Aryl Hydrocarbon-Mediated Inhibition of Osteogenesis: Reversal by Resveratrol, a Novel Aryl Hydrocarbon Receptor Antagonist

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Aryl hydrocarbon inhibition of osteogenesis: reversal by resveratrol, a novel aryl hydrocarbon receptor antagonist, by Sacha U. N. Singh. Degree: Master of Science, Department of Periodontology, Faculty of Dentistry, University of Toronto, 1999

Aryl hydrocarbon receptor ligands (AhR ligands) are environmental contaminants found in cigarette smoke and other sources of air pollution. The prototype is TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxin). There is increasing evidence linking cigarette smoking to osteoporosis and periodontal disease, but the mechanism is not well understood. By using resveratrol (3,5,4'-trihydroxystilbene), a plant antifungal compound that we recently demonstrated to be a pure AhR antagonist, we investigated the effects of TCDD on osteogenesis, hypothesizing that TCDD would inhibit bone formation in bone forming cultures and that this inhibition would be antagonized by resveratrol. We used the chicken periosteal osteogenesis (CPO) model which forms bone in vitro in a pattern morphologically and biochemically similar to that seen in vivo. The effects of drugs on various stages of bone formation such as differentiation, matrix production and mineralization can be assessed. Thus, this system is useful for studying osteogenesis through the measurement of alkaline phosphatase (ALP) activity (bone cell differentiation), and phosphate or calcium accumulation (mineralization). To confirm our findings, we also used a mammalian (rat) cell line that produces bone in vitro. The CPO data demonstrated that ALP activity was reduced by 50% (p<0.01 vs. control) in the presence of 10^{-9} M TCDD and the inhibition was completely reversed by 10^{-6} M resveratrol (p<0.05 vs. TCDD alone). TCDD-mediated inhibition of osteogenesis occurred in the osteoblastic differentiation phase (days 0-2) as later addition did not appear to have any effects. In the rat stromal bone cell line, TCDD (10^{-9} M) caused about 33% reduction in ALP activity which was abrogated by resveratrol at 3.5\times10^{-7} M. TCDD (4.12\times10^{-11} M) also induced a marked reduction in mineralization (~75%) which was completely antagonized by resveratrol. Our data also suggest that the AhR ligand effects are mediated through AhR receptors in bone. We postulate that AhR ligands are one component of cigarette smoke linking smoking to osteoporosis and periodontal disease. If so, resveratrol could prove to be a promising preventative therapy for smoking related bone loss.
The whole famn damily:

For all your support, encouragement, concern and love over the many years that it has taken to get this far, and for the comfort and security that comes from having a caring family to rely upon no matter what was needed.
Acknowledgements

I would like to thank Dr. Howard Tenenbaum for his knowledgeable guidance, insights, and friendship throughout my Master’s program; Dr. Robert Casper and his lab for their collaborative efforts to which this project is indebted; Ms. Violetta Tapia for her secretarial assistance and fabulous organization of my committee meetings; Mrs. Lori Mockler for organizing my final defense; the 6th floor pathology lab crew for their friendship and technical assistance; Dr. Sandu Pitari for generously donating the rat SBMC line; Dr. Ben Ganss for helping me figure out 5’ heads from 3’ tails; Dr. Peter Fritz for kindly donating the chicken cDNA amplicons, technical wizardry with RNA extraction and Northern analysis, and conversation which made it easy to cut up chicks but hard to decide on a car; Drs. Bruno Girard and Iona Leung for helping whenever I needed it and making the lab a fun place to be; Mr. Mark Neilson and (Dr. to be) Andie Wiseman for their caring and support; my family for everything; and most gratefully to Mr. Balram ‘strength of the prophet’ Sukhu without whose friendship, guidance, lab knowledge and filing efficiency none of this could have been accomplished.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>List of Figures</td>
<td>viii</td>
</tr>
<tr>
<td>List of Abbreviations</td>
<td>x</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Statement of Problem</td>
<td>13</td>
</tr>
<tr>
<td>Objectives</td>
<td>13</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>13</td>
</tr>
<tr>
<td>Methods:</td>
<td></td>
</tr>
<tr>
<td>General Overview</td>
<td>14</td>
</tr>
<tr>
<td>Section 1: Basic Culture Methods</td>
<td>16</td>
</tr>
<tr>
<td>1.1 CPO culture method</td>
<td>16</td>
</tr>
<tr>
<td>1.2 Rat SBMC line culture</td>
<td>17</td>
</tr>
<tr>
<td>Section 2: Assay Techniques</td>
<td>18</td>
</tr>
<tr>
<td>2.1 Pre-assay preparation of CPO</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Determination of alkaline phosphatase (AP) activity in the CPO and the rat SBMC line</td>
<td>18</td>
</tr>
<tr>
<td>2.3 Soluble protein determination in the CPO</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Determination of inorganic phosphate incorporation in the CPO</td>
<td>20</td>
</tr>
<tr>
<td>2.5 Determination of calcium incorporation in the CPO</td>
<td>20</td>
</tr>
<tr>
<td>2.6 Determination of total and radiolabeled collagen type I production in the CPO</td>
<td>21</td>
</tr>
<tr>
<td>2.7 RNA extraction and Northern Blot Analysis in the CPO</td>
<td>22</td>
</tr>
<tr>
<td>Section</td>
<td>Topic</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>2.8</td>
<td>Histological processing and analysis in the CPO</td>
</tr>
<tr>
<td>2.9</td>
<td>Determination of mineralization in the rat SBMC line</td>
</tr>
<tr>
<td>2.10</td>
<td>Whole cell competition binding assay in the rat SBMC line</td>
</tr>
<tr>
<td>2.11</td>
<td>Determination of cytotoxicity of compounds in the rat SBMC line</td>
</tr>
<tr>
<td>2.12</td>
<td>Determination of proliferation in the rat SBMC line</td>
</tr>
<tr>
<td>Section 3: Experimental Conditions</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>TCDD dose response in the CPO</td>
</tr>
<tr>
<td>3.2</td>
<td>Resveratrol dose response and antagonism of TCDD in the CPO</td>
</tr>
<tr>
<td>3.3</td>
<td>‘Window’ experiments in the CPO</td>
</tr>
<tr>
<td>3.4</td>
<td>Total and radiolabeled collagen type I analysis, RNA extraction and histological analysis in the CPO</td>
</tr>
<tr>
<td>3.5</td>
<td>Assessment of AP activity and mineralization in the rat SBMC line with a 2D-matrix</td>
</tr>
<tr>
<td>3.6</td>
<td>Whole cell competition binding assay in the rat SBMC line</td>
</tr>
<tr>
<td>3.7</td>
<td>Cytotoxicity and proliferation studies in the rat SBMC line</td>
</tr>
</tbody>
</table>

**Statistical methods**                                                                 30

**Results:**

1) **CPO model:** 31
   
   (a) TCDD dose response 31
   (b) Resveratrol dose response and antagonism of TCDD 31
   (c) ‘Window’ experiments 32
(d) Total and radiolabeled collagen type I levels ............................... 32
(e) RNA extraction and Northern analysis ........................................ 33
(f) Qualitative histological analysis .................................................. 33

II) Rat SBMC model: ........................................................................... 34
(a) AP activity ...................................................................................... 34
(b) Mineralization ............................................................................... 34
(c) Whole cell competition assays ....................................................... 35
(d) Proliferation study ......................................................................... 35
(e) Cytotoxicity study .......................................................................... 35

Discussion:

Alkaline phosphatase activity ............................................................ 58
Mineralization .................................................................................... 59
Osteogenic phase of drug activity ....................................................... 60
Collagen synthesis .............................................................................. 61
Histological assessment and culture viability ..................................... 63
Expression of mRNA for bone proteins ............................................. 64
Receptor studies ................................................................................. 65
Conclusions ........................................................................................ 66
Clinical relevance and future studies ................................................. 67

Tables ................................................................................................. 36
Figures .................................................................................................. 38
Bibliography ......................................................................................... 70
List of Figures and Tables

| Table 1:  | Soluble protein in CPO – TCDD dose response study | 36 |
| Table 2:  | Soluble protein in CPO – resveratrol dose response/TCDD antagonism study | 36 |
| Table 3:  | Soluble protein in CPO – ‘window’ experiments | 37 |
| Figure 1a: | AP activity in CPO – TCDD dose response study | 38 |
| Figure 1b: | Calcium in CPO – TCDD dose response study | 39 |
| Figure 1c: | Inorganic phosphate in CPO – TCDD dose response study | 40 |
| Figure 2a: | AP activity in CPO – resveratrol dose response/TCDD antagonism study | 41 |
| Figure 2b: | Inorganic phosphate in CPO – resveratrol dose response/TCDD antagonism study | 42 |
| Figure 3a: | AP activity in CPO – TCDD ‘window’ experiments | 43 |
| Figure 3b: | AP activity in CPO – resveratrol ‘window’ experiments | 44 |
| Figure 3c: | AP activity in CPO – compounds over days 0-6 | 45 |
| Figure 3d: | Inorganic phosphate in CPO – compounds over days 0-6 | 46 |
| Figure 3e: | Calcium in CPO – compounds over days 0-6 | 47 |
| Figure 4a: | Total collagen in CPO – compounds over days 0-6 | 48 |
| Figure 4b: | Radiolabeled collagen in CPO – compounds over days 0-6 | 49 |
| Figure 5:  | mRNA in CPO – compounds over days 0-6 | 50 |
| Figure 6:  | Histological analysis of CPO | 51 |
| Figure 7:  | AP levels in the rat SBMC line | 52 |
| Figure 8:  | Mineralization levels in the rat SBMC line | 53 |
Figure 9a: Competition binding in rat SBMC line – $^3$H-TCDD vs. TCDD..........................54
Figure 9b: Competition binding in rat SBMC line – $^3$H-TCDD vs. resveratrol..........................55
Figure 10: Proliferation in rat SBMC line.................................................................56
Figure 11: Cytotoxicity in rat SBMC line.................................................................57
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHH</td>
<td>aryl hydrocarbon hydrolase</td>
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<tr>
<td>AhR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>Arnt</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<tr>
<td>BaP</td>
<td>benzo-a-pyrene</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMD</td>
<td>bone mineral density</td>
</tr>
<tr>
<td>CPO</td>
<td>chick periosteal osteogenesis model</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome p450 1A1 enzyme</td>
</tr>
<tr>
<td>DMBA</td>
<td>dimethylbenz(a)anthracene</td>
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<tr>
<td>DMCSC</td>
<td>diluted mainstream cigarette smoke condensates</td>
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<td>DMSO</td>
<td>dimethylsulphoxide</td>
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<tr>
<td>DRE</td>
<td>dioxin response element</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>HSP90</td>
<td>heat shock protein 90</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>OCN</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>OPN</td>
<td>osteopontin</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered solution</td>
</tr>
<tr>
<td>PCB</td>
<td>polychlorinated biphenyls</td>
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<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>pN-p</td>
<td>para-nitrophenol phosphate</td>
</tr>
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<td>rat SBMC line</td>
<td>rat stromal bone marrow cell line</td>
</tr>
<tr>
<td>TCDD or dioxin</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
</tbody>
</table>
The coordinated activities of our many body systems and their component parts are required to maintain proper balance and function in the human body. The skeletal system and the bones therein perform several of these important activities. Bones provide support in the form of a rigid framework for the body, which allows movement by acting as levers for the actions of muscles, and also provides protection for the brain and other organs. Hematopoiesis occurs in the marrow space of bones, and bones serve as a store and supply of body reserves of calcium and phosphorus.

It is not surprising then that diseases affecting bone have a high impact on the quality of life and can have associated with them significant morbidity. Interventions aimed at reducing the health and socioeconomic costs of such diseases are constantly being sought. With this ultimate aim in mind, it is appropriate to single out risk factors for diseases and determine the possible mechanisms through which they exert their effects on diseases. Once understood it may be possible to identify new therapies on a more rational basis.

One such disease of bone is osteoporosis. Osteoporosis has been defined as 'a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk' (Conference 1993). Osteoporosis and osteoporotic fractures increase with age, and it is estimated that over half of all women and up to one-third of all men will experience an osteoporotic fracture during their lifetime (Jones, Nguyen et al. 1994). A better understanding of the risk factors leading to such outcomes could help us to intervene and decrease these statistics.

The pathogenesis of osteoporotic fracture involves the interaction of genetic, environmental and hormonal risk factors. Genetically, a low peak bone mass combined with
bone loss leads to a low bone mass and can result in increased bone fragility, if a person has an inherently low bone quality. Increased bone fragility can then manifest itself as fractures, which may or may not be associated with trauma (e.g. falls) (Center and Eisman 1997). Interacting with these genetic factors are several environmental and hormonal factors. Traditionally associated with osteoporosis in post-menopausal women, estrogen deficiency plays a well recognized role in bone loss (Riggs and Melton 1986). As for putative environmental factors, a wide variety have been identified, including but not limited to: dietary factors, such as calcium intake and possibly sodium and protein intake; amount of physical activity; caffeine and alcohol consumption; certain medications; and, importantly, cigarette smoking. These have all been shown to influence BMD to varying degrees (Center and Eisman 1997). Though many of the risk factors for osteoporosis have been identified, their mechanisms of action are poorly understood.

Another disease that carries with it significant morbidity is periodontal disease, a component of which involves bone loss. The periodontal tissues are those that surround the teeth and provide the support teeth require for function. Periodontitis is defined as inflammation of these supporting tissues and is usually regarded as a progressively destructive disease, which leads to a loss of bone and periodontal ligament and, ultimately, tooth loss. Clinically, there are many ways periodontal disease will manifest itself. Periodontal pocket formation (a migration of the attachment of the gingiva to the tooth toward the root of the tooth), suppuration, fibrosis, destruction of alveolar bone and periodontal ligament, mobility, and drifting of teeth are all symptoms of periodontal disease, all of which may eventually lead to tooth loss as noted above. The functional and aesthetic impact of tooth loss significantly impairs one’s quality of life, so identification and modification of the risk factors leading to this disease is constantly being pursued.
As with osteoporosis, the pathogenesis of periodontal disease is complex, involving the interaction of many factors. Offenbacher (Offenbacher 1996) reviewed the abundant literature on the pathogenesis of periodontal disease and proposed a critical pathway model of pathogenesis which highlighted the effects and interactions of these various factors. The accepted risk factors include poor oral hygiene, the presence of certain pathogenic bacteria, diabetes and, as with osteoporosis, smoking.

Investigations into understanding the risk factors for osteoporosis and periodontal disease are far from complete. However, as previously mentioned, one risk factor, which has proved to be strongly related to both of these diseases, is cigarette smoking, which shall be the focus of my studies.

Indeed, epidemiological data support the notion that cigarette smoking is a risk factor for osteopenia in men (Hollenbach, Barrett-Connor et al. 1993; Nguyen, Kelly et al. 1994; Egger, Duggleby et al. 1996; Kiel, Zhang et al. 1996; Grisso, Kelsey et al. 1997) and women (Hopper and Seeman 1994; Egger, Duggleby et al. 1996; Franceschi, Schinella et al. 1996). Further, a dose-response relationship between the number of cigarettes smoked per day and a decrease in bone mineral density has been shown to exist (Hollenbach, Barrett-Connor et al. 1993; Franceschi, Schinella et al. 1996), as has an association between smoking and increased fracture risk (Williams, Weiss et al. 1982; Aloia, Cohn et al. 1985; La Vecchia, Negri et al. 1991; Hollenbach, Barrett-Connor et al. 1993; Johansson and Mellstrom 1996; Grisso, Kelsey et al. 1997). Additionally, in a study of twins discordant for cigarette smoking, it has been demonstrated that the bone density of women who smoked, or who smoked more heavily, was significantly lower than that of their non- or lesser smoking twin sisters (Hopper and Seeman 1994). The largest difference in bone density was found in the pairs with the largest difference
in smoking. The effect of smoking on bone density was shown to be greater in current versus past smokers, suggesting that there may be a reversible component to this phenomenon (Nguyen, Kelly et al. 1994).

Similarly, smoking is accepted as one of the strongest risk factors for periodontal disease. Cross-sectional and longitudinal studies have detailed the consistent, positive association between smoking and periodontal attachment loss (Bergstrom 1989; Preber and Bergstrom 1990; Haber and Kent 1992; Horning, Hatch et al. 1992; Haber, Wattles et al. 1993; Ah, Johnson et al. 1994; Haber 1994). A meta-analysis of the literature conducted by Papapanou for the 1996 World Workshop in Periodontics (Papapanou 1996) revealed that smokers have a 2.82 times increased risk for severe periodontal disease versus non-smokers. Investigations have been unable to attribute these findings to differences in the microbiota or levels of plaque and inflammation. Smokers and non-smokers exhibit similar profiles of periodontal pathogens (Preber, Bergstrom et al. 1992; Stoltenberg, Osborn et al. 1993), and even when the levels of plaque and inflammation are the same, smokers have an increased prevalence and severity of disease (Haber, Wattles et al. 1993). Evidence also exists revealing an increased failure of dental implant osseointegration in smokers versus non-smokers (Bain and Moy 1993; De Bruyn and Collaert 1994).

As alluded to above, it is not known how cigarette smoke may exert its negative effects on bone formation or bone diseases. It is plausible that it may be certain components of cigarette smoke that negatively impact on bone health. Cigarettes are known to produce many toxins that may have an effect on the body. Though more than 4700 individual constituents have been identified in cigarette smoke (Burns 1991) (Bartecchi, MacKenzie et al. 1995) relatively little is known about how the individual components affect bone. Studies have shown that cigarette
smoking increases the levels of serum cortisol (Baron, Comi et al. 1995) and also increases the conversion of estrogen to forms with minimal estrogenic activity (Michnovicz, Hershcopf et al. 1986). It can be hypothesized that elevations in cortisol and an anti-estrogenic effect of smoking may explain such adverse consequences as osteoporosis and periodontal disease. Studies to confirm such a mechanism or to identify the component of cigarette smoke that produces those effects have not been undertaken. One study, which looked at the effect of nicotine in osteoblast-like cells isolated from chick embryo calvariae, found that nicotine inhibited collagen synthesis and alkaline phosphatase (AP) activity (Ramp, Lenz et al. 1991). Concentrations of 100-600μg nicotine/ml were shown to produce this effect. These levels are appropriate to test the effect of nicotine derived from smokeless tobacco, where the concentration of nicotine in the saliva can reach 1.56mg/ml. The authors note, however, that the circulating concentrations of nicotine attained through tobacco use (15ng/ml) are probably too low to alter osteoblastic function (Ramp, Lenz et al. 1991). Several studies have focused on the possible effects of smoking on bone healing. Nicotine has been shown to have several effects that may interfere with bone healing. Nicotine liberates adrenal catecholamines, causing vasoconstriction and decreased tissue perfusion (Reus, Robson et al. 1984), and also decreases prostacyclin production. Prostacyclin is an important component of the healing process due to its vasodilatory actions (Nadler, Velasco et al. 1983). Endothelial production of prostaglandin I₂ is reduced by nicotine, which causes delayed healing of endothelia (Effeney 1987). An alteration of fibroblast cellular metabolism, including collagen synthesis and protein secretion, results from the nonspecific binding and internalization of nicotine by fibroblasts (Chamson, Frey et al. 1982).
Carboxyhemoglobin levels are significantly higher in smokers due to the binding of carbon monoxide, which comprises 4% of cigarette smoke, to hemoglobin. This causes the oxygen-hemoglobin saturation curve to shift to the left, resulting in tissue hypoxia, a condition that could also alter bone healing (Birnstingl, Brinson et al. 1971). Cellular oxygenation may also be affected by an inhibition of cellular respiratory enzymes, which has been shown to be caused by hydrogen cyanide produced by cigarette smoke (Mosely and Finseth 1977).

One of the products of cigarette smoke which warrants particular attention but has not been studied in association with bone metabolism are the aryl hydrocarbon receptor (AhR) ligands. AhR ligands such as dioxin, benzo-a-pyrene (BaP), and polychlorinated biphenyls (PCB) are environmental contaminants that bind to the AhR, which is present in the cytosol of mammalian cells of almost all organs and tissues. These AhR ligands are present in exhaust fumes of gasoline and diesel engines, furnace gases, cooked meat and fish, dairy products, breast milk and cigarette smoke. Concern surrounding these compounds stems partly from their resistance to degradation, resulting in a biologic half-life of over 10 years in soil (Rowlands and Gustafsson 1997) and between 4 and 12 years in human blood and fat (Bock 1994). Sufficient evidence currently exists to link AhR ligand exposure to the development of atherosclerosis (Paigen, Holmes et al. 1986; Toborek, Barger et al. 1995), cancer (Bartsch, Petruzelli et al. 1992; Nebert, Puga et al. 1993; Kleman and Gustafsson 1996), immunosuppression (Esser 1994; Tonn, Esser et al. 1996; Yamaguchi, Near et al. 1997), and skin disorders (Coenraads, Brouwer et al. 1994; Anderson, Hehr et al. 1995). The amount of BaP in mainstream cigarette smoke has been estimated to be between 40-100ng per cigarette and non-smokers in a smoking environment can inhale up to 460ng of BaP per hour (Committee on Passive Smoking 1986). BaP exposure of up to 0.05mg/kg can occur daily in heavy smokers. The presence of these detrimental
compounds in cigarette smoke, combined with the confirmed association between smoking and bone loss, establishes AhR ligands as candidates for investigation. Indeed, though the specific compound(s) have not been identified, investigators have recently shown that diluted mainstream cigarette smoke condensates (DMCSC's) can bind to and transcriptionally activate AhR and estrogen receptors (ER) and cause induction of dioxin response element (DRE)- and ER-regulated genes (Meek and Finch 1999). Elucidation of the effects of AhR ligands on bone formation is clearly necessary.

The AhR itself is a 110kDa protein of the basic helix-loop-helix family of transcription factors. Found in the cytosol bound to heat shock protein 90(HSP90) the AhR dissociates from HSP90 upon binding to its ligand and this AhR-ligand complex is then translocated to the nucleus via association with an AhR nuclear translocator (Arnt) (Rowlands and Gustafsson 1997). This complex binds to DNA at dioxin response enhancers located within or upstream of a number of phase I and phase II enzymes, the most important of which is the cytochrome p450 1A1 enzyme (CYP1A1). This enzyme is responsible for the production of aryl hydrocarbon hydrolase (AHH) which metabolises AhR ligands to more pro-carcinogenic compounds (Kiyohara, Hirohata et al. 1996; Shimada, Hayes et al. 1996). AHH also increases the production of reactive oxygen species which can cause oxidative DNA damage (Park, Shigenaga et al. 1996). The phase II enzymes that are produced are responsible for the detoxification and excretion of environmental toxins, thus AhR ligand activated gene transcription initiates both toxic effects and attempts to clear the ligand from the body. The concentrations of AhR ligands in cigarette smoke are high enough to induce CYP1A1 and AHH activity in the lungs (Bartsch, Petruzzelli et al. 1992; Nebert, Puga et al. 1993), placenta (Gurtoo, Williams et al. 1983), kidney
(Bilimoria and Ecobichon 1980), ovary (Mattison and Thorgeirsson 1979), and in the endothelial cells lining blood vessels (Toborek, Barger et al. 1995).

Currently no endogenous ligand has been identified for the AhR and the only known natural ligands for this orphan receptor are methyleneindole condensation products formed after ingestion of cruciferous vegetables such as cabbage, broccoli and cauliflower (Bjeldanes, Kim et al. 1991). The compound that binds to the AhR with the highest affinity of all known compounds and is considered the prototypical AhR ligand is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) (Rowlands and Gustafsson 1997). In addition to being linked to the pathoses listed above, an anti-estrogenic effect of TCDD and other AhR ligands exists, revealed by their ability to inhibit a broad spectrum of estrogen-induced responses in rodents and breast cancer cell lines (Harris, Zacharewski et al. 1990; Safe, Astroff et al. 1991; Wang, Porter et al. 1993; Gierthy, Silkworth et al. 1994; Harper, Wang et al. 1994; Liu, Wormke et al. 1994; Krishnan, Porter et al. 1995; Safe and Krishnan 1995; Kharat and Saatcioglu 1996; Nodland, Wormke et al. 1997), and yet few studies have been undertaken to investigate the possible direct effects of AhR ligands on bone.

It has been shown that during the embryonic development of bone tissue in mice both AhR and Arnt are expressed (Abbott and Probst 1995) and that dioxin suppresses the normal differentiation of rat osteoblasts in vitro (Gierthy, Silkworth et al. 1994). This latter study showed that calvarial-derived rat osteoblasts would not upregulate expression of AP and osteocalcin (OCN) after exposure to dioxin. The authors suggested, appropriately, that this inhibition of the formation of bone tissue-like multicellular nodules intimates a direct association of dioxin and reduced bone mass.
Indeed these ligands could at least in part prove to be the compounds linking cigarette smoking to bone loss. If AhR ligands do play an important and apparently deleterious role in the inhibition of bone formation, then it would be advantageous to identify potential antagonists to those ligands. Recently, a pure AhR antagonist has been discovered and it's utility as a tool to investigate the effects of AhR ligands shown. Using a breast cancer cell line, transfected with a DNA construct containing a dioxin response element coupled to a thymidine kinase promoter and a chloramphenicol acetyl transferase reporter gene, Casper et al. (Casper, Quesne et al. 1998) screened for novel ligands as antagonists for the AhR. Approximately 50 compounds chemically similar to dioxin were investigated, since, as noted above, it is the prototypical AhR ligand. One of the compounds tested was a plant antifungal agent, which is found in certain red wines. This and other compounds found in red wine were tested due to the speculation that wine may contain compounds that could explain the so-called 'French Paradox'. Such speculation arises from recognition that despite the presence of similar risk factors for coronary heart disease (high animal fat intake, low excersise, smoking), France has a lower rate of coronary heart disease than other western countries (Renaud and de Lorgeril 1992), but also has the highest consumption of wine. Dioxin and other AhR ligands have been shown to cause endothelial cell damage and have been linked to the development of atherosclerosis (Paigen, Holmes et al. 1986; Toborek, Barger et al. 1995), thus it was hypothesized that the French paradox could be explained by a blockade of the AhR by one or more wine compounds. Using the model described above, it was found that an antifungal agent, resveratrol (3,5,4'-trihydroxystilbene), which is found in red wine, is an AhR antagonist and inhibits induction of DRE-driven transcription by TCDD in a dose response manner. Resveratrol also prevents dioxin from initiating transcription of CYP1A1 and other phase I enzymes (Casper, Quesne et al. 1998) (Ciolo, Daschner et al. 1998). Investigations to
elucidate the other effects of resveratrol have been carried out and have revealed multiple biological actions. Data are accumulating to support a role for resveratrol alone in the protection against cardiovascular disease in that it has vasodilatory effects on blood vessels (isolated rat aorta) (Chen and Pace-Asciak 1996), inhibits lipoxygenase production (Kimura, Okuda et al. 1985), thromboxane B₂ production (Shan, Yang et al. 1990), low density lipoprotein oxidation (Belguendouz, Fremont et al. 1997) (Frankel, Kanner et al. 1993) and prevents endothelial adhesion of granulocytes and monocytes (Ferrero, Bertelli et al. 1998). In addition to its putative cardioprotective effects, resveratrol may have cancer chemoprotective activity as well. Assays representing three major stages of carcinogenesis have demonstrated resveratrol has anti-initiation, antipromotion and antiprosperosis activity (Jang, Cai et al. 1997). Finally, resveratrol may have an estrogen-like action as will be discussed later (Mizutani, Ikeda et al. 1998) (Gehm, McAndrews et al. 1997).

Given the link between AhR ligands and bone metabolism, and further since resveratrol may modulate those effects through the AhR, it is essential to study the effects of these compounds on bone cells and tissue in more depth. The most practical starting point in elucidation of AhR ligand effects on bone formation is to determine the effects on in vitro bone forming systems. In vitro systems allow for greater control of the physicochemical environment and the physiological conditions can be kept relatively constant. This control results in the ability to vary the supply of a specific reagent with confidence that the effects observed are a direct result of that variation.

Though many bone forming systems exist, I shall focus on two general models, the Chick Periosteal Osteogenesis (CPO) and the Rat Stromal Bone Marrow Cell line (SBMC) model systems. The CPO model involves the removal of the ectocranial periosteal membrane from 17
day old chicken embryos (after microdissection of the excess fibrous tissue), folding this membrane such that the side of the explant originally facing bone is in apposition to itself, and then culturing this folded explant to form a layer of mineralized bone over a 6 day period (Nijweide 1975) (Tenenbaum and Heersche 1982). The developmental sequence through which the CPO progresses to reach this mineralized state includes all of the recognizable stages that occur during intra-membranous osteogenesis in vivo. In the first two days, cellular proliferation occurs with increasing organization and differentiation of the cells, including osteoblastic differentiation. Collagenous matrix production is seen over the next two days and mineralization of this matrix takes place during the final two days of culture (Tenenbaum and Heersche 1982; Tenenbaum and Heersche 1986; Tenenbaum, Palangio et al. 1986). This characteristic sequential development of the CPO model renders it useful in identification of the temporal phase of bone formation during which compounds administered to the system exert their effects (Tenenbaum and Heersche 1985). Additionally, ultrastructural analysis of the bone formed in this model has revealed it to be virtually indistinguishable from that formed in vivo (Tenenbaum, Palangio et al. 1986). Biochemical analyses of bone formation, including measurement of AP, calcium, phosphate, protein, and collagen, are possible, and mRNA can be extracted and, with the use of newly developed cDNA probes for avian proteins (Fritz, Ganss et al. 1998) effects on levels of transcription of various bone markers can be investigated. Many of the findings obtained with this avian model system (e.g. organic phosphate effects, glucocorticoid effects, tamoxifen effects and bisphosphonate effects) have been replicated in mammalian systems in vitro (Tenenbaum 1981; McCulloch and Tenenbaum 1986; Kamalia, McCulloch et al. 1992) and in vivo (Lekic, Rubbino et al. 1997; Sukhu, Rotenberg et al. 1997; Fritz, Ward et al. 1998). Thus, the CPO provides an excellent model for the comprehensive investigation of bone formation and
the results obtained can be realistically extrapolated to reflect the biological processes occurring in mammalian and probably human bone.

Despite the strengths outlined above for the CPO one must also recognize its limitations as well. Although the findings obtained with this model may be applied generally it is still, nonetheless, a non-mammalian culture system. Therefore it is important to utilize mammalian systems when possible. The rat SBMC model will express an osteogenic phenotype when treated with certain differentiation factors such as dexamethasone, vitamin C and β-glycerophosphate, which cause proliferation, differentiation and the formation of mineralized bone nodules in sequential and distinct stages. The addition of basic fibroblast growth factor (bFGF) sustains the proliferation and the formation of mineralized bone-like tissue by these cells for about one year (Pitaru, Kotev-Emeth et al. 1993; Kotev-Emeth, Pitaru et al. 1999). As indicated above, these cells will become osteoblasts, which produce distinct nodular mineralized bone-like tissue (Grigoriadis, Heersche et al. 1988; Maniatopoulos, Sodek et al. 1988; Bellows, Heersche et al. 1990; Kasugai, Todescan et al. 1991; McCulloch, Strugurescu et al. 1991; Shalhoub, Conlon et al. 1992; Malaval, Modrowski et al. 1994; Rickard, Sullivan et al. 1994; Herbertson and Aubin 1995). Again, measures of alkaline phosphatase activity and calcification provide insight into the effect compounds added to this system have on bone formation. Moreover, as will be discussed below, this cell culture model can also be used to study other questions (e.g. receptor binding assays) more readily than a tissue culture model such as the CPO.
Statement of Problem

Osteoporosis and periodontitis are diseases whose morbidity stems in large part from a loss of bone. Cigarette smoking is a major risk factor in both of these diseases. AhR ligands are an important component of cigarette smoke, and these compounds may have a negative impact on bone metabolism. If so, AhR ligands may be one component of cigarette smoke linking smoking to osteoporosis and periodontitis. An AhR antagonist, if it blocks the negative effects of AhR ligands, could form the basis for a promising therapy in the treatment and prevention of smoking related bone loss.

Objectives

- To determine if AhR ligands inhibit osteogenesis
- To determine if an AhR antagonist will antagonize the effect of AhR ligands on osteogenesis

Hypothesis

TCDD, the prototypical AhR ligand, will inhibit osteogenesis in bone forming cultures and resveratrol, a novel AhR antagonist, will antagonize this effect.
MATERIALS AND METHODS

As discussed in the introduction, two different models of osteogenesis were used, the CPO model and the rat SBMC line. Although utilization of the CPO model provides reliable insight into effects on osteogenesis of the compounds in question, confirmation of these findings in a mammalian system (i.e. the rat SBMC line) confers increased validity to the results.

The experiments and analyses carried out on the CPO were:

1. A dose response study using TCDD at doses of $10^{-9} \text{M}$ and $10^{-10} \text{M}$ to determine the dose required for inhibition of bone formation (section 3.1).

2. A dose response study using the dose of TCDD determined above to inhibit bone formation plus resveratrol at doses of $10^{-5} \text{M}$, $10^{-6} \text{M}$, and $10^{-7} \text{M}$ to determine the dose of resveratrol required to inhibit TCDD effects on bone formation (section 3.2).

3. ‘Window’ experiments administering TCDD or resveratrol in the doses determined above over days 0-2, 2-4 or 4-6 of culture to determine the phases of osteogenesis affected by the drugs (section 3.3).

4. Determination of collagen I levels (total and radiolabeled) in CPO cultures after administration of TCDD, resveratrol, and TCDD + resveratrol in the doses determined above (section 3.4).

5. Northern analysis of 6-day CPO cultures after administration of TCDD, resveratrol, and TCDD + resveratrol in the doses determined above using probes for collagen type I, alkaline phosphatase, and osteopontin (section 3.4).
6. Qualitative histological evaluation of 6-day CPO cultures after administration of TCDD, resveratrol, and TCDD + resveratrol in the doses determined above (section 3.4).

Using the rat SBMC line, the following studies were done:

1. Dose-response studies on AP activity and mineralization for both TCDD and resveratrol in combination in 96-well plates using a 2-dimensional matrix (section 3.5)

2. Competition assays with intact cells using $^3$H-TCDD vs. cold TCDD or resveratrol to determine the presence of the AhR in this bone forming culture and whether resveratrol competes with TCDD for the latter's cognate receptor (section 3.6)

3. A study to determine the effect of TCDD, resveratrol and TCDD + resveratrol on proliferation of cells (section 3.7)

4. A study to determine the cytotoxic effects of TCDD, resveratrol and TCDD + resveratrol on these cells (section 3.7)
Section 1 – Basic Culture Methods

1.1 – CPO culture method

Culture was carried out under sterile technique in a laminar flow hood. All instruments (beakers, forceps, scissors, dishes etc.) were sterile and instruments were kept in 70% ethanol when not being used. Before each use instruments were dipped in PBS. Holding the beak of a 17-day-old White Rock Meat chicken embryo with a pair of curved forceps, the skin of the head was grasped above the ear with a pair of straight forceps and peeled back. Microdissection to remove the outer fibrous layer was accomplished by gently scraping along the suture areas of the skull with the tip of a pair of pointed forceps. Two triangles of calvaria were removed by cutting along the central suture, transversely along the posterior suture and laterally above the eye. The triangles of calvaria were transferred to a petri dish containing PBS + 1% antibiotic. The ectocranial periosteum (outer curved surface) was peeled off using forceps under microscopic magnification and folded so that the surface originally facing bone was in apposition to itself. The folded explant was then placed on a Millipore filter (HA 0.45μm), which was transferred onto a stainless steel grid resting in the centre well of an organ culture dish (Falcon Plastics, Lincoln Park, NJ, USA) that was filled with culture media to a level such that the culture was supported at the gas-liquid interface of the media (~1.5ml of media). The outside well of the tissue culture dish was filled with 1ml of dH2O to maintain humidity. Three cultures were placed in each dish which was then covered and incubated at 37°C in a humidified atmosphere containing 5% CO2 in air.

Media changes occurred on days 2 and 4. The CPO medium was composed of a solution optimized for bone tissue growth (BGJb medium (Fitton-Jackson Modification) with L-glutamine
(Gibco, Grand Island, NY, USA)) and supplemented with 2% antibiotic (10,000 units/ml Penicillin G sodium, 10,000 µg/ml Streptomycin sulfate in 0.85% saline) (Gibco), 10% fetal calf serum (Gibco), 10⁻⁷M dexamethasone (Sigma, St. Louis, MO, USA) (Tenenbaum and Heersche 1985) (McCulloch and Tenenbaum 1986), 10mM β-glycerophosphate (Tenenbaum, McCulloch et al. 1989), and 300µg/ml L-ascorbate (Gibco) (Tenenbaum and Heersche 1986). To this was added the compounds under investigation in the appropriate concentrations (see below). On day 6 the culture dishes were placed on ice and individual cultures were transferred to separate test tubes and stored at -20°C for further analysis.

1.2 – Rat SBMC line culture

Cells for SBMC were obtained from Dr. Sandu Pitaru in Israel and maintained in T₂₅ tissue culture flasks (Sarstedt, Newton, NC, USA) in a medium composed of αMEM+RNA+DNA+antibiotic supplemented with 10% fetal calf serum, 10mM β-glycerophosphate, 10⁻⁴M dexamethasone, 50µg/ml vitamin C and 3ng/ml fibroblast growth factor. The media were changed every 48 hours and the cells subcultured when almost confluent (approximately every 72 hours). Using 0.01% trypsin at 37°C for about 5 minutes to release the cells from their substrate, the cells were subcultured at 1:10 (~0.4 x 10⁶ cells/cm²) in a T₂₅ tissue culture flask.
Section 2 – Assay Techniques

2.1 – Pre-assay preparation of CPO

required for assays of alkaline phosphatase activity, protein production, calcium incorporation, inorganic phosphate incorporation

CPO cultures in test tubes were kept on ice. 1ml of bicarbonate buffer (3mM NaHCO₃ in 15.0 mM NaCl pH 7.4) was added to each test tube. The cultures were homogenized by grinding on ice using a Polytron® homogenizer (Kinematica GMBH, Switzerland) at high speed (25 000 rpm) for 1 minute (intervals of 10 second grinding and 5 second rest period). The homogenized cultures were then subjected to centrifugation for 25 minutes at 4°C and 3180g. The supernatant fraction was assayed for alkaline phosphatase activity and protein production. The pellet was kept and assayed for calcium and inorganic phosphate incorporation after hydrolysis in 0.5N HCl.

2.2 – Determination of alkaline phosphatase (AP) activity in the CPO and the Rat SBMC line

The sample used for the assay of AP activity was either the supernatant produced by the centrifugation of the homogenized CPO culture or cultured SBMC cells. The assay for AP activity is based on the enzymatic cleavage of phosphate from para-nitrophenol phosphate (pN-p) to produce para-nitrophenol (pN). This reaction results in the substrate changing from a colourless liquid to a yellow liquid. The intensity of this colour change was evaluated via colorimetric analysis using a Titertek® Multiskan® MCC/340 Spectrophotometer (Flow Laboratories, Mississauga, ON, Canada) and compared to a standard curve obtained from a serial dilution of 10mM pN with bicarbonate buffer (pH 7.4).
Using a flat bottomed Titertek 96-well plate, 50μl of sample and 100μl of reagent were added per well. The reagent was prepared in advance and composed of a 3:7 ratio of 0.02M pN-p: 0.1M NaBarbital, pH 9.3. Each sample was done in duplicate. A single well blank was also prepared using 50μl of bicarbonate buffer, pH 7.4 instead of sample. The plate was mixed well and then incubated at 37°C with 5% CO₂ for 30 – 60 minutes. The reaction was stopped with 100μl of 0.2N NaOH. The colour change was then read at 405nm on the Titertek.

2.3 - **Soluble protein determination in the CPO**

Soluble protein content was assayed in the supernatant produced by the centrifugation of the homogenized CPO culture. The assay for soluble protein determination was accomplished by using the Bio-Rad Protein Assay® (Bio-Rad Laboratories, CA, USA) based on the Bradford dye-binding procedure (Bradford 1976). This involves the colour change of Coomassie brilliant blue g-250 dye in response to various concentrations of protein. The intensity of this colour change was evaluated using the Titertek and compared to a standard curve obtained by reacting bovine serum albumin standards. Using a flat bottomed Titertek 96-well plate, 80μl of sample and 20μl of reagent were added per well. Each sample was done in duplicate. A single well blank was also prepared using 80μl of bicarbonate buffer, pH 7.4 instead of sample. The plate was mixed well and the reaction was complete in 5 minutes. The colour change was read at 620nm on the Titertek.
2.4 – Determination of inorganic phosphate incorporation in the CPO

Inorganic phosphate incorporation was measured in the pellet produced from centrifugation of the homogenized CPO culture. 500μl of 0.5N HCl were added to hydrolyze the pellet and allowed to sit overnight at 4°C. The samples were vortexed and then centrifuged for 10 minutes at 3180g and 4°C. The supernatant was saved and became the sample. The assay for inorganic phosphate incorporation is based on the colour change that occurs when a phosphomolybdate complex is reduced by ascorbic acid. The intensity of this colour change was evaluated via colorimetric analysis using the Titertek and compared to a standard curve obtained from a serial dilution of 1mM inorganic phosphate with 0.5N HCl. Using a flat bottomed Titertek 96-well plate, 30μl of sample and 270μl of reagent (a 1:6 ratio of 10% Ascorbic acid: 0.42% ammonium molybdate 4H2O in H2SO4) were added per well. Each sample was done in duplicate. A single well blank was also prepared using 30μl of 0.5N HCl instead of sample. The plate was mixed well and incubated at 37°C for 1 hour. The colour change was read at 620nm on the Titertek.

2.5 – Determination of calcium incorporation in the CPO

As for inorganic phosphate, calcium content was measured in the pellet produced from centrifugation of the homogenized CPO culture and hydrolyzed as noted above. The samples were vortexed and then the post-hydrolysis supernatant was analyzed via atomic absorption spectrophotometry. A 1:20 dilution of the sample with 10mM lanthanum chloride was carried out and duplicate measures of calcium concentration were made with the Perkin Elmer Atomic Absorption Spectrophotometer® (Perkin-Elmer, Norwalk, CT, USA) using the 422.7nm wavelength filter for calcium.
2.6 – Determination of total and radiolabeled collagen type I production in the CPO

Radiolabeled (and total) collagen was measured in cultures incubated with $^{14}$C-glycine (Amersham, 59mCi/mMole) added at a concentration of 10μci/ml media in each group over days 4-6. The cultures were hydrolyzed in 0.1 ml of 0.1N HCl and vortexted. After 15 minutes, the supernatant was removed and saved in a 2ml eppendorf tube. The culture was transferred to a 0.5ml eppendorf tube containing 0.1ml of pepsin (50μg/ml in 0.1% glacial acetic acid) to digest the cultures. These were maintained in a 15°C water bath for 5 hours, vortexing the samples every hour. The supernatant was transferred to a new 0.5ml eppendorf tube, covered with parafilm, and frozen on dry ice. The parafilm was perforated several times and the sample was then lyophilized overnight. The amount of collagen was determined by gel electrophoresis as described hereunder.

The lyophilized samples were reconstituted with 0.1ml of a solution made of 70% sample buffer (10mM Tris•HCl and 1mM EDTA, pH 8.0) (Sigma-Aldrich Canada Ltd., Oakville, Canada), 2.5% SDS (Sigma), 5% β-mercaptoethanol (Sigma) and 0.01% bromophenol-blue (Sigma). The samples were boiled for 5 minutes, centrifuged for 10 minutes at 10 000g and the resulting supernatant used to run the gel. 2μl of supernatant were loaded in each lane of an 8-lane 7.5% homogenous SDS gel (Amersham Pharmacia Biotech, Inc, Quebec). The gel was then run on the PhastSystem™ (Pharmacia, Quebec) at 250V, 10.0mA, 3.0W, 15°C for 20 minutes (~2/3 the length of the gel). The gel was stained with coomassie blue for 1 hour. The filtered stain solution was 0.1% coomassie brilliant blue, 30% methanol, and 10% acetic acid in dH₂O. The destain solution was 30% methanol, and 10% acetic acid in dH₂O, and the preserving solution was 2.5% glycerol and 10% acetic acid in dH₂O. Measurement of the intensity of
staining of the collagen bands was carried out with a laser densitometer (Pharmacia) to quantify the total collagen content of the samples.

Fluorography was used to analyze the amount of $^{14}$C-labeled collagen in each sample. Each gel was coated with 20% PPO in DMSO. The gels were then exposed to photographic film (Kodak, Rochester, NY, USA) for 2 days at -80°C, and the film then developed. The intensity of the resultant bands on the film was quantified using a laser densitometer to give a measure of the amount of $^{14}$C-labeled collagen in each sample.

2.7 – RNA extraction and Northern Blot Analysis in the CPO

Isolation of RNA was accomplished using the RNeasy Mini Kit™ from Qiagen (QIAGEN Inc., Mississauga, Canada). The RNeasy Mini Protocol for Isolation of Total RNA from animal tissues was followed with minor modifications developed in our laboratory to optimize results in the CPO (noted in italics). 12 CPO explants were required per group to obtain an adequate amount of mRNA to perform Northern Blot analysis. The 12 explants were pooled in one test tube to which was added 600μl Buffer RLT™ (denaturing guanidinium isothiocyanate (GITC)- containing buffer), 150μl 0.5M EDTA (pH 8.0) and 7.5μl 14.5M β-mercaptoethanol (β-ME). The explants underwent 3 x 15sec polytron homogenization on ice with a 10 sec pause between cycles. The lysate was then pipetted into a QIAshredder™ column sitting in a 2 ml collection tube and centrifuged for 1 minute at 14,000rpm and room temperature. Nest, the lysate was centrifuged for 3 minutes at 2,000rpm in a microfuge and the supernatant was used in the subsequent steps. 600μl of 70% ethanol was added to the cleared lysate and mixed well by pipetting. 700μl aliquots of the sample were loaded successively to a RNeasy™ mini spin column sitting in a 2ml collection tube and centrifuged for 15 sec at 10,000rpm. Next 700μl of Buffer RW1™ was pipetted onto the RNeasy™ column and
centrifuged for 15 sec at 10,000 rpm to wash. *This step was then repeated.* The RNeasy™ column was transferred to a new 2 ml collection tube and 500µl of Buffer RPE™ pipetted onto the column and centrifuged for 15 sec at 10,000 rpm to wash. This wash was repeated with a 2 minute centrifuge at maximum speed to dry the RNeasy™ membrane. Finally the RNeasy™ column was transferred into a new 1.5ml collection tube and 30µl of RNase-free water pipetted directly onto the RNeasy™ membrane. This was centrifuged for 1 min at 10,000 rpm to elute the RNA.

The concentration of RNA extracted was determined by measuring the absorbance at 260 nm (A$_{260}$) in a spectrophotometer. The equivalent of 10 µg of RNA was added to sufficient dH$_2$O to make each sample 10 µl total. Reprecipitation in 20 µl of 2% potassium acetate in 100% EtOH at -20°C for 20 minutes was followed by centrifugation at 4°C and 14,000 rpm for 10 minutes. The supernatant was removed and the pellet denatured by adding 1x MOPS running buffer, 50% formamide, and 2.5M formaldehyde in dH$_2$O and heating for 10-15 min at 60°C. The sample was stained with bromophenol blue and run on an agarose gel at 60V until the bromophenol blue migrated 2/3 the length of the gel. The gel was examined on a transilluminator to visualize the RNA and photographed with a ruler laid along side so the band positions could later be identified on the membrane. The gel was then transferred overnight to a Hybond™-NX nylon membrane optimized for nucleic acid transfer (Amersham LIFE SCIENCE, Buckinghamshire, England). Finally, a 4 minute UV exposure was used to bind the RNA to the membrane.

Northern blot analysis began with prehybridization of the nylon membrane in ExpressHyb™ Hybridization solution for 2 hours at 68°C. During this time the probe to be used was prepared. 1 µl of the appropriate PCR product (chicken collagen type I, alkaline
phosphatase, or osteocalcin cDNA amplicon), 28µl of the corresponding primer sequence, 6µl of dH₂O, 1.5µl of 50mM MgCl₂, 5µl of 10x PCR buffer, 4µl of 0.25mM GTA mix, 5µl of 0.25mM dCTP-³²P and 1µl of Taq polymerase were added together. This was amplified with PCR (94°C for 3 min; 5 cycles of 94°C for 20 sec, 45°C for 20 sec, 72°C for 30 sec; then cooled to 4°C). 1µl of 0.5M EDTA (pH 8.0) was added to the probe to stop the PCR reaction. The probe was then loaded on a Probe Quant™ G-50 spin column (Micro Columns, Amersham pharmaciabiotech, Buckinghamshire, England) and spun at 3,000g for 2 min. 1µl of the probe was added to 5ml of scintillation fluid and counted to determine the activity of the probe. The probe was boiled for 5 minutes, cooled on ice for 2 minutes and then added to 5ml of prehybridization solution and hybridized for 2 hours. The membrane was then washed in 1L of 2x SSC and 0.05% SDS for 1 hour at 50°C. The membrane was then exposed on BioMax™ Film (Eastman Kodak, Rochester, New York) overnight at -70°C. The film was developed and scanned on an Epson Expression 636 flatbed scanner. The bands were quantified using ImageQuant™ version 1.2 for Macintosh (Molecular Dynamics, Sunnyvale, California).

2.8 – Histological Processing and Analysis in the CPO

CPO cultures on filter paper were transferred to a Tissue-Tek™ biopsy cassette (Miles Inc., Elkhart, IN, USA) and fixed in 10% formalin. The cultures were subjected to dehydration in increasing concentrations of ethanol (70% - 100%) and then 100% xylene, and finally embedded with wax. The cultures were sectioned to a 5µm thickness using a microtome, put onto plain slides, and placed in a slide drier (60°C-80°C). The sections were then stained with hematoxylin (10 minutes) and eosin (2 minutes), dehydrated, cleared and mounted. This resulted in staining the nuclei blue, calcified bone pink, and osteoid light pink.
2.9 – *Determination of mineralization in the Rat SBMC line*

Mineralization levels in the SBMC were determined via staining with Alazarin Red. The media were removed from the SBMC cells, that had been cultured in 96-well plates. The cells were washed once with 100\(\mu\)l of PBS. Cell fixation was accomplished by adding 100\(\mu\)l of 10% neutral formalin for 5 minutes. After washing 3x with 100\(\mu\)l of dH\(_2\)O, 100\(\mu\)l of 2% Alazarin Red-S (Sigma) was added, again for 5 minutes. Final washing 3x with 100\(\mu\)l of dH\(_2\)O was done and reading the optical density on the Titertek at 525nm assessed the intensity of staining.

2.10 – *Whole cell competition binding assay in the Rat SBMC line*

Four replicates were used in conducting the whole cell competition binding assay. 10,000 cells were plated per well in a Titertek 96-well plate and left overnight to attach. The rat SBMC medium (see section 1.2) was removed from the 96-well plate by aspiration and the various concentrations of cold drug were prepared and added to the wells in a volume of 90\(\mu\)l. The cold drug was left for a 1 hour pre-incubation period (pilot studies having shown that pre-incubation was required to detect competition). One set of wells was cultured without cold drug to determine total binding. \(^3\)H-TCDD at 10,000 cpm/well in a volume of 10\(\mu\)l was added and incubated for 4 hours at room temperature. The media were aspirated and the cells washed 4x with 100\(\mu\)l of PBS. 100\(\mu\)l of 0.1N sulphuric acid was added and the cells were incubated at 37\(^\circ\)C overnight to break the cells open. 80\(\mu\)l of the lysate was added to 5ml of scintillation fluid and counted in a beta counter.

2.11 – *Determination of cytotoxicity of compounds in the Rat SBMC line*

Trypan blue staining and a hemocytometer were used to determine total cell counts and viable cell number. This method is based on the principle that live (viable) cells do not take up the large molecular weight dye, whereas dead (non-viable) cells with leaky membranes do.
Three replicates were used in conducting the cell cytotoxicity assay. 3,000 cells were plated per well in a Titertek 96-well plate and cultured in rat SBMC medium (see section 1.2) under different concentrations of the drugs being investigated. After a 3 day culture period the media were removed from the 96-well plate by aspiration and the cells were washed with 200μl PBS. 100μl of cell dissociation buffer was added and the cells incubated at 37°C until they lifted off of the plate. 30μl of cells and 20μl of 0.4% Trypan blue solution were combined in a new 96-well plate and mixed well. After 10 minutes the number of staining (dead) and non-staining (viable) cells were counted using a hemocytometer.

2.12 – Determination of proliferation in the Rat SBMC line

The MTT assay was used to determine proliferation in the SBMC line. This test is based on the enzymatic reduction of the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide) in living, metabolically active cells but not in dead cells. The reaction was carried out in a 96-well plate in triplicate, and the reaction product, a purple-coloured formazan soluble in dimethylsulfoxide, was measured colorimetrically. 3,000 cells were plated per well in a Titertek 96-well plate and cultured in rat SBMC medium (see section 1.2) under different concentrations of the drugs being investigated. After a 3 day culture period 30μl of the MTT dye were added to each well and the cells incubated for 4 hours at 37°C. The media were then aspirated and 100μl of DMSO added to each well. The plate was well mixed and left for 5 minutes before reading the optical density on the Titertek at 570nm to assess the colour change.
Section 3 – Experimental Conditions

3.1 – TCDD dose response in the CPO

6-day CPO cultures (section 1.1) were treated with $10^{-9}$ M TCDD, $10^{-10}$ M TCDD or vehicle control media. The compounds were administered over the entire 6-day culture period with media changes every 48 hours. Each group was comprised of 9 cultures. One organ culture dish could accommodate 3 cultures therefore each group required 3 organ culture dishes with approximately 1.5 ml of media in each culture dish per media change. TCDD, originally obtained in a concentration of 10 pg/ml in 100% toluene (Supelco, Bellefonte, PA, USA), was diluted to a stock solution of 1 pg/ml in DMSO. Thus this stock was $3 \times 10^{-7}$ M in 90% DMSO and 10% toluene. The appropriate amount of stock was added to CPO medium (see section 1.1) to obtain the desired concentrations. The amount of vehicle in the vehicle control was determined by calculating the concentration of vehicle in the most concentrated TCDD treatment group, $10^{-9}$ M TCDD, which was 0.3% DMSO, and 0.03% toluene. The media in the control group were adjusted to these levels.

3.2 – Resveratrol dose response and antagonism of TCDD in the CPO

6-day CPO cultures (section 1.1) were treated with TCDD $10^{-9}$ M, resveratrol $10^{-5}$ M ± TCDD $10^{-9}$ M, resveratrol $10^{-6}$ M ± TCDD $10^{-9}$ M, resveratrol $10^{-7}$ M ± TCDD $10^{-9}$ M, vehicle control or plain control media. The compounds were administered over the entire 6-day culture period with media changes every 48 hours. When given together, TCDD and resveratrol were added simultaneously to assess their effects in direct combination. The appropriate amount of the various stock solutions was added to CPO medium (see section 1.1) to obtain the desired final concentrations. A $3 \times 10^{-7}$ M TCDD stock was prepared as in section 3.1 and the initial stock solution of resveratrol, prepared from powder form to $10^{-2}$ M in 34% ethanol and 66% PBS, was
used to prepare the resveratrol $10^{-5}$M solution. This stock was diluted in PBS to obtain two secondary stock solutions: resveratrol $10^{-3}$M and $10^{-4}$M, which was used to prepare the resveratrol $10^{-6}$M and $10^{-7}$M solutions respectively. The amount of vehicle in the vehicle control was determined and adjusted as in section 3.1.

3.3 – 'Window' experiments in the CPO

6-day CPO cultures (section 1.1) were treated with TCDD $10^{-9}$M, resveratrol $10^{-5}$M, or vehicle. All cultures were maintained for the entire 6-day period. The compounds, however, were administered over four different temporal phases: a) days 0-6; b) days 0-2; c) days 2-4 or d) days 4-6 (again, all cultures were stopped at 6 days). Results were compared to e) vehicle alone over days 0-6.

The cultures received vehicle control when not receiving resveratrol or TCDD and the media were changed every 48 hours.
3.4 - Total and radiolabeled Collagen type I analysis, RNA extraction and histological analysis in the CPO

6-day CPO cultures (section 1.1) were treated with TCDD $10^{-9}$M, resveratrol $10^{-6}$M ± TCDD $10^{-9}$M or vehicle. Each group was comprised of 9 cultures for the collagen type I analysis, 12 cultures for the RNA extraction, and 3 cultures for the histological analysis.

3.5 - Assessment of alkaline phosphatase activity and mineralization in the Rat SBMC line with a 2-dimensional matrix

The cultured rat SBMC line (section 1.2) was trypsinized with 0.01% trypsin for 5 minutes at 37°C and resuspended in rat SBMC medium (see section 1.2). 4000 cells were added to each well of a Titertek 96-well plate (12 columns x 8 rows) and a 2-dimensional dilution and co-incubation matrix was set up to permit analysis of the drugs in combination. Media was changed every 48 hours. One set of cells was maintained for 3 days and then assessed for alkaline phosphatase activity (section 2.2), while another set was maintained for 14 days and then assessed for mineralization (section 2.9).

3.6 - Whole cell competition binding assay in the Rat SBMC line

The competition binding assays were carried out in separate Titertek 96-well plates. 10,000 cells/well were plated in a Titertek 96-well plate and grown at 37°C in a humidified atmosphere containing 5% CO₂ in air. The next day media was carefully aspirated and the cells in each plate were pre-incubated with either cold TCDD or cold resveratrol for 1 hour at room temperature. $^3$H-TCDD at 10,000 cpm/well was then added to every well and incubated for 4 hours at room temperature. The media were aspirated out and the cells washed 4x with 100μl of PBS. 100μl of
0.1N sulphuric acid was added and the cells incubated at 37°C overnight to break the cells open. 80μl of the lysate was added to 5ml of scintillation fluid and counted in a beta counter.

### 3.7 – Cytotoxicity and proliferation studies in the Rat SBMC line

The cytotoxicity and proliferation studies were carried out in triplicate in Titertek 96-well plates. Cells were treated for 3 days with $10^{-9}$M TCDD, $10^{-6}$M resveratrol, $10^{-9}$M TCDD + $10^{-6}$M resveratrol (co-added) or vehicle control. Media were changed every 48 hours and after 3 days the cells underwent either a cytotoxicity assay (section 2.11) or a cell proliferation assay (section 2.12).

**Statistical Methods**

All CPO experiments were carried out with 9 cultures per group (n=9) except for the mRNA extraction in which 12 cultures per group were utilised (n=12) and the histological assessment with 3 cultures per group (n=3). The rat SBMC line experiments were carried out with 3 replicates per group (n=3) in all cases. In all experiments the results from each treatment group were compared only against the group which received vehicle-containing media (denoted as ‘vehicle’ in graphs). In the resveratrol dose response experiments a separate ‘control’ group, which received media without vehicle, was reported in order to reveal any vehicle-induced effects. All of the experiments were repeated either in whole or, as was more often the case, in part as a component of a succeeding experiment. Comparisons were made only between experimental and control groups (i.e. no multiple comparisons) and so it was thought that Students’s t-test would be appropriate to test for significant differences (p<0.05).
RESULTS

CPO Model

TCDD dose response in the CPO

AP activity in cultures treated with $10^{-9}$M TCDD was reduced by 85% compared to control ($p<0.05$), while treatment with $10^{-10}$M TCDD did not significantly alter AP activity (fig. 1a). TCDD ($10^{-9}$M) treatment caused a significant reduction in calcium incorporation (to 16% of control) but the reduction with $10^{-10}$M TCDD was not significant (fig. 1b). Inorganic phosphate incorporation in the $10^{-9}$M TCDD treated cultures and the $10^{-10}$M TCDD treated cultures was not significantly different from control cultures although a definite trend was noted for reduced inorganic phosphate uptake in the presence of $10^{-9}$M TCDD (fig. 1c). Soluble protein content in the $10^{-9}$M TCDD treated cultures and the $10^{-10}$M TCDD treated cultures was not significantly different from control (table 1).

Resveratrol Dose Response and Antagonism of TCDD in the CPO

TCDD ($10^{-9}$M) treatment reduced AP activity by over 80% ($p<0.05$ vs. vehicle) while vehicle had no effect. Treatment with resveratrol alone did not alter AP activity significantly in any of the concentrations used ($10^{-7}$M, $10^{-6}$M and $10^{-5}$M). AP activity was significantly greater in all three groups treated simultaneously with TCDD and resveratrol versus cultures treated with TCDD alone. Compared to treatment with $10^{-9}$M TCDD alone, AP activity was almost doubled in the $10^{-9}$M TCDD + $10^{-7}$M resveratrol treatment group and approximately tripled in the $10^{-9}$M TCDD + $10^{-6}$M resveratrol and $10^{-9}$M TCDD + $10^{-5}$M resveratrol treatment groups. However, complete reversal (i.e. vs. control) of the inhibition by TCDD using resveratrol was not observed (fig. 2a).
Despite obvious effects on AP and calcium, there were no significant effects on inorganic phosphate incorporation (fig. 2b) or protein production (table 2) compared to control.

‘Window’ experiments in the CPO

Consistent with the previous results, treatment with $10^{-9}$M TCDD alone significantly reduced AP activity compared to vehicle when administered over the entire 6-day culture period. Administration of $10^{-9}$M TCDD alone during days 2-4 or 4-6 did not significantly alter AP activity but administration during days 0-2 reduced AP activity by 53% ($p<0.05$ vs. vehicle) (figs. 3a, 3c). No significant change in AP activity was noted when $10^{-6}$M Resveratrol alone was added over days 0-6, days 0-2, days 2-4, or days 4-6 (figs. 3b, 3c).

Inorganic phosphate incorporation was not significantly different from control in any of the treatment groups over days 0-6 (fig. 3d), nor was calcium incorporation (fig. 3e) or protein production (table 3).

Total and radiolabeled collagen type I levels in the CPO

Total collagen type I levels (reported as Absorbance Units (AU) x mm) were increased 2-fold in the treatment groups receiving $10^{-9}$M TCDD alone and $10^{-6}$M resveratrol alone compared to vehicle ($p<0.05$). Co-treatment with TCDD and resveratrol did not significantly change the total collagen type I levels (fig. 4a).

Though there was no statistically significant alteration of levels of radiolabeled collagen type I in any of the treatment groups, the trend observed in the results obtained was similar to the results found for alterations in AP activity. A reduction with $10^{-9}$M TCDD treatment alone was seen compared to vehicle, while treatment with $10^{-6}$M resveratrol alone did not alter radiolabeled
collagen type I levels. Co-treatment with TCDD and resveratrol resulted in a mild increase in the level of radiolabeled collagen type I vs. both vehicle and TCDD alone (fig. 4b).

**RNA extraction and Northern Analysis in the CPO**

Northern analysis revealed modest effects of the various treatments on mRNA levels. mRNA levels in the control group with vehicle alone were taken as 100% and each lane was normalized against 18S rRNA. TCDD (10^{-9} M) treatment caused a 7.5% reduction of mRNA for AP, and a 17% reduction in mRNA for collagen type I. There was a negligible reduction in message for osteopontin (OPN) (2%). Resveratrol (10^{-6} M) reduced mRNA for AP (16%) but induced a mild increase in mRNA for collagen type I (4%) and for OPN (12%). In combination, TCDD + resveratrol again reduced mRNA for AP (7%) but increased the mRNA for collagen type I (28%) and for OPN (19%) (fig. 5).

**Qualitative histological analysis of the CPO**

Histological observation of all treatment groups suggested that the CPO cultures were viable and continued to produce bone (fig. 6). Though strict morphometric analyses of the CPO cultures were not carried out, the mineralized area noted in the TCDD alone treatment group was distinctly less than in any other group. Sparse and discrete with TCDD alone, these mineralized areas (bordered by a mineralization front and exhibiting osteocytes, osteoblasts, an area of osteoid and an outer fibrous layer in all groups) were large and uniform in all other groups.
**Rat SBMC line**

**Alkaline phosphatase activity in the Rat SBMC line**

TCDD (3.3x10^{-9}M and higher) caused a 33% reduction in AP activity versus control and this reduction was not reversed by administration of resveratrol at various concentrations. A 33% reduction in AP activity was also shown following treatment with a lower concentration of TCDD (1.1x10^{-9}M). In this case, however, increasing concentrations of resveratrol (4x10^{-8}M to 1x10^{-4}M) abrogated the effects of TCDD and brought AP activity back to that seen in control cultures. This pattern was repeated in the cultures receiving lower levels of TCDD. The initial reduction in AP activity due to TCDD was dose dependant (3.3x10^{-9}M to 4.12x10^{-11}M). The abrogation of the effect of TCDD by increasing resveratrol concentrations was exhibited at each level of TCDD administration until a concentration of 4.12x10^{-11}M TCDD was reached. At this concentration, AP activity was not affected (i.e. similar to control) and the increasing concentration of resveratrol had no effect either. At several of the TCDD concentrations the addition of the highest levels of resveratrol resulted in an increase in AP activity above that found in control (fig. 7).

**Mineralization in the Rat SBMC line**

There was a dose dependant reduction in mineralization in the presence of 4.12x10^{-11}M to 1.52x10^{-12}M TCDD. Mineralization was decreased by approximately 75% in the presence of TCDD and resveratrol reversed this. Interestingly, at the highest concentration of resveratrol, not only were TCDD effects attenuated but there appeared to be a 3-fold increase relative to control levels. At TCDD concentrations higher than 4.12x10^{-11}M there was a reduction
mineralization, but increasing concentrations of resveratrol could not antagonize the effects of these higher doses (fig. 8).

**Assays for whole cell competition of $^3$H-TCDD in the Rat SBMC line**

The whole cell competition assays demonstrated the existence of the AhR receptor in this culture system. Increasing concentrations of TCDD effectively competed with $^3$H-TCDD for its cognate receptor and reduced binding to background levels at $3.3 \times 10^{-9}$ M. Approximately 50% competition was achieved at $3.7 \times 10^{-9}$ M TCDD (fig. 9a). Resveratrol competed with $^3$H-TCDD in a similar fashion. Resveratrol ($1.37 \times 10^{-9}$ M) reduced binding to background levels and 50% competition occurred at $\sim 1.8 \times 10^{-10}$ M resveratrol, a dose 20x lower than the dose of TCDD producing this level of competition (fig. 9b).

**Proliferation study in the Rat SBMC line**

On the basis of the relative optical densities in the MTT assay TCDD, resveratrol or TCDD + resveratrol did not disturb proliferation in this culture system (fig. 10).

**Cytotoxicity study in the Rat SBMC line**

As with the proliferation study, treatment with TCDD, resveratrol or TCDD + resveratrol did not appear to be cytotoxic. The viable cell count in the $10^{-9}$ M TCDD cultures was $\sim 2 \times 10^4$ cells/ml, $\sim 3 \times 10^4$ cells/ml in the $10^{-6}$ M resveratrol cultures and $\sim 3.5 \times 10^4$ cells/ml in the $10^{-9}$ M TCDD + $10^{-6}$ M resveratrol cultures, none of which were significantly different than control ($\sim 2.2 \times 10^4$ cells/ml) (fig. 11).


**TABLES**

**TCDD dose response**

*Table 1 – Soluble protein content (measurements in µg protein/ml)*

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<thead>
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<th>Control</th>
<th>10^{-9}M TCDD</th>
<th>10^{-10}M TCDD</th>
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<tr>
<td></td>
<td>23.534±4.351</td>
<td>34.611±5.922</td>
<td>23.707±2.995</td>
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</tbody>
</table>

**Resveratrol dose response and TCDD antagonism in the CPO**

*Table 2 – Soluble protein content (measurements in µg protein/ml)*

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<th>alone</th>
<th>+ 10^{-9}M TCDD</th>
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<tr>
<td>Control</td>
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<td>---</td>
</tr>
<tr>
<td>Vehicle</td>
<td>34.90±3.93</td>
<td>---</td>
</tr>
<tr>
<td>10^{-9}M TCDD</td>
<td>40.84±5.25</td>
<td>---</td>
</tr>
<tr>
<td>10^{-7}M Resveratrol</td>
<td>51.04±4.97</td>
<td>41.98±4.75</td>
</tr>
<tr>
<td>10^{-6}M Resveratrol</td>
<td>32.98±2.09</td>
<td>56.39±5.51</td>
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<tr>
<td>10^{-5}M Resveratrol</td>
<td>45.15±3.92</td>
<td>52.95±5.87</td>
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</table>
Table 3 - Soluble protein content (measurements in µg protein/ml)

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<thead>
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<th></th>
<th>Day 0-6</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>$10^{-9}$ M TCDD</td>
<td>25.89±2.86</td>
</tr>
<tr>
<td>$10^{-6}$ M Resveratrol</td>
<td>23.14±3.16</td>
</tr>
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Fig. 1a - Alkaline phosphatase activity in 6-day CPO cultures with and without various doses of TCDD. Treatment with $10^{-9}$ M TCDD resulted in an 85% reduction in AP (p<0.05 vs. control) activity, while treatment with $10^{-10}$ M TCDD did not significantly alter AP activity from control. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.

* p<0.05
Fig. 1b - Calcium incorporation in 6-day CPO cultures treated with and without various doses of TCDD. $10^{-9}$M TCDD treatment resulted in a 6-fold reduction in calcium incorporation compared to control ($p<0.05$ vs. control). The reduction with $10^{-10}$M TCDD was not statistically significant.

Each bar represents the mean of 9 cultures ($n=9$) and the vertical line shows the standard error of the mean.

* $p<0.05$
Fig. 1c - Inorganic phosphate incorporation in 6-day CPO cultures treated with and without various doses of TCDD. Although there is a trend to reduced phosphate at $10^{-9} \text{M}$ TCDD, there were no significant differences found in inorganic phosphate incorporation in any of the treatment groups. Each bar represents the mean of 9 cultures ($n=9$) and the vertical line shows the standard error of the mean.
Fig. 2a - Alkaline phosphatase activity in 6-day CPO cultures treated with various doses of resveratrol with and without 10⁻⁹M TCDD. Treatment with vehicle did not significantly alter AP activity vs. control, nor did resveratrol alone in any of the concentrations used. 10⁻⁹M TCDD alone reduced AP activity by over 80% (p<0.05 vs. control). Addition of resveratrol resulted in a 2- to 3-fold increase in AP activity (p<0.05) vs. TCDD alone.

Each bar represents the mean of 9 cultures (n=9) and the vertical lines show the standard error of the mean.
Fig. 2b - Inorganic phosphate incorporation in 6-day CPO cultures treated with various doses of resveratrol with and without 10⁻⁹M TCDD. No significant differences were noted in any of the groups. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 3a - Alkaline phosphatase activity in 6-day CPO cultures treated with $10^{-9}$M TCDD over varying time periods. Administration of TCDD over days 0-6 and also over days 0-2 reduced AP activity by 45-53% (p<0.05 vs. vehicle). Dosing the cultures over days 2-4 or 4-6 did not significantly alter AP activity. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 3b - Alkaline phosphatase activity in 6-day CPO cultures treated with $10^{-6}$M resveratrol over varying time periods. Administration of resveratrol did not significantly alter AP activity over any of the dosing periods. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 3c - Alkaline phosphatase activity in 6-day CPO cultures treated with $10^{-9}$ M TCDD with and without $10^{-6}$ M resveratrol over days 0-6. Treatment with TCDD alone significantly reduced AP activity but the addition of resveratrol brought AP activity back to control levels ($p > 0.05$ vs. control). Each bar represents the mean of 9 cultures ($n=9$) and the vertical line shows the standard error of the mean.
Fig. 3d - Inorganic phosphate incorporation in 6-day CPO cultures treated with $10^{-9}$M TCDD with and without $10^{-6}$M resveratrol over days 0-6. The levels of inorganic phosphate incorporation were not significantly altered in any of the groups. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 3e - Calcium incorporation in 6-day CPO cultures treated with $10^{-9}$M TCDD with and without $10^{-6}$M resveratrol over days 0-6. Though a trend was exhibited towards reduced calcium incorporation with TCDD treatment and a return to control levels with TCDD + resveratrol, no significant differences were found between groups. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 4a - Total collagen type I levels in 6-day CPO cultures treated with $10^{-9}$M TCDD with and without $10^{-6}$M resveratrol over days 0-6. A 2-fold increase in total collagen type I levels resulted from treatment with both TCDD alone and resveratrol alone (p<0.05 vs. control), but combination treatment with TCDD + resveratrol did not significantly alter total collagen type I production. Each bar represents the mean of 9 cultures (n=9) and the vertical lines show the standard error of the mean.
Fig. 4b - Radiolabelled (\(^{14}\)C) collagen type I levels in 6-day CPO cultures treated with 10\(^{-9}\)M TCDD with and without 10\(^{-6}\)M resveratrol over days 0-6. Though a trend was exhibited towards reduced radiolabelled collagen type I levels with TCDD treatment and a return to control levels with TCDD + resveratrol, no significant differences were found between groups. Each bar represents the mean of 9 cultures (n=9) and the vertical line shows the standard error of the mean.
Fig. 5 - Levels of mRNA for AP, col (I) and OPN in the CPO (normalized against 18S rRNA)
Levels in vehicle treated cultures taken as 100%. Treatment with TCDD alone reduced mRNA levels for all three proteins. Resveratrol alone and in combination with TCDD resulted in a decrease in mRNA for AP but increased mRNA for col(I) and OPN. mRNA was extracted from 12 cultures per group (n=12).
Fig. 6 - Histological evaluation of the CPO. TCDD treatment resulted in a substantial reduction in bone formation which was reversed by the addition of resveratrol. For all treatments, there is a well defined mineralized area (M) bordered by a mineralization front, the presence of osteocytes (●), osteoblasts (▲), an area of osteoid (O) and an outer fibrous layer (f). Representative sections obtained from 3 cultures per group are shown.
Fig. 7 - Relative alkaline phosphatase levels in the rat SBMC line. Each graph represents one TCDD concentration. Each tick on the x-axis of each graph represents [Resveratrol] going from $4 \times 10^{-8}$ M to $1 \times 10^{-5}$ M. A 33% reduction in AP activity resulting from the higher doses of TCDD decreased with lower concentrations of TCDD. At each dose of TCDD the addition of increasing levels of resveratrol caused a steady increase in AP activity, returning AP activity to that seen in control. Each point represents the mean of measures done in triplicate ($n=3$).
Fig. 8 - Mineralization Levels in the Rat SBMC Line. Each graph represents one TCDD concentration. Each tick on the x-axis of each graph represents [Resveratrol] going from $4 \times 10^{-8}$ M to $1 \times 10^{-5}$ M. TCDD caused a reduction in mineralization with less of an effect at lower doses of TCDD. At each TCDD concentration resveratrol induced a dose dependant abrogation of the TCDD-mediated reduction in mineralization. Each point represents the mean of measures done in triplicate (n=3).
Fig. 9a - Rat SBMC line whole cell competition binding assay, $^3$H-TCDD vs. TCDD (background subtracted, % of total binding). Binding was reduced to background levels at $3.3 \times 10^{-8}$ M TCDD and 50% competition was produced by $\sim 3.7 \times 10^{-7}$ M TCDD. Each point represents the mean of measures done in triplicate (n=3) and the vertical line shows the standard error of the mean.
Fig. 9b - Rat SBMC line competition binding assay. $^{3}$H-TCDD vs. resveratrol. (background subtracted, % of total binding). Binding was reduced to background levels by $1.37 \times 10^{-9}$ M resveratrol and 50% competition occurred at $\sim 1.8 \times 10^{-10}$ M.

Each point represents the mean of measures done in triplicate (n=3) and the vertical line shows the standard error of the mean.
Fig. 10 - Proliferation study in rat SBMC line (relative OD$_{570}$ produced by MTT assay).
No significant differences were noted in any group vs. vehicle. Each bar represents the mean of measures done in triplicate (n=3) and the vertical line shows the standard error of the mean.
Fig. 11 - Cytotoxicity study in rat SBMC line (0.4% trypan blue uptake)
Similar to the proliferation study, no significant differences were noted between the groups in the cytotoxicity study. Each bar represents the mean of measures done in triplicate (n=3) and the vertical line shows the standard error of the mean.
DISCUSSION

The studies I employed were designed to test the effects of aryl hydrocarbon receptor ligands on bone formation in vitro. Using two different in vitro bone forming models it was shown that TCDD, the prototypical aryl hydrocarbon receptor ligand, does inhibit bone formation and that a novel aryl hydrocarbon receptor antagonist, resveratrol, partially and in some cases completely reverses this inhibition.

Alkaline phosphatase activity

Utilizing the CPO model, \(10^{-9}\text{M}\) TCDD administration over days 0-6 (i.e. the entire culture period) consistently caused a significant reduction in alkaline phosphatase (AP) activity. Similarly, the ability of \(10^{-6}\text{M}\) resveratrol to antagonize the effects of TCDD on AP activity was consistently observed. AP has a number of functions and may act as an inorganic phosphate provider or as a modulator of matrix macromolecules (Golub 1996), as well as in other diverse manners (see below). In any case, synthesis of AP is accepted as a hallmark of an osteoblastic phenotype and bone formation (Termine 1993). The doses of TCDD and resveratrol shown to have a significant affect in the CPO model were consistent with doses used by other investigators using differing in vitro bone forming systems (Gierthy, Silkworth et al. 1994) (Mizutani, Ikeda et al. 1998) and in a breast cancer cell line (Casper, Quesne et al. 1998). The findings obtained for AP activity using the CPO model were paralleled in the rat SBMC line. The effects of TCDD on AP activity were clearly observed and were dose dependent, and the ability of resveratrol to antagonize these effects were likewise shown to be obvious and dose dependent. Given similar findings in the two models one may conclude that TCDD inhibits osteoblastic AP activity and that resveratrol antagonizes this effect in a manner that is neither species nor model specific.
*Mineralization*

Further corroboration of these results was obtained by investigating mineralization in the rat SBMC line. As seen with AP activity, TCDD had a dose dependent effect and caused a drastic reduction in mineralization at higher doses. Resveratrol antagonized the TCDD-mediated inhibition of mineralization and this occurred in a dose dependent fashion, similar to its effect on TCDD-mediated inhibition of AP activity. However, when the concentration of TCDD exceeded 4.12x10^{-11}M, resveratrol could not antagonize its effect. Understanding that TCDD has the highest binding affinity to the AhR of all known compounds (Rowlands and Gustafsson 1997), it would appear that resveratrol was unable to out compete TCDD for the latter's cognate receptor at these high doses. Assessment of mineralization in the CPO initially showed that TCDD administration caused a significant reduction in calcium incorporation, however subsequent experiments revealed only a trend towards a decrease in calcium incorporation but did not produce significant differences. TCDD did not affect inorganic phosphate uptake in any of the CPO experiments. These findings are difficult to explain especially in light of our other data. One explanation may be related to the fact that although the rat SBMC line and CPO models did generally seem to produce comparable results in these experiments, the two models are not identical. For example, whereas the rat SBMC line relies absolutely on the presence of dexamethasone for osteodifferentiation (Grigoriadis, Heersche et al. 1988) (Herbertson and Aubin 1995) (Kasugai, Todescan et al. 1991) (Maniatopoulos, Sodek et al. 1988) (Rickard, Sullivan et al. 1994) (Shalhoub, Conlon et al. 1992), the CPO does not (McCulloch and Tenenbaum 1986). Therefore, in the CPO there are dexamethasone dependent and independent osteogenic cells. It is conceivable that TCDD may not affect the dexamethasone independent osteogenic cells as much as the dexamethasone dependent ones. If this was the case, one might
see altered responses in post-differentiation events such as mineralization. It is also noteworthy that the CPO is highly technique sensitive. As it is closer to being like an in vivo system than cell culture (Tenenbaum, Palangio et al. 1986), it is our experience that the CPO is open to more variability than the cell culture systems and this alone may explain the apparent differences in responses seen. Nonetheless, there still was a notable trend showing that in large part, TCDD inhibits mineralization in the CPO while there was clear and statistically significant evidence that TCDD inhibits mineralization in the rat SBMC line. In both cases, and particularly with the rat SBMC line, resveratrol inhibits those effects.

Osteogenic phase of drug activity

The temporal or ‘window’ experiments suggested that TCDD’s inhibitory effects on osteodifferentiation, as reflected by alkaline phosphatase activity, were exerted mainly during the early phase of culture in the CPO model (i.e. over days 0-2). As noted above, the CPO model passes through various stages of osteodifferentiation and osteogenesis ranging from differentiation (approximately days 0-2), matrix production (approximately days 2-4), and mineralization (approximately days 4-6) (Tenenbaum and Heersche 1982) (Tenenbaum and Heersche 1986) (Tenenbaum, Palangio et al. 1986). Since TCDD inhibited parameters of osteogenesis when present at days 0-2, and not when added later, this would suggest that this agent interferes to some degree with osteoblastic differentiation but has little effect on already differentiated osteoblasts. If, as noted above, there is a cohort of cells in the CPO model that is insensitive to TCDD, this might also explain the somewhat equivocal effects on mineralization discussed above since this is a later event modulated by more differentiated osteoblasts.

A second interesting finding in the CPO ‘window’ experiments was the increase in AP activity induced by administration of resveratrol over days 4-6. These data, combined with the
finding that resveratrol induces a dose dependent increase in AP activity and mineralization above control levels in the rat SBMC line, a mature osteoblastic cell-line that is capable of producing mineralized bone-like tissue (Pitaru, Kotev-Emeth et al. 1993) (Kotev-Emeth, Pitaru et al. 1999) intimate a stimulatory effect of resveratrol. This effect is observed only when resveratrol is administered during the late stages of osteogenesis (days 4-6 in the CPO), pointing toward a resveratrol responsive cell population that is more fully differentiated (Tenenbaum 1981) (Tenenbaum and Heersche 1982) (Tenenbaum and Heersche 1985). This phenomenon has recently been observed and reported elsewhere in the literature. Indeed, Mizutani et al. showed that resveratrol caused a dose dependent increase in AP activity when administered to a mature osteoblastic cell line (osteoblastic MC3T3-E1 cells) (Mizutani, Ikeda et al. 1998). Further, the authors found that in the presence of tamoxifen, an anti-estrogenic agent, the stimulatory effects of resveratrol were blocked, suggesting resveratrol’s effects to be mediated through an estrogen-like action. Other investigators have also discovered resveratrol to exhibit variable degrees of estrogen receptor agonism (Gehm, McAndrews et al. 1997). Thus a stimulatory effect of resveratrol (possibly estrogenic in nature), in addition to its AhR antagonistic effects, is feasible and warrants further investigation.

Collagen synthesis in the CPO

Changes in levels of collagen type I were expected to follow the pattern of changes seen in AP activity and a trend in this direction was observed in the level of 14C-labeled collagen type I in the CPO. Though not statistically significant, preliminary studies revealed that administration of TCDD did reduce levels of 14C-labeled collagen type I vs. control whereas combination with resveratrol resulted in a slight increase in 14C-labeled collagen type I levels vs. control. Thus, the expected trends were shown with radiolabeled collagen but clear cut findings
were not observed. Surprisingly, either TCDD or resveratrol increased the level of total collagen significantly when administered alone. In the former case, it may be that TCDD inhibited the turnover of collagen type I, allowing it to accumulate over the culture period. Turnover and remodeling of collagen is known to occur continuously during both physiologic and pathologic conditions and appears to be accomplished through the action of a number of proteolytic enzymes and lysosomal phagocytosis (Everts, van der Zee et al. 1996). Notably, an association between an upregulation of AP activity and increased collagen phagocytosis was demonstrated by Hui et al., where it was suggested that AP may play an integral role in collagen degradation by upregulating phagocytosis (Hui, Tenenbaum et al. 1997). In explanation of our findings, since TCDD decidedly decreases AP activity, it is conceivable that collagen phagocytosis could also be reduced thereby leading to increased total collagen type I in the TCDD treated cultures. Even the somewhat equivocal findings shown with radiolabeled collagen production could have been affected by this.

The increase in total collagen type I in response to resveratrol administration is in accordance with the results of a study referred to earlier investigating the effects of resveratrol on osteoblastic MC3T3-E1 cells (Mizutani, Ikeda et al. 1998). In this study $10^{-6}$-10^{-5}$M resveratrol accelerated prolyl hydroxylase activity, indicating that collagen synthesis would be increased by resveratrol in osteoblastic cells, as we observed with a dose of $10^{-6}$M resveratrol in the CPO. Caution must be exercised in interpretation of these results, however, as confirmation of these preliminary findings would require repeated, in depth experimentation, focused on both synthesis and degradation of collagen as well as assessment of translational and post-translational events (see below). Indeed the discordance of the collagen results with our other data, which support a TCDD-mediated inhibition of bone formation, bears witness to the inherent complexity of the
CPO model. In this mixed tissue system changes in collagen usually reflect changes occurring mainly in bone but this can also be confounded by collagen synthesis in the outer fibrous layer. Thus changes in collagen synthesis may not be reflective of differences solely in bone formation in all experiments. Bone formation was more clearly assessed through histological analysis of the CPO. When obtaining representative sections of the different groups it was consistently discovered that it was easy to find bone in all groups save the TCDD treated cultures. In this group it was always necessary to section deeper into the culture to find the area of bone formation and this area was greatly reduced compared to all other groups (see below). A more representative assessment of TCDD’s effects on collagen synthesis might be obtained by utilising autoradiographic techniques. This would allow grain counts to be measured in the bone vs. the outer fibrous layer, revealing more precisely where changes in collagen are occurring in this complex bone forming system.

**Histological assessment and culture viability**

The TCDD-mediated reduction in bone formation was not related to toxicity. Histologic evaluation showed that bone formation and the cellular characteristics of viable CPO cultures were exhibited in all groups. Though morphometry was not done to measure the amounts of bone formed in the CPO cultures, gross observations revealed the amount of mineralized bone was greatly reduced in the TCDD group compared to all others, which were approximately the same, supporting the contention that TCDD inhibits bone formation. Further, the somewhat equivocal results regarding mineralization in the CPO are supported by the finding that TCDD does not completely shut down bone production and the increases seen in collagen production could be explained by an increased collagen:bone ratio in TCDD treated cultures. Future studies aimed at strict morphometric analysis of the effects of TCDD and resveratrol in the CPO could
provide greater detail of these possible effects. Conclusions regarding the effects of TCDD and resveratrol on mineralization cannot be made by assessing hematoxylin and eosin stained sections and would require either a von Kossa stain or ultrastructural analysis. Such studies might reveal if TCDD causes a disruption of mineralization itself. Total soluble protein production in the CPO was also unaffected by administration of any of the drugs used, suggesting the effects of TCDD were non-toxic. Similarly, cytotoxicity and proliferation studies in the rat SBMC line confirmed that TCDD was not cytotoxic nor did it reduce the proliferative capacity of the cells.

Expression of mRNA for bone proteins in the CPO

In the hopes of elucidating the underlying mechanism of TCDD affects on bone formation, mRNA levels for several bone proteins in the CPO were analyzed. The bone proteins investigated included collagen type I, AP and osteopontin (OPN). Collagen type I, the most abundant protein in the bone matrix (~90%), is synthesized by osteoblasts and deposited into an osteoid seam which then mineralizes (Cowles, DeRome et al. 1998). AP, as discussed earlier, is the hallmark of an osteoblastic phenotype and bone formation (Termine 1993). Though the actual function of AP is unknown, several possible roles have been proposed, which include: acting as a transporter/provider of inorganic phosphate; increasing calcium uptake by cells by acting as a calcium binding protein; functioning as a Ca\(^{2+}\)/Mg\(^{2+}\)-ATPase; hydrolyzing inorganic pyrophosphate which may act as an inhibitor of calcification; or functioning as a tyrosine-specific phosphoprotein that conditions the skeletal matrix for ossification (Whyte 1994). Other novel roles for AP have been investigated and include the initiation of pathological mineralization in non-osteogenic tissues (Hui, Li et al. 1997; Hui and Tenenbaum 1998), roles in cellular differentiation and proliferation (Hui, Hu et al. 1993) (Hui, Sukhu et al. 1996) and, as
previously mentioned, AP levels may influence collagen degradation (Hui, Tenenbaum et al. 1997). OPN mRNA levels have been shown to be maximal in osteogenesis during the period of rapid tissue growth and is found at the mineralization front of fetal porcine calvarial bones. Its function has been proposed as controlling mineralization by inhibiting crystallization (Cowles, DeRome et al. 1998). Administration of TCDD had an inhibitory effect on the levels of mRNA for AP, OPN and collagen type I. However, these effects were very mild suggesting that it is unlikely that TCDD was causing a reduction in parameters of bone formation by affecting transcription of these proteins. Similarly, tamoxifen and anti-TGF-β1 have been shown to decrease protein levels of AP, OPN and collagen type I in rat bone marrow cell culture, yet this occurred without altering levels of the mRNA transcripts for these proteins (Sukhu, Rotenberg et al. 1997) (Binkert, Demetriou et al. 1999). It is postulated that a similar phenomenon may have occurred here, however further experimentation would help elucidate this more clearly, as would Northern analysis in the rat SBMC line (underway in our laboratory).

Receptor studies in the Rat SBMC line

It was postulated that the effects of TCDD were receptor mediated. Using the rat SBMC line, the whole cell competition binding assay revealed a competition binding curve indicating that TCDD effectively competed with ³H-TCDD for its cognate, and presumably cytoplasmic, receptor. Similarly, resveratrol also competed with ³H-TCDD for the receptor, demonstrating that resveratrol is also binding to the AhR. The presence of the AhR in the rat SBMC line is consistent with the literature wherein all the known effects of TCDD have been reported to be mediated through the AhR (Birnbaum 1994) (Lavin, Hahn et al. 1998). The AhR has been identified in both mammalian and non-mammalian species. In humans it has been found in cultures of lung, liver, kidney, placenta, tonsils, B-lymphocytes, and ovarian and breast cancer
cells (Rowlands and Gustafsson 1997) (Lavin, Hahn et al. 1998). No endogenous ligands for this receptor have been identified and so its physiological function remains unclear. Data suggesting the necessity of the AhR for the normal development of many tissues exists, and research into understanding the AhR function, finding the normal endogenous ligand and determining how disruptions in its activity may affect normal developmental processes is ongoing (Gasiewicz 1997). The AhR complex has been well characterized (Hankinson 1995) and binding of TCDD to the AhR occurs through a high affinity bond. The ramifications of this powerful interaction were reflected in our experimental method inasmuch as pre-incubation with cold drug was necessary to attain competition for the receptor. The necessity of pre-incubation further increased the probability that the results attained were due to receptor competition and not just differential uptake of the drug by living cells. This latter scenario is a possibility when using a whole cell competition assay as opposed to a classic cytosolic preparation but, as indicated above, the necessity to pre-incubate would argue against uptake differences alone. Previous studies have shown that the results obtained with assays of radiolabeled hormone uptake using ROS 17/2.8 cells were highly comparable to direct cytosol binding assays (Tenenbaum, Kamalia et al. 1995) and have been used successfully to study steroid binding kinetics (Sukhu, Rotenberg et al. 1997).

Conclusions

The evidence gathered from these experiments supports the hypothesis that TCDD, the prototypical AhR ligand, inhibits bone formation. This inhibition appears to be a receptor mediated effect and occurs during the early phases of bone formation (i.e. cellular proliferation and osteoblastic differentiation). Resveratrol, a novel AhR antagonist, competes for the AhR and
partially or fully antagonizes these effects depending on the model or parameters being used.

**Clinical relevance and future studies**

As discussed in the introduction, cigarette smoking is an important risk factor in osteoporosis (Rudgren and Mellstrom 1984; Pocock, Eisman et al. 1989; La Vecchia, Negri et al. 1991; Hollenbach, Barrett-Connor et al. 1993; Hopper and Seeman 1994; Nguyen, Kelly et al. 1994; Egger, Duggleby et al. 1996; Franceschi, Schinella et al. 1996; Kiel, Zhang et al. 1996; Grisso, Kelsey et al. 1997) and periodontitis (Bergstrom 1989; Preber and Bergstrom 1990; Haber and Kent 1992; Horning, Hatch et al. 1992; Preber, Bergstrom et al. 1992; Haber, Wattles et al. 1993; Stoltenberg, Osborn et al. 1993; Ah, Johnson et al. 1994; Haber 1994), both of which involve a loss of bone. Recognizing that over 4700 individual constituents have been identified in cigarette smoke (Burns 1991) (Bartecchi, MacKenzie et al. 1995) it is likely that more than one component of cigarette smoke, through more than one mechanism, contributes to smoking related bone loss. Indeed, as previously described, nicotine (Ramp, Lenz et al. 1991) (Reus, Robson et al. 1984) (Nadler, Velasco et al. 1983) (Effeney 1987) (Chamson, Frey et al. 1982), carbon monoxide (Birnstingl, Brinson et al. 1971), and hydrogen cyanide (Mosely and Finseth 1977), may all have deleterious effects on bone and other tissues through various perturbations of the components required for bone formation and tissue healing. However, knowing that AhR ligands are important components of cigarette smoke and based on our experimentation (i.e. TCDD inhibits bone formation), there is now evidence that AhR ligands similar to TCDD may be one component of cigarette smoke causing bone loss. Indeed, our finding that a compound known to be an AhR antagonist, i.e. resveratrol (Casper, Quesne et al. 1998) (Ciolo, Daschner et al. 1998), reverses TCDD-mediated effects on bone formation further substantiates the link between exposure to AhR ligands and bone loss. Many studies need to be done to validate this
conclusion. BaP and dimethylbenz(a)anthracene (DMBA), being the most abundant AhR ligands in cigarette smoke (Committee on Passive Smoking 1986), should be tested to determine their effects in the systems we employed followed by determination of the effects of AhR ligands in in vivo models. Another avenue to explore is the effect of AhR ligands on bone resorption.

We have shown that TCDD inhibits bone formation most likely through an effect on early cellular proliferation and osteoblastic differentiation. It should be recognized that these compounds might also contribute to bone loss by increasing bone resorption. Thus, testing AhR ligands in an osteoclastic cell-mediated bone resorption model is warranted. Similarly, it has been recognized that osteoblasts and osteoclasts interact and the processes of bone formation and resorption are interrelated (Hattersley, Owens et al. 1991) (Weir, Horowitz et al. 1993) (Sugimoto, Kanatani et al. 1993) (Galvin, Cullison et al. 1994). Recent studies focused on this interaction have revealed a heretofore unrecognized regulation of osteoclast differentiation and function by osteoblasts. Namely, osteoprotegerin ligand (OPGL), a novel member of the TNF-ligand family, is expressed by osteoblasts as a membrane associated factor. Osteoclastic precursors and mature osteoclasts, both of which possess receptor activator of NF-kB (RANK), a TNF-receptor family member, are activated via a cell-to-cell interaction with osteoblasts expressing OPGL. In the former case, this interaction causes osteoclastic precursors to differentiate into osteoclasts and in the latter case, osteoclastic bone resorbing activity is induced (Burgess, Qian et al. 1999) (Takahashi, Udagawa et al. 1999). A potential role of Cbfα1, an essential osteoblast differentiation transcription factor (Komori, Yagi et al. 1997; Otto, Thornell et al. 1997), in osteoclastogenesis has also been suggested as studies have shown Cbfα1 upregulates osteoclastic expression of mRNA for OPGL. Thus the importance of investigating the effects of AhR ligands in a system where osteoblasts and osteoclasts can interact should not
be overlooked. Indeed, Loomer et al. (Loomer, Ellen et al. 1998) have developed a co-culture system that could be employed to elucidate more fully the effects of AhR ligands on bone metabolism. The mechanism through which the AhR ligands exert their effects can also be more thoroughly investigated by conducting functional assays for bone specific proteins such as OPN, bone sialoprotein, osteocalcin, osteonectin, and Cbfa1 and possibly relating these to effects on mRNA levels for these proteins (not only in the CPO but also in the rat SBMC line). Similarly, functional assays to detect changes in levels of IL-1β and TNF-α in response to AhR ligands in cultures known to produce theses pro-inflammatory mediators could help determine if these cytokines play a role in the observed effect of AhR ligands on bone metabolism. IL-1β and TNF-α directly and indirectly inhibit bone formation (Birkedal-Hansen 1993; Kuroki, Shingu et al. 1994) and are linked to periodontitis (Yavuzylmaz, Yamalik et al. 1995) (Kornman, Crane et al. 1997). In light of the anti-estrogenic properties of TCDD (Harris, Zacharewski et al. 1990; Safe, Astroff et al. 1991; Wang, Porter et al. 1993; Gierthy, Silkworth et al. 1994; Harper, Wang et al. 1994; Krishnan, Porter et al. 1995; Safe and Krishnan 1995; Kharat and Saatcioglu 1996; Nodland, Wormke et al. 1997) and the possible estrogenic activity of resveratrol (Gehm, McAndrews et al. 1997) (Mizutani, Ikeda et al. 1998) studies to elucidate the contribution of these effects in bone metabolism should be also be performed (and are underway in our laboratory).

Given these findings, the potential therapeutic effects of resveratrol require further study, not only in smokers, but in those who live in industrialized urban areas as well. The possibility that the deleterious effects of AhR ligands on bone metabolism could be antagonized by resveratrol needs to be explored. Ultimately, resveratrol could prove to be effective in the treatment and prevention of smoking related bone loss.


