CHAPTER 3

General Cell Culture Procedures

This chapter deals with cell culture maintenance and general methodology. Chemicals and reagents used were of analytical grade and purchased from Sigma Chemical Co (St. Louis, MO, USA). Specialised reagents, kits and apparatus and the suppliers thereof are cited in the text. Detailed accounts of specific experimental protocols are given in the appropriate chapters.

3.1 Cell cultures

3.1.1 Cell lines

MG-63 (human osteoblast-like, osteosarcoma-derived) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Nontransformed MC3T3-E1 mouse calvaria fibroblasts (established from the calvaria of an embryo/fetus C57BL/6 mouse) described to differentiate to osteoblasts,[285] were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

3.1.2 Maintenance of cell cultures

Cells were grown at 37ºC in a humidified atmosphere containing 5% CO_2 in a Forma Scientific incubator (USA). Growth medium was replaced with fresh medium at one to three day intervals. All cell cultures were propagated in large tissue culture flasks until a confluent monolayer had formed. When confluent, the medium was discarded and the cells rinsed with phosphate-buffered saline (PBS). Cells were detached from the tissue culture flask by adding 3 ml 10% trypsin/EDTA (disodium ethylene diaminetetraacetate) for 2-5 minutes at 37ºC and gently shaking the flask. Three milliliters growth medium (see below) was then added to neutralise the trypsin. The

trypsin solution was removed by centrifugation at 200g for five minutes. Cells were subsequently resuspended in fresh medium and seeded into new flasks or cell culture cluster plates. When required, surplus cells plus 1.5 ml of the appropriate freeze medium per cryotube were frozen at -70ºC. The cooling rate during the freezing process is estimated at ± 1ºC/min. Cell lines were kept in continuous culture or frozen at -70ºC. Growth media and freeze media were used as recommended by the suppliers of the various cell lines. The composition of growth culture media and freeze media for the respective cell lines is given in Table 3.1.

Aseptic techniques were applied throughout, with all work being carried out in an Labotec laminar airflow cabinet (Labotec (Pty)Ltd., Halfway House, SA). All solutions used for cell culture maintenance or freezing were sterilised by passage through a 0.22 µm filter. Non-sterile equipment was steam sterilised in a Butterworth auto-clave (England).

Table 3.1. Composition of maintenance culture media and freeze media used.

Maintenance growth media	
MG-63 cells	90% Dulbecco's modified Eagle's medium (DMEM) 10% heat-inactivated fetal calf serum (FCS) 2 mM L-glutamine gentamycin (25 µg/ml)
MC3T3-E1 cells	90% alpha modification of Eagle's minimal essential medium (α-MEM) 2 mM L-glutamine 10% heatinactivated FCS gentamycin (25 µg/ml)
Freeze media	
MG-63 cells	95% DMEM 5% dimethylsulphoxide (DMSO)
MC3T3-E1 cells	70% α-MEM 20% heat-inactivated FCS 10% DMSO

3.1.3 Choice and preparation of cell culture media

Growth media recommended for cell culture maintenance and experimental conditions differ widely. The suppliers of MC3T3-E1 cells recommend the use of α-MEM for culturing, however, others use DMEM successfully.[252]

3.1.3.1 Preparation of cell culture media for oestrogen exposure

Although it is controversial whether the pH indicator phenol red has oestrogenic activity[286] or not[287] it was decided to use culture media without phenol red for all experiments investigating the effects of exposure of cells to low oestrogen levels. Whilst phenol red free α-MEM was not readily available and MC3T3-E1 and MG-63 cells both tolerated DMEM well (as tested in our laboratory), it was decided to use phenol red free DMEM, supplemented with 2 mM L-glutamine, for experiments investigating effects of oestrogen.

Using an adaptation of Horwitz et al's (1987) method,[288] dextran-coated charcoal was used to remove endogenous oestrogens from FCS as follows:
One gram dextran-coated charcoal was added to 100 ml FCS. The mixture was then placed in a shaking water bath at 45ºC for 30-45 minutes, keeping the charcoal in suspension. At the end of incubation the charcoal was removed by centrifugation (1500-2000 rpm for 30-45 minutes) and the supernatant carefully poured off. After cooling to room temperature, the FCS was filtered by passage through a 0.22 µm filter into sterile containers and stored at -20ºC until needed.

3.1.3.2 Media for growth (proliferation) studies

Ascorbic acid has been shown to stimulate proliferation of MC3T3-E1 cells.[289] As α-MEM contains ascorbic acid in its formula, it was decided to rather use DMEM, which is ascorbic acid free, for all experiments investigating proliferation. Fetal calf serum contains various growth factors, which reportedly also affect cell proliferation.[289,290] To limit the proliferative effect of high FCS levels, FCS content in

the culture media was limited to 5%. To minimize any differences in culture conditions, both MC3T3-E1 and MG-63 cell lines were treated in the same way.

3.1.3.3 Osteogenic supplemented media

It is customary to use osteogenic supplemented media when investigating mineralisation properties of cells.[291-293] This supplemented medium contains ascorbic acid, ß-glycerophosphate as well as the synthetic glucocorticoid dexamethasone. It was, however, decided to omit dexamethasone, as it was proven in rat bone marrow stromal cells that dexamethasone per se acts at multiple points in the differentiation process to stimulate osteoblastic maturation.[53,294-296] It has also been suggested that dexamethasone inhibits prostaglandin synthesis.[140,214,255,256] Since PGE_2 is a down-stream product of AA investigated in this study, it is important not to interfere with PGE_2 production. When an osteogenic medium was required, α-MEM supplemented with 50 μg/ml L-ascorbic acid and 10 mM ß-glycerophosphate was used.[291]

3.1.4 Trypan blue exclusion test for cell viability

Whenever cells were seeded for experiments cell viability was determined by making use of the trypan blue exclusion test. Cells in logarithmic growth phase (refer to figure 3.1)[297] were dissociated and stained 1:1 with 0.2% trypan blue in Hanks' balanced salt solution (Hanks' BSS). Unstained, and thus viable, cells were counted with a haemocytometer. Ten µl of the cell suspension was introduced under the slip of the haemocytometer on each side and cells in the eight 1 mm^2 squares were counted. The concentration of cells per ml of cell suspension was calculated by dividing the total by 4 and multiplying by 10^4.

3.2 Preparation of stock solutions

Table 3.2 indicates the preparation and storage conditions of compounds used throughout this study. Stock solutions were prepared according to the manufacturer's recommendations.

Table 3.2 Preparation and storage conditions of compounds used.

Compound	Preparation and storage of stock solution
Polyunsaturated fatty acids: Arachidonic acid (AA)* Docosahexaenoic acid (DHA)*	100 mg/ml in 100% ethanol. Aliquots stored at -70ºC in the dark.
Oleic acid*	100 mM in 100% ethanol. Aliquots stored at -70ºC in the dark.
PTH	10^{-4} M in distilled water + 0.1% bovine serum albumin (BSA). Aliquots stored at -70ºC.
Oestrogen (17-β estradiol)**	$2x10^{-3}$ M in DMSO. Kept at room temperature.
PGE_2 *	1 mg/ml in 100% ethanol. Stored at -20ºC.
PGE_2 antibody, lyophilised	Reconstituted in distilled water. Aliquots stored at -70ºC.
NS-398**	0.1 M in DMSO. Aliquots stored at -70ºC.
Indomethacin**	0.1 M in DMSO. Freshly prepared when needed.
Anti-COX-1 antibody, lyophilised	2500 μg/ml in distilled water. Aliquots stored at -70ºC.
Anti-COX-2 antibody, lyophilised	5000 μg/ml in distilled water. Aliquots stored at -70ºC.
Osteoprotegerin (OPG)	100 μg/ml in PBS + 0.1% BSA. Aliquots stored at -70ºC.
Anti-OPG capture antibodies	500 μg/ml in PBS. Aliquots stored at -70º C.
Anti-OPG detection antibodies	50 μg/ml in tris-buffered saline (TBS) + 0.1% BSA. Aliquots stored at -70ºC.
Ascorbic acid	50 mg/ml in sterile ddH_2O. Aliquots stored at -70ºC.
$1,25(OH)_2D_3$ (Calcitriol)*	50 mg/ml in 100% ethanol. Aliquots stored at -70ºC in the dark.

*** When diluted, ethanol concentrations in cell culture medium did not exceed 0.2% (v/v).**

****When diluted, DMSO concentrations in cell culture medium did not exceed 0.05% (v/v).**

Ethanol (final concentration 0.2%) and DMSO (final concentration 0.05%) per se had no toxic effects on cells tested in our laboratory.

3.3 Standardisation of a method for quantification of cell number

Cells seeded into culture do not divide initially. During this phase, known as the *lag-phase* of cell growth, cells are preparing for mitosis and there is little change in cell number. When cells divide, the increase in cell number is logarithmic. This phase is known as the *log phase* of cell growth. The final phase of cell growth is referred to as the *plateau* or *stationary phase.*[297] (Figure 3.1).[297]

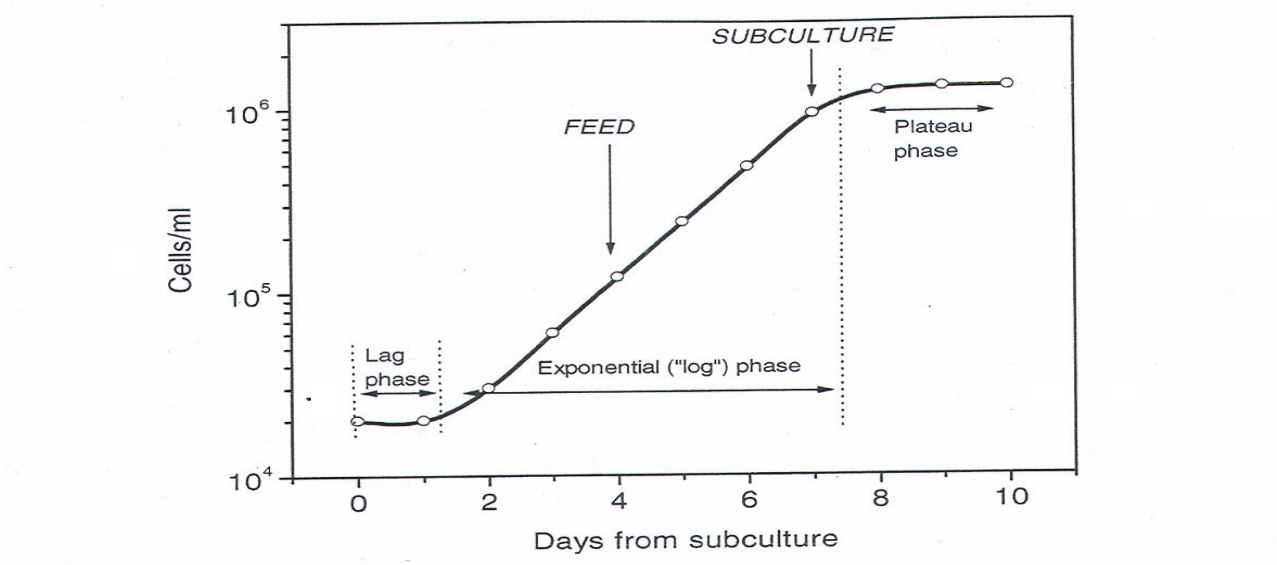


Figure 3.1 Growth curve and cell maintenance.

Semilog plot of cell concentration versus time from subculture, showing the lag phase, the exponential phase, and a plateau, and indicating times at which subculture and feeding should be performed. *(Freshney RI. Culture of animal cells: a manual of basic technique. 4th ed. New York: Wiley-Liss; 2000. p.184.)*[297] *©2000 (Wiley-Liss, Inc., A Wiley Company) Reproduced with permission of John Wiley & sons, Inc.*

For some experiments it is essential to quantify the number of cells in culture. By applying an adaptation of the crystal violet staining procedure[290,298,299] a method was developed to quantify cell number. Following trypan blue exclusion, MG-63 and MC3T3-E1 cells were seeded in quadruplicate at densities from 1.563 x 10^3 to 50 x 10^3 cells per well (in DMEM with 5% FCS) in 24-well cluster plates. After 24 hours with cells still in the lag phase, the experiment was terminated by fixing the cells in 300µl/well of 1% glutaraldehyde in PBS for 15 minutes. Thereafter 300µl/well crystal violet (1% in PBS) was added for 30 minutes. Subsequently the plates were

immersed in running tap water for 15 minutes. After the plates had dried, 500µl of 0.2% Triton X-100 was added to each well. The plates were then incubated at room temperature for 90 minutes and 150 µl of the liquid content transferred to 96-well microtiter plates. Absorbance (optical density) (O.D.) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 570nm; 0.2% Triton X-100 in water was used as a blank. Crystal violet is a basic dye, which stains cell nuclei.[298] Spectrophotometer readings of colour intensity are therefore an indication of DNA content and therefore cell numbers. Results were analysed using a linear standard curve (Figure 3.2). Intra and interassay variability was 4% and 11% respectively.

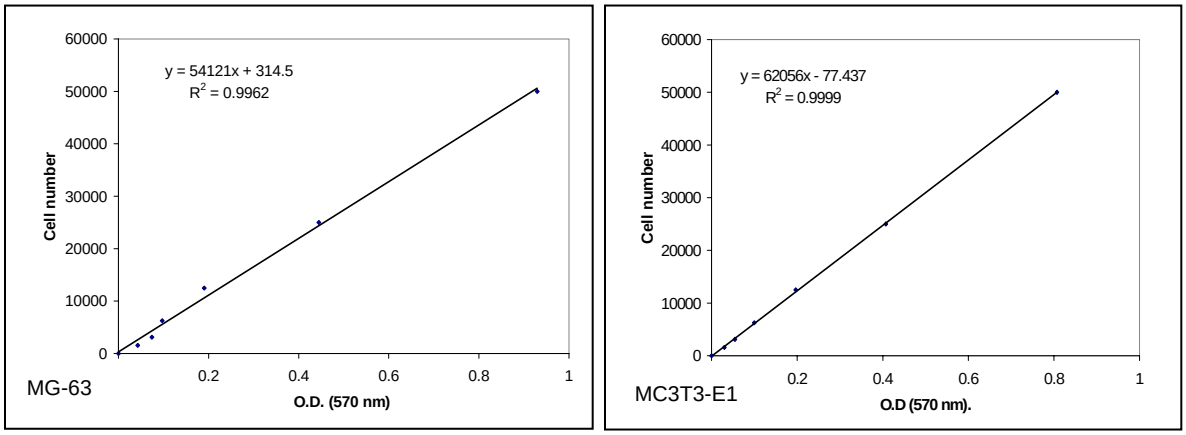


Figure 3.2 Correlation between crystal violet-derived absorbance and cell number.

Cells (MG-63 and MC3T3-E1) at densities ranging from 1.563 x 10^3 to 50 x 10^3 were seeded in 24-well cluster plates. After 24 hours the cultures were fixed with 1% glutaraldehyde, and subsequently stained with 1% crystal violet. Dye was extracted with 0.2% Triton X-100 and the absorbance read at 570 nm. Each data point represents the mean for quadruplicate cultures.

3.4 Prostaglandin E_2-related experiments

3.4.1 Radioimmunoassay (RIA) of prostaglandin E_2 in cell culture media

The amount of PGE_2 produced by cells and released into media was assessed using a competitive binding radioimmunoassay, adapted from a method described by Raisz and Simmons (1985).[237] In this assay, 100 µl unlabeled PGE_2 samples or

standards diluted in working buffer (0.01 M TRIZMA®BASE in 0.14 M NaCl, pH 7.6, containing 2 mg gamma globulin/ml) in duplicate were incubated overnight at 4ºC with radiolabeled PGE_2 ([^{3}H]-PGE_2, New England Nuclear™ Life Science Products, Inc., Boston, MA, USA) and PGE_2 antibody (provided by Dr Laurence Levine, Brandeis University, Waltham, Massachusetts, USA). The samples were put on ice and subsequently, 400µl dextran-coated charcoal (4 g/100 ml cold dH_2O) was added to each polystyrene tube. Free PGE_2 was removed by centrifuging for 15 minutes at 3000 rpm at 4ºC with a Rotixa 120R Hettich centrifuge (Tuttlingen, Germany). Thereafter, 400µl supernatant was transferred to a counting vial and 4.5 ml Liquiscent (NEN) scintillation cocktail (Packard Bioscience B.V., Groningen, The Netherlands) added. Samples were counted for 10 minutes each on the ^{3}H channel using a Beckman L55000CE Liquid Scintilation counter (Beckman instruments, Nuclear systems operations, Fullerton, CA, USA). The percentage of bound versus unbound [^{3}H]-PGE_2 was compared to a standard curve (region used for analysis: 150 to 1000 pg/ml) to calculate the amount of PGE_2 in the original samples (Figure 3.3). The intra and interassay coefficients of variation were 3% and 4.4% respectively.

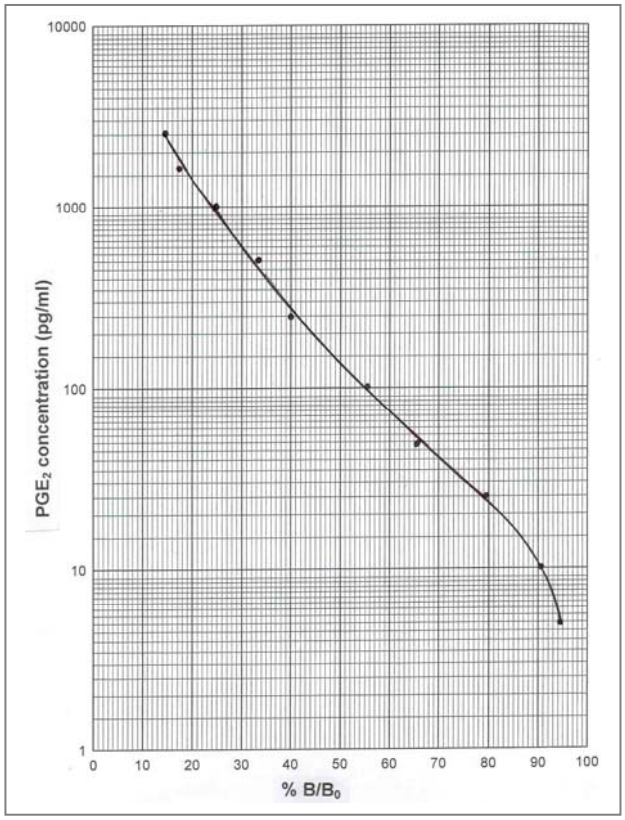


Figure 3.3 Example of a standard curve for the calculation of the amount of prostaglandin E_2 in cell culture samples using RIA.

3.4.2 Indirect immunofluorescence for detection of COX-1 and COX-2 in MG-63 cells

The presence of COX-1 and COX-2 in MG-63 cells after exposure to vehicle (0.2% ethanol) and AA were visualised by indirect immunofluorescence. Cells were seeded at 150 000/well onto heat-sterilised coverslips in 6-well culture plates. At the end of the experiment, after discarding the medium, cells were fixed in 10% formaldehyde (v/v) in (2 mM EGTA in PBS) for 10 minutes at room temperature. The coverslips were then transferred to a staining dish and rinsed thrice for five minutes with PBS at room temperature. Thereafter cells were permeabilised in ice cold 97% methanol containing 2 mM EGTA in PBS at –20°C for four minutes and then rinsed thrice for five minutes with PBS. Primary antibody (25 μl mouse monoclonal antibody against human COX-1 or COX-2 (1:1000 in PBS))(Cayman Chemical Company, Ann Arbor, MI, USA) was added to each coverslip and incubated for 60 minutes at 37ºC in a humidified environment. Cells were rinsed thrice for five minutes with PBS. Subsequently, the cells were incubated with the secondary antibody (25 μl 1:100 biotin-conjugated anti-mouse IgG (Fab-specific, developed in goat) in Fluroisotiocianate-conjugate (FITC-conjugate) diluent) (Diagnostic and Technical Services, Johannesburg, SA.), for 60 minutes at 37ºC in a humidified environment. Following rinsing, cells were finally incubated with ExtrAvidin®-FITC conjugate (1:200 in FITC-conjugate diluent)(Sigma Chemical Co, St. Louis, MO, USA) for 60 minutes. The coverslips were then mounted with a glycerol-based mounting fluid after the final 3x5 minutes wash step. Photographs were taken with 400 ASA film on a fluorescent microscope (Nikon Optiphot microscope equipped with an episcopic-fluorescence attachment and an excitation-emission filter with an average wavelength of 495 mm for FITC).

3.5 Proliferation studies

Proliferation can best be evaluated over an extended period of time; therefore it was decided to evaluate the effects of the different agents over a 72 hour exposure period. Longer periods of time are not suitable as cells then often reach confluency, which causes contact inhibition.

MG-63 and MC3T3-E1 cells were seeded at 3000 cells/well in sterile 96-well culture plates (n=8). After 72 hours at the end of culture, after exposure to vehicle and test agents, proliferation was determined by making use of an adaptation of the crystal violet staining procedure.[300] Hundred µl crystal violet (1%, in PBS) per well was added to fixed cells for 30 minutes; thereafter the culture plates were immersed in running tap water for 15 minutes. After the plates had dried, 200 µl of 0.2% Triton X-100 was added to each well. The plates were incubated at room temperature for 90 minutes and subsequently 100 µl of the liquid content was transferred to 96-well plates. Absorbance (OD) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 570nm; 0.2% Triton X-100 in water was used as a blank. Colour intensity is an indication of DNA content, thus cell growth.[298] Results are presented as percentage relative to control. Three independent experiments (each conducted in eightfold) were conducted to validate this procedure.

3.6 Haematoxylin and eosin (H&E) cell staining

Previous studies detected morphological effects of PUFAs on cultured osteoblasts after 24 to 48 hours exposure.[301] Cells were therefore seeded onto heat-sterilised coverslips in 6-well culture plates. After 48 hours at the end of culture, after exposure to vehicle and test agents, the experiment was terminated by removing the coverslips from the cluster plates, inserting them into coverslip holders followed by exposure to Bouin's fixative (Accustan™ Bouin's solution, Sigma) for 30 minutes. Thereafter, cells were stained with haematoxylin and eosin (H&E) using the method of Kiernan (1990).[302]

Cells were left in 70% ethanol for one hour; rinsed with tap water; left in Mayer's haemalum for 15 minutes; rinsed in running tap water; rinsed with 70% ethanol and subjected to 1% eosine for two minutes. Thereafter, cells were dehydrated by rinsing twice for five minutes with each of the following: 70% ethanol; 96% ethanol and 100% ethanol. Finally, coverslips were rinsed twice with xylol for five minutes, mounted to microscope glass slides with rapid mounting resin (Entellan) and left to

dry. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Opthiphot microscope (Nikon, Tokyo, Japan).

3.7 Hoechst 33342 (HOE) and propidium iodide (PI) staining for detection of apoptosis

Various methods for the detection of apoptosis in cell cultures have been developed, including terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL). HOE and PI staining for apoptosis detection has previously been standardised in our laboratory and was therefore applied in this study. Previous studies reported apoptotic effects within 24 to 48 hours of exposing cells to apoptotic agents.[42] Similar to H&E staining, testing for apoptosis was limited to 24 hours to 48 hours PUFA exposure.

Cells were seeded onto heat-sterilsed coverslips in 24-well cluster plates. At the end of culture, after treatment with vehicle and test agents, growth medium was discarded and the cells gently rinsed with PBS. Thereafter 500 μl/well Hoechst No 33342 (HOE) (0.5 μg/ml in PBS) was inserted into each well. After 30 minutes incubation at 37ºC, 125μl of a propidium iodide (PI) solution (0.5 mg/ml in PBS) was added directly to each well. Within five minutes, coverslips were mounted on microscope slides with mounting fluid (90% glycerol, 4% N-propyl-gallate, 6% PBS). Photographs were taken with 400 ASA film on a Nikon Optiphot microscope (Nikon, Tokyu, Japan) with UV-light and a blue filter.

While all cells take up HOE, only cells with intact cell membranes can exclude PI. Cells stained pink with PI are therefore classified as necrotic, while apoptotic cells only stain blue, indicating that these cells still have functional cell membranes capable of excluding PI, although they may have an aberrant appearance.[303]

3.8 Quantification of alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) is a membrane-bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell. An increase in ALP activity reflects the maturation from an earlier to a more mature stage of osteoblast differentiation. The level of ALP activity is therefore used in *in vitro* experiments as a marker of osteoblast differentiation.[41] As $1,25(OH)_2D_3$ (vit D_3) induces cellular ALP activity,[241,292,299] 10^{-8} M vit D_3 was used as a positive control in some experiments.

At the end of the culture period, after treatment with vehicle and test agents, cells were washed with PBS and fixed in 300 μl/well 4% formaldehyde in PBS (v/v) for 15 minutes. Using a colorimetric assay, ALP activity was assayed as the release of ρ-nitrophenol (ρ-NP) from ρ-nitrophenylphosphate (ρ-NPP) substrate.[291] Fixed cells were incubated with 300 μl/well ALP assay buffer (5 mM ρ-NPP; 0.5 mM $MgCl_2$; 0.1% Triton X-100 in 50 mM TBS, pH 9.5) for 60 minutes at 37ºC. Thereafter 100 μl reaction product per well was transferred to a 96-well microtiter plate and absorbance (O.D.) was read on an ELX800 Universal Microplate Reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA) at a wavelength of 405 nm. Assay buffer was used as a blank. Cells were washed again with distilled water, and cell number determined using a standard curve established with crystal violet assay, as previously described. Three independent experiments were executed in quadruplicate. Specific ALP activity was quantified against a standard curve of 0-400 μM ρ-NP and expressed as nanomoles of ρ-NP per hour per 10^4 cells (Figure 3.4).

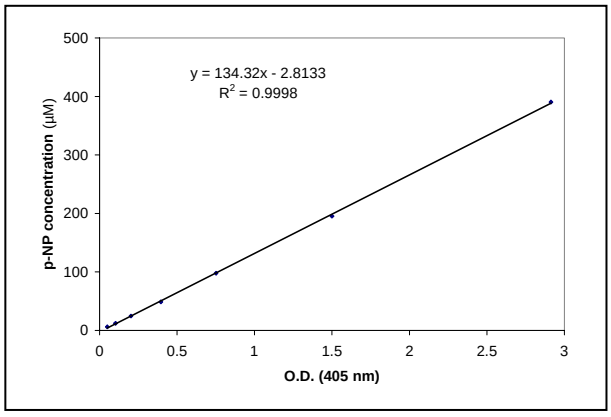


Figure 3.4 Standard curve for the calculation of alkaline phosphatase (ALP) activity in cell cultures using colorimetric para-nitrophenol hydrolysis.

3.9 Detection of mineralising properties

The terminal differentiation of osteoblasts *in vitro* is characterised by the formation of mineralising plaques.[304] To examine the capability of MG-63 cells and MC3T3-E1 cells to mineralise *in vitro*, formation of mineralising plaques were visualised by a modified von Kossa staining technique for phosphate detection (black) on fixed cell layers.[305,306]

Cells were rinsed thrice in 0.9% NaCl solution (pH 7.2) and fixed in 300 μl/well 4% (v/v) paraformaldehyde in 0.9% (w/v) NaCl solution at room temperature for 10 minutes. After fixation, the cells were rinsed thrice in ddH_2O, incubated with 400 μl/well 3% (w/v) $AgNO_3$ in the dark for 30 minutes, and exposed to ultraviolet light (254 nm) for 30 minutes. Cells were then washed again in ddH_2O and counterstained with Toluidine Blue for five minutes. Cells were then photographed making use of a Zeiss Axiovert 200 microscope attached to a Nikon DXM 1200 digital camera (Nikon, Tokyo, Japan). Alternatively, rinsing twice each with 70% ethanol, 96% ethanol, 100% ethanol and xylol cells were dehydrated. Coverslips were then mounted to microscope glass slides with resin, left to dry and photographed with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan) using 400 ASA film.

3.10 Assay of adipocytogenesis

3.10.1 Quantification of adipocytogenesis by Oil red O staining

Oil red O is a dye that is soluble in lipids and specifically stains triglycerides and cholesterol oleate but no other lipids. To determine whether osteoblasts accumulate lipid droplets in the cytoplasm when treated with PUFAs, an adaptation of the Oil red O staining procedure developed by Ramirez-Zacarias *et al* (1992) was performed on these cells.[307] Previous studies reported the accumulation of large quantities of triglycerides in the cytoplasm, after exposing osteoblasts to 100 μM oleic acid for a six-day period.[56]

At the end of the culture period, after treatment with vehicle and test agents, cells were rinsed with PBS, fixed in 300 μl/well 10% paraformaldehyde in PBS (v/v) for 15 minutes, and washed with PBS. Cells were then stained for 30 minutes with Oil red O by complete immersion in 300 μl/well working solution*. Thereafter, cells were rinsed briefly with 60% propanol, rinsed exhaustively with dH_2O and air-dried. In order to determine the extent of adipose conversion, 500 μl/well isopropanol was added to the stained cultures for 10 minutes. One hundred and fifty μl/well of the extracted dye was removed by gentle pipetting and transferred to a 96-well cluster plate. Using a GLR 1000 microplate reader (Genelabs Diagnostics, UK) absorbance was monitored spectrophotometrically at 490 nm. Isopropanol was used as blank. After washing with 70% ethanol, cell numbers were determined by crystal violet assay, as previously described. Three independent experiments were executed in quadruplicate to validate the assay.

*An Oil red O stock solution was prepared by dissolving 1.0 g of Oil red O in 100 ml isopropanol. After stirring overnight at room temperature, the solution was filtered through analytical filter paper. Oil Red O working solution was prepared by diluting the stock solution with dH_2O at a ratio of 7:3 and filtering twice before application.

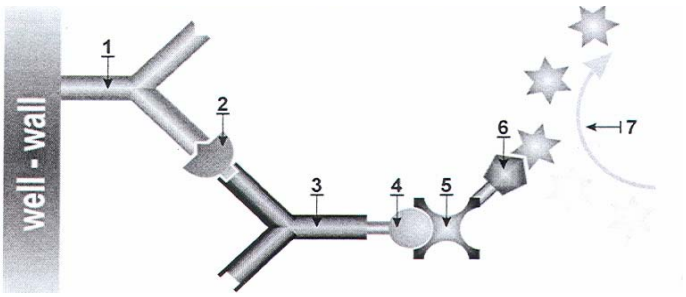
3.10.2 Microscopic visualisation of lipid accumulation

In order to visualise the accumulation of lipid droplets in cells after treatment with PUFAs, MG-63 and MC3T3-E1cells were seeded aseptically onto heat-sterilised coverslips in 6-well culture plates. At the end of culture, after exposure to vehicle and test agents, cells were rinsed with PBS, fixed in 10% paraformaldehyde in PBS for 15 minutes, and washed with PBS. The coverslips were then removed from the cluster plates, inserted into coverslip holders, stained with Oil red O for 30 minutes and rinsed briefly with 60% isopropanol. The cells were then counter-stained with hematoxylin for five minutes,[110] and mounted to microscope glass slides with rapid mounting resin and left to dry. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan). (Original magnification 400x)

3.11 Measurements of osteoprotegerin and RANKL secretion

3.11.1 Enzyme linked immunosorbent assay (ELISA) quantification of osteoprotegerin concentrations in MG-63 conditioned media

Levels of OPG in the conditioned media were determined using an adaptation of the sandwich ELISA protocol developed by Brändström *et al* (2001)[139] The principle of the assay is explained in Figure 3.5.[308]



1 OPG capture antibody
2 Sample/standard
3 OPG detecting antibody
4 Peroxidase-conjugated streptavidin
5 TMB liquid substrate
6 Stop (H_2SO_4)
7 Enzyme catalyzed colour change

Figure 3.5 Principle of the osteoprotegerin ELISA assay.

(Reproduced with permission from Osteoprotegerin. Enzyme immunoassay for the quantitative determination of osteoprotegerin in EDTA plasma, heparin plasma, serum or cell culture supernatants. ELISA kit protocol. Cat. No. BI-20402. Biomedica Medizinprodukte GmbH&Co KG, Vienna.)[308]

A MaxiSorb microtiter plate (NUNC™ Brand Products, Roskilde, Denmark) was coated with 50 µl/well of 2µg/ml monoclonal mouse anti-human OPG capture antibody (R&D Systems Inc, Minneapolis, MN, USA) and incubated overnight at 4ºC. The plate was then blocked using 250µl/well PBS containing 1% BSA, 5% sucrose and 0.05% NaN_3 for one hour at room temperature. (**) Fifty µl/well of samples (diluted 1:100), and standards (ranging from 31.25 to 5000 pg/ml) (human recombinant OPG, Amgen Inc, Thousand Oaks, California, USA) diluted in dilution buffer (PBS containing 1% BSA and 0.02% NaN_3) in duplicate were added and incubated for two hours at 37ºC. (**) Thereafter 50 µl/well of 0.2µg/ml biotinylated goat anti-human OPG detecting antibody (R&D Systems) was added. After two hours incubation at 37ºC (**), 50µl/well peroxidase-conjugated streptavidin (DAKO

A/S, Glostrup, Denmark) (1:2000 dilution in PBS containing 0.05% Tween^{R}20 (MERCK, Schuchardt, Germany)) was added and incubated for 30-40 minutes at room temperature. (**) Two hundred µl/well ready to use 3'3',5,5' tetramethylbenzidine (TMB) liquid substrate (Sigma-Aldrich, Inc., Saint Louis, MO, USA), was added and the plate incubated at room temperature with shaking (300 rpm) for a period of 10 to 20 minutes until colour developed. The reaction was terminated by the addition of 50µl/well of 0.9M H_2SO_4. Optical density was then read at 450/620 nm using an ELX800 Universal microplate reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA). Results were analysed using a linear standard curve (Figure 3.6). (**) Between each step of the ELISA the plate was washed three times with PBS containing 0.05% Tween^{R}20. Intra and interassay variability was 5% and 11% respectively.

3.11.2 Enzyme linked immunosorbent assay (ELISA) quantification of osteoprotegrin concentrations in MC3T3-E1 conditioned media

For measurement of murine OPG, a sandwich ELISA protocol similar to the one used for the detection of human OPG was developed. A monoclonal anti-mouse OPG antibody (R&D Systems Inc, Minneapolis, MN, USA) was used as capture antibody, while a biotinylated anti-mouse OPG antibody (R&D Systems) was used as detection antibody. Undiluted samples and standards (31.25 to 5000 pg/ml) (recombinant mouse OPG/Fc chimera) (R&D Systems) in duplicate were prepared as described above. Intra and interassay variability was 6.5% and 16% respectively.

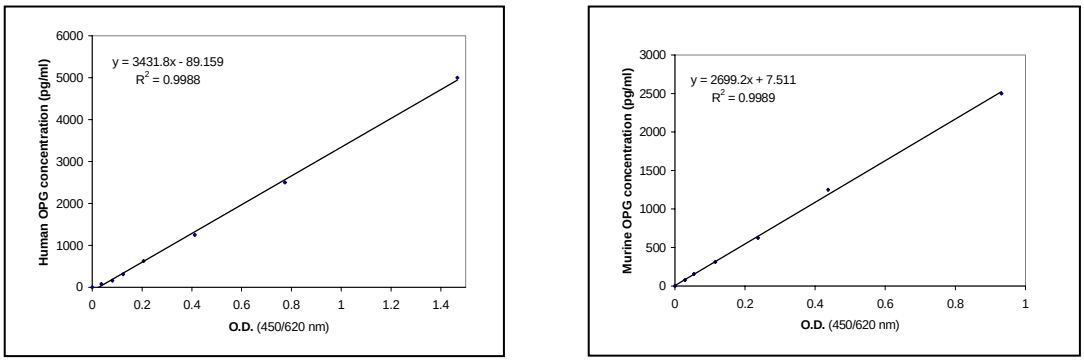


Figure 3.6 Examples of standard curves for the calculation of the amount of osteoprotegerin in the conditioned media from MG-63 cells and MC3T3-E1 cells.

3.11.3 Enzyme linked immunosorbent assay (ELISA) quantification of free soluble RANKL (sRANKL) concentrations in MC3T3-E1 conditioned media

A commercial sandwich ELISA kit (Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria) was used for the quantitative determination of free sRANKL in the conditioned media of the MC3T3-E1 cells. In short, recombinant murine OPG in duplicate was used as 'capture antibody' while a polyclonal biotinylated anti-mouse sRANKL antibody was used as detection antibody. Recombinant mouse sRANKL ranging from 12.5 to 100 pmol/liter was prepared in DMEM and used as standards. Cell culture medium was used as blank. Streptavidin-horseradish peroxidase was used as conjugate and TMB liquid as substrate for colour development. The reaction was terminated by the addition of H_2SO_4. Optical density was read at 450/620 nm using an ELX800 Universal microplate reader (Bio-Tek Instruments Inc., Analytical Diagnostic Products, Weltevreden Park, SA). Results were analysed using a linear standard curve that was constructed from the standard values. RANKL concentrations were then expressed as (pg/ml)/10 000 cells. (1 pg/ml = 0.05 pmol/liter). Intra and interassay variability was 4.2% and 9% respectively.

3.12 Detection of oestrogen receptors in MG-63 cells

For the detection of oestrogen receptors, 250 000 MG-63 cells were seeded onto sterilised coverslips in 6-well plates and left to adhere for 24 hours. MCF-7 human breast carcinoma cells (Highveld Biological (Pty) Ltd, (Sandringham, SA)), as positive control,[309,310] were treated similarly. Cells were then fixed and permeabilised as previously described for COX-detection. Cells were incubated sequentially for one hour at 37°C in each of the following, with incubations separated by 3 x 5 minutes washing steps: primary monoclonal antibody against oestrogen receptor (developed in rabbit (1:1000 in PBS) (Sigma-Aldrich, Inc., Saint Louis, MO, USA); goat anti-rabbit IgG peroxidase conjugate (1:200 in PBS) (Cappel™ Research Reagents, ICN, Aurora, Ohio, USA) and ExtrAvidin®-peroxidase conjugate (1:15). Antigen detection was achieved by adding 3,3' diaminobenzidine as substrate

(60 mg in 200 ml PBS, containing 0.1% hydrogen peroxide). Coverslips were left in the dark for five minutes and subsequently mounted with a glycerol-based mounting fluid. Photographs were taken with 400 ASA film with a Nikon camera (Nikon, Tokyo, Japan) attached to a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

3.13 Statistics

For each of the cell lines tested, three independent experiments (n=4) were conducted unless otherwise stated. Data were expressed as mean ± SD. Statistical analysis was performed using statistics for Windows software (version 2, Tallahassee, Florida, USA). Student's paired *t*-test was used to evaluate differences between the sample of interest and its respective control. For analysis of dose responses, means of a group were compared by one way analysis of variance (ANOVA) and significance was determined by post-hoc testing using Bonferroni's method. A *P* value less than 0.05 was considered to be statistically significant.