosphatase (TRAP), hydroxyproline (HxP) and urinary calcium (U-Ca) reflects osteoclastic activity and/or collagen degradation. Osteoclasts express multiple lysosomal enzymes, including tartrate-resistant acid phosphatase (TRAP), a characteristic marker protein which has been used since years as a marker of osteoclastic bone resorption (Minkin, 1982). TRAP is secreted at the ruffled border by osteoclasts which in turn dephosphorylate osteopontin (bone matrix protein; TRAP substrate) (Christenson, 1997). It serves as an excellent marker for studying potential target of agents that act by modulating osteoclastogenesis. Chronic PHT and SVP treatment significantly (p<0.001) enhanced TRAP activity in lumbar bones suggesting increased bone resorption (Table 23) while LTM did not affect TRAP activity. RLX and CVD treatment significantly reversed the enhanced TRAP activity (Table 25, 26). Our results are consistent with the findings on RLX reducing osteoclastic activity through reduction of receptor
activator of nuclear factor kappa B ligand (RANKL) and increased osteoprotegerin (OPG) consequently reducing TRAP activity (Luvizuto et al., 2011).

HxP, another bone resorption marker, is a modified form of an amino acid constituting about 13% of collagen content (Delmas, 1993). Post-translational hydroxylation of proline of a peptide chain generates HxP. The urinary and/or tissue (bone) level of HxP reflects osteoclastic activity and collagen degradation. Clinical utility of urinary HxP as a marker has several limitations such as lack of tissue specificity and extensive metabolic degradation. About 85-90% of released HxP from bone is broken down into free amino acid which is filtered by kidney, reabsorbed and completely oxidised by liver so that only 10-15% appears in urine (Swaminathan, 2001). Therefore in order to make it more specific, the HxP content was estimated in bone. Our results show that the HxP content was decreased in mice lumbar vertebral bone after PHT and SVP treatment indicating enhanced osteoclastic activity. This could be explained by the fact that AEDs might have altered the nature of collagen and stabilized the cross-linking as observed previously (Viguet-Carrin et al., 2006). RLX treatment significantly (p<0.001) restored the bone HxP content in both preventive and therapeutic groups (Table 25, 26). Again, both the doses (100 and 200 mg/kg) of LTM did not produce any significant change in the HxP content of lumbar (L2-L4) bones. Thus, the biochemical changes were thus reflective of our results observed in BMD and histopathology.

Despite lack of specificity, urinary calcium (U-Ca) has been used as a bone resorption marker to screen altered bone turnover. Increased calcium / creatinine ratio has been reported in postmenopausal women indicating enhanced bone turnover (Sachdeva et al., 2005). Chronic PHT and SVP treatment significantly (p<0.001) raised the urinary calcium:creatinine (Ca/Cr) ratio as compared to control. Although LTM treatment (higher dose) also produced some increase in urinary calcium (though not as significant as PHT and SVP), probably the changes were not sufficient to cause significant changes in DXA. The raised U-Ca/Cr ratio could be correlated to hyperparathyroidism induced by PHT as a result of vitamin D deficiency (Maclay et al., 1978) and fanconi syndrome associated with SVP treatment (Dhillon and Hogler, 2011). Preventive (RLX and CVD) and therapeutic (RLX and CVDD) treatment significantly normalized the raised U-Ca/Cr ratio. The reversal could be explained directly through upregulation of TGF-β3 expression and its deposition on bone matrix followed by subsequent mineralization as well as indirectly through increasing
serum estradiol level which enhances the calcium deposition on bone (Yang et al., 1996; Uebelhart et al., 2004; Narayana Murthy et al., 2006).

5.3 Effect of AEDs on serum estradiol and lumbar TGF-β3 content and modulation by raloxifene/CVD/CVDD

PHT and SVP treatment significantly reduced serum estradiol (E2) levels in mice. Our findings on serum estradiol are in agreement with previous reports where PHT or SVP treatment reduced serum estradiol levels in both female and male rats (Sveberg et al., 2002; Heinicke et al., 1984). The deprived estrogen deficiency following PHT treatment could be attributed to microsomal catabolism of estrogen resulting in increased levels of sex hormone binding globulin that in turn lowers testosterone and other adrenal androgens that are aromatized to estrogens (Khanna et al., 2009). Whereas, polycystic ovarian syndrome as well as direct inhibition of estradiol release after chronic treatment with SVP might explain the reduced serum estradiol levels following SVP (Tauboll et al., 2003; Hu et al., 2011). Further, both PHT and SVP are reported to inhibit aromatase by 50%, and thus inhibiting the conversion of testosterone to estradiol (Jacobsen et al., 2008) reducing estradiol levels. We did not observe reduced serum estradiol levels following chronic treatment with LTM and hence, our findings are in agreement with the observation that though LTM inhibited the forskolin-stimulated estradiol secretion, it showed no effect on basal estradiol secretion (von Krogh et al., 2010).

Both RLX and CVD/CVDD restored the depleted estradiol levels. RLX binds to tissue-specific estrogen receptors and forms a RLX-estrogen receptor complex that enters the nucleus and activates gene transcription via recruiting various co-activators and co-repressors and hence promoting well-known protective effect of estrogen on bone (Miki et al., 2009). The probable explanation for CVD reversing estrogen levels could be substantiated as follows: vitamin D is known to regulate estrogen synthesis in human osteoblasts through usage of promoters I.4 and I.3 (Enjuanes et al., 2003). Further, an activation of aromatase promoters has been reported in brains glioma cells (Yague et al., 2009). Since deficiency of vitamin D reduces the expression of aromatase (Kinuta et al., 2000), vitamin D might be expected to increase expression of aromatase thereby enhancing estradiol levels.
Figure 19: Hypothetical depiction of the chain of events that might have happened following PHT and SVP-induced bone loss and its reversal by RLX

AEDs (Sodium valproate, SVP and Phenytoin, PHT) cause estrogen deficiency either by reducing the secretion and biosynthesis of estrogen from C19 steroids by inhibiting aromatase complex or by enhancing catabolism of estradiol (thereby increasing SHBG level). The deprived estrogen can directly reduce TGF-β3 and induce bone loss or indirectly, may downregulate antioxidant pathways and/or increase production of reactive oxygen species thereby enhancing osteoclastogenesis. Decreased TGF-β3 (following estradiol depletion) associated bone loss (as presented in the present study) could have mediated bone loss via an increase in Receptor Activator of Nuclear Factor Kappa B (RANKL) expression leading to osteoclastogenesis or decrease osteoprotegerin (OPG) on osteoblast cell surface. The bone protective effects of Raloxifene is mediated partly, by direct estrogenic action and increased estrogen level and partly, by upregulating TGF-β3 expression in bone which inturn promotes differentiation of osteoblast and apoptosis of osteoclast.

Transforming growth factor β (TGF β) is a protein that controls proliferation and cellular differentiation in most cells. It is present in abundant amount in bone matrix and its
production increases in response to factors that stimulate osteoclastic bone resorption (Chenu et al., 1988; Cox, 1995; Oreffo et al., 1989). Estrogen deprivation and ovariectomy has been previously reported to reduce TGF β3 in rat bones (Finkelman et al., 1992; Yang et al., 1996; Narayana Murthy et al., 2006). In line with this, we found that reduced estradiol levels following PHT and SVP were associated with reduced TGF-β3 content in lumbar bones of mice. Since TGF β3 is known to potently stimulate osteoblastic bone formation and also reduce osteoclastic activity by inducing apoptosis of osteoclasts (Quinn et al., 2001), the reduction possibly contributed to alterations in BMD and BTMs causing bone loss. Thus, decreased TGF-β3 (following estradiol depletion) associated bone loss (as presented in the present study) could have mediated bone loss via an increase in Receptor Activator of Nuclear Factor Kappa B (RANKL) expression leading to osteoclastogenesis or decrease osteoprotegerin (OPG) on osteoblast cell surface. In addition, vitamin D deficiency following enzyme-inducer PHT could also possibly reduce TGF-beta as it has been reported that vitamin-D deficient bones contain less TGF-β than vitamin-D enriched bones (Finkelman et al., 1991). In our study, we also observed a reduction in lumbar TGF-β3 following chronic LTM even though it did not affect estradiol levels. This was surprising and hence the reduction in lumbar TGF-β3 doesn’t appear to be a consequence of depleted estradiol, at least in case of LTM and was possibly attributed to its ability to attenuate TGFβ-inducible early response gene-1 (TIEG1) expression as a previous study reported the down regulation of TIEG1 gene in hippocampus (Christensen et al., 2010). RLX treatment significantly reversed the AEDs-induced reduction in TGF-β3 content possibly through its agonistic effect on TGF-β3 promoter or indirectly through increase in estradiol level. Indeed, a previous study demonstrated stimulation of TGF-β3 gene expression in rat bone as a potential mechanism of bone maintenance (Yang et al., 1996). Though CVD/ CVDD treatment also increased TGF-β3 content, the effect was not as pronounced as in case of RLX.

Thus, the findings of the present study collectively suggest that alterations in estrogen mediated TGF β3 content could account at least partly for the bony deficits induced by PHT and SVP. RLX, being an SERM having tissue selective estrogenic action on bone and TGF-β agonistic action, reverses the bony changes induced by these AEDs without affecting the antiepileptic efficacy of these drugs. However, our results are preliminary and further studies are needed to delineate any conclusion regarding the chronic incorporation of RLX in AED regimen.