

**THE ROLE OF PHENYTOIN (5,5-DIPHENYLHYDANTOIN)
AND STRUCTURALLY RELATED COMPOUNDS IN
WOUND HEALING**

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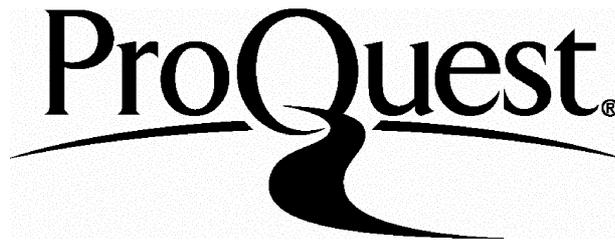
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Abstract

Phenytoin (PHT; 5,5-diphenylhydantoin), a commonly used drug for the control of epilepsy, has also been recently reported to promote wound healing, however its mode of action in the latter was yet undetermined.

This research studied the effects of PHT *in vitro* on cells derived from normal skin, and *in vivo* using a porcine full thickness wound model. Additionally, its influence on fibroblasts grown from Recessive Dystrophic Epidermolysis Bullosa (RDEB) skin was also investigated. These experiments targeted specific wound healing events such as fibroplasia (cell proliferation and migration) and matrix remodelling (contraction).

Low concentrations of PHT (5-10 μ g/ml) were found to upregulate the proliferation of some fibroblast and keratinocyte cell lines, but not others. Micro Chemotaxis Chamber assays revealed PHT (5-50 μ g/ml) to be a chemoattractant for both normal and RDEB fibroblasts and keratinocytes, indicating that it may facilitate the recruitment of cells into the wound space.

Prolonged treatment with PHT (20 μ g/ml) reduced the contraction of both the normal and the hypercontractile RDEB fibroblasts *in vitro* as measured by the reduction in surface area of untethered fibroblast-populated collagen lattices or directly by the Culture Force Monitor. Good correlations were observed with this finding *in vivo* in reducing wound contraction of pigs without impairment of healing after two or three weeks post-operatively (with either PHT powder or incorporated into Fibrin Sealant).

The effects of compounds structurally related to PHT were also investigated to see if a structure-activity relationship could be determined which would provide clues for the design of new drugs for wound healing. Both Micro Chemotaxis Chamber assays and Culture Force Monitor measurements indicated that metabolites of PHT were more active than the parent compound. Structure-activity analysis of hydantoin derivatives in these test systems suggested that substitution with a hydroxyl or methyl group at the meta or para position on the phenyl ring of PHT favoured maximum activity.

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List of Abbreviations

ALLAN	Allantoin
BMZ	basement membrane zone
BSA	bovine serum albumin
CFM	Culture Force Monitor
CO₂	carbon dioxide
COL	Colchicine
CYP	cytochrome P450
CYTO	Cytochalasin B
DAD	drug administration device
DEJ	Dermo-Epidermal Junction
DHD	dihydrodiol or 5-(3,4-dihydroxy-1,5-cyclohexadien)-5-phenylhydantoin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
EB	Epidermolysis Bullosa
Ebf-n	Epidermolysis Bullosa fibroblast cell line number n
EGF	epidermal growth factor
EH	epoxide hydroxylase
ER	endoplasmic reticulum
FCS	foetal calf serum
Fig.	Figure
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FN	fibronectin
FPCL	fibroblast-populated collagen lattice
FS	Fibrin Sealant

HPLC	High Pressure Liquid Chromatography
HYD	Hydantoin
HYDAC	Hydantoic acid
HYDACAC	Hydantoin acetic acid
IL	interleukin
IMF	integrin-mediated force
KGF	keratinocyte growth factor
KMK-2	Keratinocyte Medium kit-2
k-n	keratinocyte cell line number n
m-HPPH	meta-hydroxyphenyl-5-phenylhydantoin or 5-(3-hydroxyphenyl)-5-phenylhydantoin
Me-PH	5-methyl-5-phenylhydantoin
(MePh)-PH	5-(4-methylphenyl)-5-phenylhydantoin
MMF	microtubule-mediated force
MMP	matrix metalloproteinase
MUH	4-methyl umbelliferyl heptanoate
Nf-n	normal fibroblast cell line number n
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PF	Peak Force
ΔPF_0	difference in Peak Force between the control and experimental contraction curves at the point of drug addition
ΔPF_1	change in Peak Force immediately after drug addition (force changes associated with mechanical disturbance)
ΔPF_2	drug-induced difference in Peak Force

ΔPF_3	difference in Peak Force between the control and experimental contraction curves after drug addition
ΔPF_4	difference in Peak Force between the control and experimental contraction curves at the end of the experiment
p-HPPH	para-hydroxyphenyl-phenylhydantoin or 5-(4-hydroxyphenyl)-5-phenylhydantoin
PHT	Phenytoin or 5,5-diphenylhydantoin
PIGO	Phenytoin-induced gingival overgrowth
PT	Peak Time
RDEB	Recessive Dystrophic Epidermolysis Bullosa
RGDS	Arg-Gly-Asp-Ser
RIT	residual internal tension
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
SC	Stratum Corneum
SD	standard deviation
SG	Stratum Granulosum
ΔT_1	time lag associated with ΔPF_1
ΔT_2	time lag associated with ΔPF_2
TAX	Taxol
TGF	transforming growth factor
TRITC	tetramethylrhodamine B isothiocyanate
u-PA	urokinase-type plasminogen activator
u-PAR	urokinase-type plasminogen activator receptor
UV	ultraviolet

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I would like to dedicate this thesis to my little girl, whose name is Sophie, meaning "Wisdom".

CHAPTER 1. INTRODUCTION

1.1. Preface

This thesis investigates a well-known anti-epileptic drug, phenytoin (5,5-diphenylhydantoin, PHT) and its potential use in wound healing (Fig. 1.1). Despite many clinical trials carried out world-wide, this drug's mechanism of action in promoting wound healing is still unknown. The aim of this research was to study the *in vitro* and *in vivo* effects of PHT and thereby gain an insight into its mode of action. The effects of some compounds structurally related to PHT were also investigated to establish a structure-activity relationship, which may lead to the design of a new drug for wound healing.

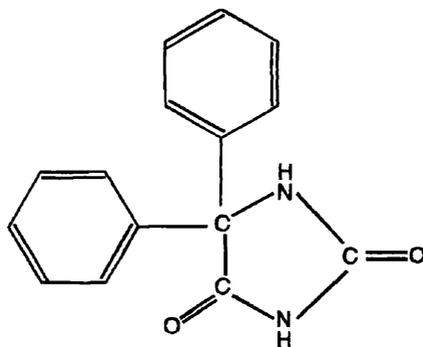
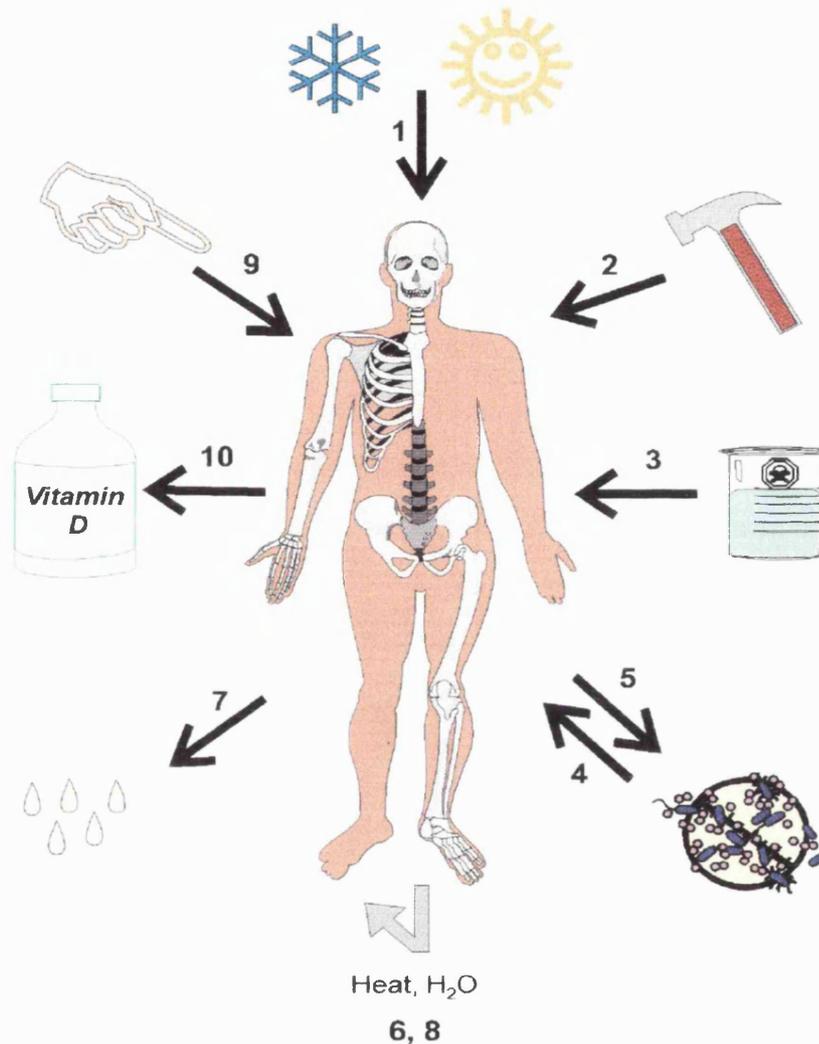


Figure 1.1. Structure of PHT. Phenytoin or 5,5-diphenylhydantoin are both generic names for 5,5-diphenyl-2,4-imidazolidinedione. Other trade names include Alepsin, Aleviaton, Citrullamon, Dentyl sodium, Difhydan, Dihydan soluble, Dilantin sodium, Dilantin, Dintoina, Diphantoine, Diphenine sodium, Diphentoin, Epamin, Epanutin, Epelin, Eptoin, Hidantal, Idantoin, Leptoin sodium, Minetoin, Phenytoin, Phenhydan, Solantoin, Solantyl, Tacosal and Zantropil (Smith *et al.*, 1988a; Hassell, 1981).

1.2. General introduction - The structure and function of skin

The skin or integument, has a surface area of around 2m² and weighs about 4kg, making it one of the largest organs of the body (Odland, 1983). It acts as a barrier (Cork, 1997) and is actively involved in the maintenance of homeostasis of the body (Wheater and Burkitt, 1987; Fig. 1.2).

The importance of skin is evidenced by the high mortality associated with large burns (Ryan *et al.*, 1998) and toxic epidermal necrolysis (Green *et al.*, 1993).



1. Protection against cold, heat and radiation
2. Protection against pressure, shock and friction
3. Protection against the effects of noxious chemicals
4. Protection against the invasion of germs (acidic surface film)
5. Immunologic defence against invading micro-organisms
6. Protection against loss of heat and moisture
7. Secretion of sweat (cooling function)
8. Circulatory and thermoregulation by cutaneous circulation
9. Detection of temperature, pressure and pain
10. Production of Vitamin D

Figure 1.2. The functions of skin.

Many different types of injury to the body result in damage to the skin. The body is usually capable of closing these wounds spontaneously to restore the protective functions of the skin. In lower vertebrates complete tissue regeneration can occur, while in higher vertebrates the loss of normal architecture results in a fibroproliferative response yielding a scar. This process is called wound healing and involves a variety of cells in the skin layers and some originating from blood. In order to understand these events taking place during the course of wound healing, a brief look at the structure of healthy skin (Holbrook and Wolff, 1993) is necessary.

Anatomically the skin can be divided into three layers (Fig. 1.3):

- a. the epidermis, which is a superficial outer protective layer,
- b. the dermis, which provides a tough flexible foundation,
- c. the subcutaneous adipose tissue located underneath.

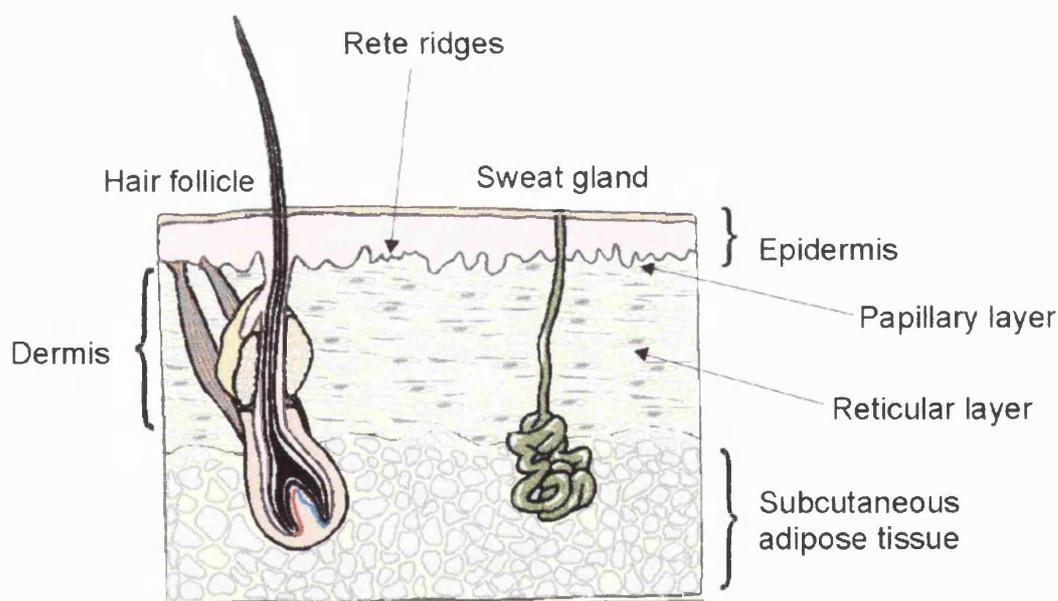


Figure 1.3. The structure of skin.

The epidermis is a multilayered, horny, squamous epithelium. It consists mainly of keratinocytes, but melanocytes and Langerhans cells are also present. The former is important in the protection against UV light, while the latter plays an important part in the immunologic defence of skin.

The epidermis can be divided into four layers (Holbrook and Wolff, 1993). The actively dividing keratinocytes from the Stratum Basale migrate upwards and undergo morphological changes into highly differentiated, non-dividing cells. In the Stratum Spinosum they begin to secrete keratin and form intercellular bridges or "spines". Ultrastructurally, these are composed of desmosomes, extensions of the intracellular keratin. In the Stratum Granulosum (SG) the cells acquire additional keratin, keratohyaline and lamellar granules. These cells also contain degradative enzymes responsible for the breakdown of nuclei and intracytoplasmic organelles. The dead cells of the Stratum Corneum (SC) represent an effective physical barrier (Cork, 1997).

The dermal side of the epidermis is characterised by rete ridges. The epidermal layers are fed from the underlying dermis, which is full of blood vessels. There are also so called skin appendages (hair follicles and glands) which extend upward from the dermis through the epidermis. The crypts of hair follicles are important in that they are in continuity with the epidermis and therefore can be a source of epidermal regeneration.

The interface between the epidermis and the dermis is termed the basement membrane zone (Cleary, 1996). Ultrastructurally it can be divided into three layers (Fig. 1.4).

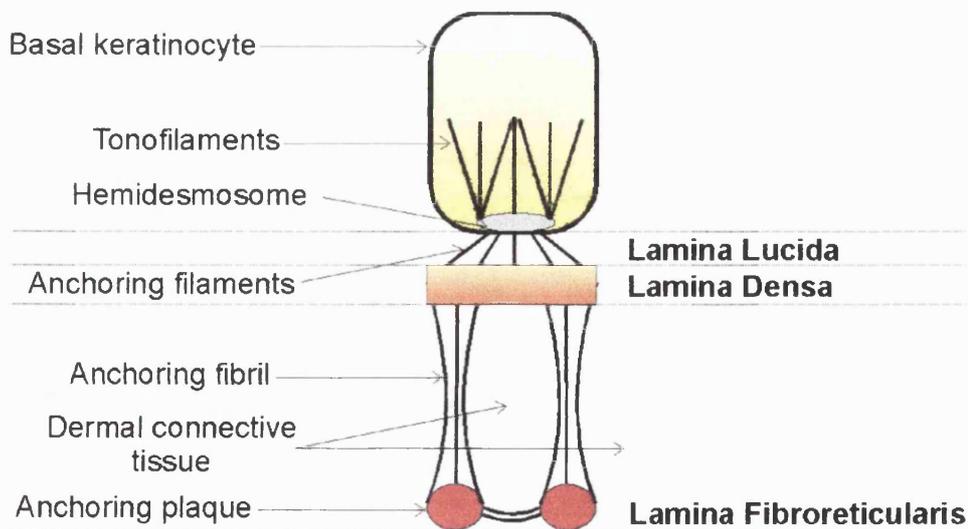


Figure 1.4. Schematic representation of the Dermo-Epidermal Junction.

The Lamina Lucida is traversed by vertical anchoring filaments that connect the basal cell membranes with the Lamina Densa and, finally, thick fibrous strands of dermal origin complete the connection with the dermis in the Lamina Fibroreticularis.

The basement membrane fundamentally serves as glue between the epidermis and the dermis and is vital in the maintenance of a healthy skin structure. Each of the planes of the dermo-epidermal junction (DEJ) has specific structural, biochemical, and antigenic properties. The DEJ is also the primary site of blister formation in many skin defects (e.g. Epidermolysis Bullosa) associated with its different molecular components (Marinkovich, 1993).

The dermis is composed of two parts: the Stratum Papillare is the top layer and connects to the epidermis via connective tissue papillae. The Stratum Reticulare is the dominant region of skin in general and is located adjacent to the subcutaneous tissue below. The distinctions of these two zones are based largely on their differences in connective tissue organisation, cell density, vascular and nerve patterns. This papillary region is characterised by a high density of fibroblasts that proliferate more rapidly and are more metabolically active than those found in the reticular region (Beyth and Culp, 1985; Schafer *et al.*, 1985; Azzarone and Macieira-Coelho, 1982; Tajima and Pinnel, 1981; Harper and Grove, 1979). Mature elastic fibres are not usually found here, but it is abundant in small-diameter collagen fibres and oxytalan elastic fibres. These structural characteristics allow the skin to accommodate impact. In contrast, the reticular region contains fewer cells and its dense interwoven large-diameter collagen fibrils run parallel to the body's surface. Between its meshes ground substance fills the space and mature branching elastic fibres contribute to the skin's extensibility. These fibres' diameters increase in size progressively towards the hypodermis.

1.3. General introduction - Wound healing

A wound is defined as a skin injury in which tissues are separated or destroyed. Wound healing is a complex process involving the collaborative efforts of heterogeneous groups of cells. During this highly organised sequence of events, keratinocytes, endothelial cells, fibroblasts and inflammatory cells communicate with each other via cytokines and other soluble mediators, but in

some cases, cell-cell contacts and cell-matrix interactions also act as regulatory factors. The wound healing process is usually divided into three overlapping phases (Fig. 1.5): inflammation, granulation tissue formation and remodelling (Clark, 1996).

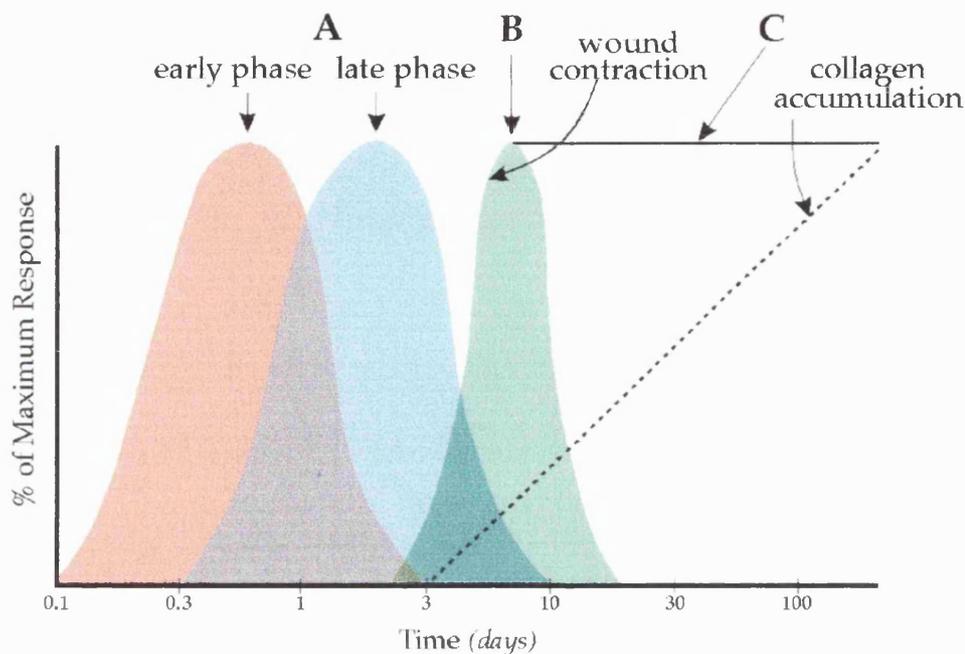


Figure 1.5. The course of events in wound healing (adapted from Clark, 1996). The healing of the wound has been arbitrarily divided into three overlapping phases: inflammation (A), re-epithelialisation and granulation tissue formation (B) and matrix formation and remodelling (C).

In this overview, the current ideas regarding wound repair are briefly summarised following this chronological order of wound repair.

1.3.1. Inflammation

After injury, a temporary repair is achieved by the formation of a fibrin clot or thrombus, which fills the discontinuity, and a series of events are initiated to regenerate the missing part. The substances released from the tissue debris result in the classical characteristic inflammatory reactions: redness, heat, swelling, pain and functional disturbance. The thrombus acts as a scaffold to which inflammatory cells (neutrophilic granulocytes, macrophages and

phagocytic cells) attach and migrate through (Haslett and Henson, 1996; Riches, 1996). It also serves as a reservoir of growth factors and cytokines, which attract these inflammatory cells to the injured site, but moreover influence re-epithelialisation, granulation tissue contraction, and angiogenesis later on. The recruited inflammatory cells absorb and enzymatically degrade any foreign material, thereby cleaning the wound and release further soluble factors amplifying the earlier signals. Early inflammation is characterised by neutrophil-rich, whilst late inflammation by mononuclear cell-rich infiltrates.

1.3.2. Granulation tissue formation

Approximately four days after injury, new stroma begins to replace the thrombus. This granulation tissue formation entails connective tissue deposition and angiogenesis. Macrophages, fibroblasts and blood vessels move into the wound space simultaneously and are interdependent (Hunt, 1980). Macrophages provide the cytokines necessary for fibroplasia and angiogenesis (Riches, 1996), while fibroblasts deposit the new extracellular matrix (ECM) supporting cell ingrowth. New blood vessels, on the other hand, deliver oxygen and nutrients for the sustenance of continued repair. Granulation tissue formation can be thus further divided into fibroplasia and neovascularisation.

1.3.2.1. Fibroplasia

Fibroplasia is characterised by fibroblasts and the ECM produced by them. However, a complex, dynamic reciprocity exists between fibroblasts and the ECM (Welch *et al.*, 1990; Kurkinen *et al.*, 1980). The early ECM provides a scaffold for contact guidance (fibronectin and collagen), low impedance for cell mobility (hyaluronic acid; Toole, 1991) and acts as a reservoir of cytokines (Nathan and Sporn, 1991). Cytokines generated at the wound site induce fibroblast proliferation, migration, and ECM production. Fibroblasts, on the other hand, remodel, newly synthesise and deposit the very ECM influencing them (Clark *et al.*, 1995; Grinnell, 1994; Mauch *et al.*, 1988). Granulation tissue deposition occurs in an ordered sequence, it is initially fibronectin (FN) and hyaluronic acid, which is followed by type III collagen and finally type I collagen (Clark and Denver, 1985).

In order to migrate into the wound space, fibroblasts penetrate the blood clot, which is mainly composed of fibrin and, to a lesser extent, FN and vitronectin (VN). It has been suggested that fibroblasts use this provisional FN matrix to move around the wound, as it has been shown to be the case *in vitro* (Hsieh and Chen, 1983). They may also translocate by binding to either fibrin or VN, as fibroblasts were able to attach to both of these matrix proteins *in vitro* (Singer *et al.*, 1988; Dejana *et al.*, 1984). Binding takes place via specialised cell membrane receptors, called integrins, to the Arg-Gly-Asp-Ser (RGDS) tetrapeptides within the cell binding domains of these ECM molecules.

Cell migration, as defined by the locomotion of a cell over an ECM matrix, includes the alteration of cell-cell and cell-ECM adhesion together with changes in the organisation of the cytoskeleton (Erickson, 1990; Zigmond, 1989). It also requires a functional cytoskeletal system (Liao *et al.*, 1995; Cunningham *et al.*, 1991; Zigmond, 1989), comprised of actin, myosin, tubulin, intermediate filaments and adhesion plaques. The process of cell movement (reviewed by Palecek *et al.*, 1997; Lauffenburger and Horwitz, 1996; Williams *et al.*, 1994; Lee *et al.*, 1993; Condeelis, 1993; Hynes, 1992; Ginsberg *et al.*, 1992) involves extension at the leading edge and retraction at the trailing edge. Inside these migrating cells the cytoskeleton is continuously reconstituted, primarily by the assembly of the actin filaments at the leading edge and disassembly at the trailing edge. Outside the cells, binding of integrins to their ECM ligands at the leading edge results in signalling from the integrins to the cell interior, anchoring of the newly polymerised actin to them and the formation of focal adhesion sites. It is thought that tractional forces exerted by these attachments sites, together with the mechanical forces generated by the contraction of actin filaments, result in the cell body being pulled forward. This is followed by retraction, caused partly by the trailing edge being ripped and partly by the dissociation of integrin-ligand complexes, which is regulated by intracellular signals.

Fibroblast migration may involve chemotactic, haptotactic and contact guidance mechanisms. Chemotaxis takes place, when cells are directed to move toward a chemotactic gradient of a soluble attractant, by extending their lamellopodia toward the stimulus. Accelerated random migration of cells, on the other hand, is termed as chemokinesis (Wilkinson and Lackie, 1988).

Haptotaxis occurs when there is a directed response of the cells to a surface-bound adhesion gradient (Harris *et al.*, 1980; Carter, 1970). In this case the orientating cues may be particular matrix components and translocation occurs by the random extension of lamellopodia. In the absence of such an adhesion gradient, the fibrils of the ECM determine the alignment and the direction of fibroblast migration. This process of contact guidance, initially recognised by Weiss (Weiss, 1985), is related to haptotaxis and refers to the tendency of cells to align to the discontinuities in the substrata they are attached to. Contact guidance has been observed of cultured fibroblasts, which migrated along and not across the FN fibres (Wojciak-Stothard *et al.*, 1996; Hsieh and Chen, 1983) and scratched surfaces (Curtis and Clark, 1990; Dunn and Brown, 1986).

A number of chemoattractants have been identified in wounds, some produced by inflammatory cells and some the result of tissue breakdown. Fibroblasts have been shown to respond *in vivo* to various growth factors at different concentrations (Pierce *et al.*, 1991). FGF, TGF- β and PDGF were all found to be chemoattractants for fibroblasts *in vitro* (Buckley-Sturrock *et al.*; 1989; Postlethwaite *et al.*; 1987; Seppa *et al.*, 1982) and can upregulate integrin receptors (Gailit *et al.*, 1995; Ahlen and Rubin 1994; Heino *et al.*, 1989). Additional chemotactic signals are provided by fibrin, FN and collagen degradation products (Postlethwaite *et al.*, 1981; Gauss-Muller *et al.*, 1980; Ali and Hynes 1978; Postlethwaite *et al.*, 1978).

To facilitate movement through the cross-linked fibrin clot and a tight meshwork of ECM, a variety of fibroblast- and serum-derived enzymes cleave a path for migration. These include interstitial collagenase (MMP-1), gelatinase (MMP-2), stromelysin (MMP-3) and the plasminogen activators (Stetler-Stevenson *et al.*, 1989; Saus *et al.*, 1988; Grant *et al.*, 1987; Wilhelm *et al.*, 1987). Additionally, chemotactic factors, such as PDGF and TGF- β may also upregulate matrix metalloproteinase (MMP) and plasminogen activator (PA) production and secretion (Overall *et al.*, 1989; Laiho *et al.*, 1986).

Once migrating fibroblasts reached the wound, they gradually become secretory, whose major function is protein synthesis (Welch *et al.*, 1990). The previously retracted endoplasmic reticulum (ER) and golgi apparatus becomes dispersed throughout the cytoplasm and a loose matrix is produced, which is

mainly composed of FN and type III collagen (Grinnell *et al.*, 1981; Kurkinen *et al.*, 1980). Ultimately, this profibrotic phenotype takes over, which is characterised by an abundance of rough ER and golgi apparatus, secreting newly synthesised collagen (Welch *et al.*, 1990) in response to highly expressed TGF- β . Notwithstanding, TGF- β fails to upregulate further collagen deposition, once a matrix has been deposited (Clark *et al.*, 1995). It is also thought that IL-4 released by mast cells induces a modest increase in types I and III collagen together with FN (Postlethwaite *et al.*, 1992). Mast cells furthermore produce tryptase (a serine esterase) in abundance, which has been shown to upregulate fibroblast proliferation (Ruoss *et al.*, 1991).

The stimuli responsible for fibroblast proliferation and matrix synthesis (TGF- α , TGF- β and PDGF) have been extensively investigated *in vitro* (Sporn and Roberts, 1992; Ross and Raines, 1990; Derynck, 1988). Their action was confirmed by *in vivo* manipulation of wounds as well (Pierce *et al.*, 1991; Schultz *et al.*, 1991; Sprugel *et al.*, 1987). γ -Interferon on the other hand was demonstrated to have a negative effect on the mitogenic and synthetic potential of fibroblasts *in vitro* and *in vivo* (Granstein *et al.*, 1987; Duncan and Berman, 1985). In addition, the collagen matrix itself can suppress these activities (Clark *et al.*, 1995; Grinnell, 1994), whilst fibrin or FN matrix has little or no suppressive effect (Clark *et al.*, 1995).

Interestingly, many fibroblasts undergo apoptosis (programmed cell death) in day-10 healing wounds (Williams, 1991), thereby marking the transition from a fibroblast-rich granulation tissue to a scar tissue with reduced cell density.

1.3.2.2. Neovascularisation

Angiogenesis, or blood vessel growth, occurs concurrently with fibroplasia. For this complex process to take place, it is imperative that there is an alteration in the endothelial phenotype (Ausprunk and Folkman, 1977). The induction of proliferation and migration occurs in the presence of a suitable ECM together with the so-called "free edge effect" (the absence of neighbouring endothelial cells; Schwartz *et al.*, 1982). Additionally, the presence of

macrophages in a hypoxic environment is also needed for vessel ingrowth (Knighton *et al.*, 1981).

The exact soluble factors that prompt angiogenesis are not known; however, numerous candidates are suggested both from activated macrophages and tissue components (Folkman and Klagsbrun, 1987). In response to these, the endothelial cells begin to migrate from the nearest venule into the perivascular space. This is a highly protease and integrin dependent event (Madri *et al.*, 1996). The remaining cells in the parent vessel begin to proliferate, providing a further supply of cells. It is thought that capillary bud formation and extension occurs in response to chemotactic factors and endothelial replication is secondary to this (Folkman, 1982). Eventually, capillary loops and plexus are formed and blood flow begins.

1.3.3. Re-epithelialisation

Re-epithelialisation begins within 24-48 hours after injury. Rapid skin coverage by new epithelium reduces both morbidity and mortality. A wound is clinically termed re-epithelialised if the moist vascular granulation bed is covered by a sheet of dry layer of epithelium (Woodley, 1996). This is achieved by the rapid migration of keratinocytes from the wound edge or epidermal appendages. Epithelial migration is induced in response to a number of chemoattractants and loss of contact inhibition from neighbouring cells. Although wounding stimulates both migration and proliferation, the former is considered to be of greater importance. Experimental blockage of cell division did not have an effect on the rate of wound closure (Gipson *et al.*, 1982; Dunlap and Donaldson, 1978; DiPasquale, 1975). The migrating cells either arise from the wound periphery or from pilosebaceous or eccrine remnants (Cotsarelis *et al.*, 1990; Pang *et al.*, 1978).

Two mechanisms have been suggested for epithelial wound closure depending on the state and character of the epithelium affected (Stenn and DePalma, 1988). The sliding model suggests that epithelial cells migrate as a sheet: the cells at the margin appear to be motile, while the cells behind are dragged along (Vaughan and Trinkaus, 1966). This type of wound closure has been directly demonstrated in tissue culture (Vaughan and Trinkaus, 1966), in the so-called embryonic purse-string re-epithelialisation process (Martin, 1997)

and corneal wound healing (Fujikawa *et al.*, 1984; Buck 1979). However, the movement of keratinocytes of the stratified epidermis in skin is much more complex. Winter (1962), on the other hand, proposed the “leap frog” model. According to this, submarginal cells crawl over the recently adhered basal cells in a leap frog fashion.

During migration the basal keratinocytes dramatically change from their stationary phenotype as observed by electron microscopy (Odland and Ross, 1968). In its unwounded state, basal keratinocytes are characterised by polarity, whereas the migratory phenotype is flat, elongated with lamellopodia and ruffled cytoplasmic projections (Stenn and Depalma, 1988; Odland and Ross, 1968). This metamorphosis involves the dissolution of the characteristic intercellular desmosomes (cell-cell links) and hemidesmosomes links between cell-matrix junctions (Krawczyk and Wilgram, 1973). Also, the tonofilaments are withdrawn toward a perinuclear location. Meanwhile, contractile proteins, such as actin and myosin appear, thus equipping epithelial cells for motility (Gabbiani *et al.*, 1978; Odland and Ross, 1968). These migrating keratinocytes are similar but not identical to basal cells of stratified epidermis based on the keratin proteins they contain (Mansbridge and Knapp, 1987), although they lack filaggrin, and synthesise involucrin and transglutaminase, substances characteristic of cells in the SG. An interesting feature of migrating epithelial cells is that they do not terminally differentiate. Similar phenotypic changes could be achieved in culture by reduced calcium concentrations, as normal levels drive terminal differentiation (Hennings *et al.*, 1980).

The specific signals for the initiation of re-epithelialisation are yet to be identified, although there is evidence for both physical and chemical stimuli. It is thought that “contact inhibition” i.e. physical cell-cell contact alone can prevent cell movement. The surrounding connective tissue itself influences the activity of keratinocytes, such as proliferation, attachment, migration and spreading (Woodley *et al.*, 1988a, 1990). Furthermore, keratinocytes synthesise their own basement membrane (Woodley *et al.*, 1980ab, 1988b), including laminin, type IV and VII collagens and anchoring filament-associated components, which all have an influence on cell motility. During the course of wound healing, when the basement membrane is disrupted, keratinocytes use a meshwork of mainly FN and fibrin to move along the wound bed (Clark *et al.*, 1982).

This originates mainly from serum coagulum but is also manufactured both by fibroblasts and keratinocytes (O'Keefe *et al.*, 1984, 1985; Kubo *et al.*, 1984). Furthermore it has been shown that addition of exogenous FN aided re-epithelialisation in human dermal and corneal wounds (Scheel *et al.*, 1991; Wysocki *et al.*, 1988; Kono *et al.*, 1985; Nishida *et al.*, 1985). Wound keratinocytes also express functional integrin receptors for FN, normally lacking in epidermal cells (Toda *et al.*, 1987). Another such plasma-serum component, that acts as provisional matrix, is VN, or, as it is otherwise known, epibolin. It was identified as the factor in serum that induces keratinocyte outgrowth from explants (epiboly; Stenn, 1981). In the absence of VN, keratinocytes are unable to spread on FN, collagen or laminin (Stenn, 1987; Stenn and Dvoretzky, 1979). An integrin-mediated process is responsible for adhesion of keratinocytes to the RGDS sequences in both FN and VN (Kim *et al.*, 1994, 1992). Although collagen also does contain RGDS sequences, keratinocyte motility in this case is mediated by a collagen receptor, $\alpha 2\beta 1$ (Kim *et al.*, 1992). Laminin on the other hand acts as a restraint for keratinocytes (Woodley *et al.*, 1988a), and is associated with a stationary state. It appears late during epidermal healing together with type VII collagen (Cornelius *et al.*, 1986). Thus basement membrane proteins synthesis follows a very ordered sequence of events (Clark *et al.*, 1982).

Soon after injury, epithelial cell proliferation begins at the wound margins (Krawczyk, 1971). The soluble factors responsible for the initiation of epidermal outgrowth are considered to be EGF (Brown *et al.*, 1989), TGF- β (Mustoe *et al.*, 1987) and IL-1 (Chen *et al.*, 1995). KGF/FGF-7, produced by fibroblasts, is a paracrine mediator of epithelial cell proliferation also inducing cell migration and the upregulation of urokinase-type plasminogen activator activity (Tsuboi *et al.*, 1993). U-PA is an important factor in the induction of epidermal migration (Grondahl-Hansen *et al.*, 1988), as it activates both plasminogen and collagenase. Keratinocytes secrete collagenases that degrade type I and IV collagen (Woodley *et al.*, 1986) and its degree of activation reflects the migratory capacity of the cells (Petersen *et al.*, 1989). These keratinocytes are highly phagocytic (Takashima and Grinnell, 1984) and dissect under the scab and non-viable tissue by releasing proteases ingesting wound debris, although phagocytosis itself does not stimulate their migration (Woodley *et al.*, 1988a).

Once the wound is re-epithelialised, the basement membrane is re-established and the epidermal cells revert to their normal phenotype firmly attached through hemidesmosomes and anchoring fibrils (Gipson *et al.*, 1988).

1.3.4. Tissue remodelling

In acute wounds, this phase overlaps with granulation tissue formation and encompasses matrix remodelling, cell maturation and apoptosis. Moreover, tissue remodelling has already started at the wound margins while granulation tissue is still invading the wound space (Kurkinen *et al.*, 1980). As soon as the granulation tissue has filled the wound and is covered with a neo-epidermis, the wound begins to simultaneously contract and the epidermal cells begin to differentiate in order to re-establish its barrier function. Finally, programmed cell death takes place resulting in a scar with low cell density. Paralleling this event, the appearance of the scar becomes pale due to low vascular perfusion and recesses.

The ECM and its timely reorganisation serve numerous crucial functions for effective wound repair. After granulation tissue formation, the ECM continuously changes for many months (Compton *et al.*, 1989). Initially FN provides a support for migrating cells (fibroblasts, keratinocytes, and endothelial cells) together with hydrated hyaluronan ensuring that parenchymal cells easily penetrate the matrix. FN substratum furthermore provides a linkage for myofibroblasts and a template for collagen fibrillogenesis (McDonald *et al.*, 1982; Kurkinen *et al.*, 1980). Initially collagen types I, III and V are laid down to provide some tensile strength. As time progresses, FN and hyaluronan disappear, the collagen bundles enlarge (type III collagen is gradually replaced by type I) thus increasing wound-breaking strength (Gabbiani *et al.*, 1976; Diegelmann *et al.*, 1975). Collagen turnover is a highly balanced process involving the healthy maintenance of synthesis and degradation by fibroblasts, cytokines, matrix metalloproteinases, and their inhibitors. The final deposition of proteoglycans and elastin (Davidson *et al.*, 1993) also ensures resilience to deformation and elasticity. The matrix constituents present in a wound site will, however, ultimately depend on the cellular activity present in that particular microenvironment (Igotz and Massague, 1986; Roberts *et al.*, 1986).

Fibroblasts also reorganise their matrix during wound contraction. Wound contraction begins early in the repair process, when the wound is not fully epithelialised. Scar contracture is distinct from wound contraction since it occurs late in the remodelling phase, involving the compaction of scar tissue. The rapid closure of a wound is important in the prevention of body fluid loss and infection (Rudolph, 1979). Much of this is attributed to wound contraction, where the edges of a wound are drawn to the centre by forces generated internally (Mast *et al.*, 1992). This is thought to be a cell-mediated event, however it is disputed whether the contractile forces involved are generated by the locomotion of fibroblasts (tractional forces; Erlich, 1988), or by specialised fibroblasts, called the myofibroblasts (Gabbiani *et al.*, 1972).

Fibroblasts-mediated contraction has been studied extensively *in vitro* (Brown *et al.*, 1998, 1996; Eastwood *et al.*, 1998ab; 1996, 1994; Kolodney and Elson, 1995; Kolodney and Wylsolmerski, 1992; Lambert *et al.*, 1992; Tranquillo and Murray, 1992; Delvoye *et al.*, 1991; Erlich and Rajaratnan, 1990; Finesmith *et al.*, 1990; Guidry and Grinnell, 1985; Bell *et al.*, 1979) by the use of fibroblast-populated collagen lattices (FPCL). The myofibroblast has been implicated in mediating the contraction of such collagen lattices *in vitro* (Gabbiani *et al.*, 1972) and of granulation tissue *in vivo* (Garana *et al.*, 1992; Vande Berg *et al.*, 1984; Gabbiani *et al.*, 1978). Rudolph and co-workers using a porcine model has shown, that during active wound contraction myofibroblasts are present throughout the granulation tissue (Rudolph *et al.*, 1977). Although myofibroblasts ultrastructurally combine characteristics of both fibroblasts and smooth muscle cells, they are derived from the former and not the latter (Darby *et al.*, 1990; Eddy *et al.*, 1988). Myofibroblasts express α -smooth muscle actin, in the form of stress fibres parallel to the long axis of the cell and display convoluted nuclear membranes. Interestingly, application of topical smooth muscle contraction inhibitors also inhibited wound contraction (Madden *et al.*, 1974). It is proposed that myofibroblasts link up via cell-to-cell contacts and undergo synchronised, multicellular contraction, which translocates the surrounding collagen fibrils (Majno *et al.*, 1971). Mechanical forces, growth factors, and extracellular components are good candidates for myofibroblast stimulation. However, little is known about the factors, which regulate their disappearance. Currently much effort is focussed on elucidating its role in scar

function and pathological wound healing process (Desmouliere and Gabbiani, 1996; Erlich *et al.*, 1994).

Erlich and Rajaratnan for the role of myofibroblasts present an entirely different hypothesis on the other hand. Based on *in vitro* FPCL studies and an *in vivo* noncontracting animal wound model it is proposed that the presence and abundance of myofibroblast during wound contraction is incidental (Erlich and Rajaratnan, 1990; Bell *et al.*, 1979). Moreover, Erlich suggests that myofibroblasts are involved in the resolution of fibroplasia and may be in the process of leaving the wound environment (Erlich and Rajaratnan, 1990). Also, myofibroblasts are difficult to discern in full-excision rat wounds any earlier than 7 days, by which time the wound has contracted by 50 percent (Majno *et al.*, 1971). This alternative theory also suggests that wound contraction is due to tractional forces of normal migrating fibroblasts, as demonstrated by Harris and co-workers. Fibroblasts plated onto a polymerised silicone film wrinkle the surface as they move across it, yet retain their bipolar morphology (Harris *et al.*, 1981). In this case, solitary fibroblasts are proposed to reorganise and compress their surrounding matrix, thus pulling the surrounding skin with them. Therefore, contraction is achieved without the need to invoke a specific contractile fibroblast. Thus, the major difference between these two theories is that myofibroblasts are said to be acting as multicellular units that undergo contraction (Majno *et al.*, 1971), whereas the tractional-fibroblast theory suggest, that the movement of individual fibroblasts causes the rearrangement of the surrounding matrix. Grinnell (Grinnell, 1994) in his review has attempted to combine these two controversial hypotheses, argued recently by Erlich and Rajaratnan (Erlich and Rajaratnan, 1990) and Rudolph *et al.* (Rudolph *et al.*, 1992), proposing that mechanical tractile forces, generated *in vivo* by locomotion of fibroblasts, may potentially stimulate myofibroblast differentiation.

Thus at the end of the wound contraction phase, at around three weeks, the wound has regained around 20% of its final strength. Thereafter it continues to gain tensile strength slowly, by the remodelling of existing collagen bundles into larger ones and forming intermolecular crosslinks, rather than by the deposition of further new collagen (Bailey *et al.*, 1975). However, scar tissue fails to attain the same tensile strength of its uninjured state, and at best is only 70% as strong as normal, intact skin (Leveson *et al.*, 1965).

Scars in general contain no hair, no pigment and no glands, therefore do not represent full structural regeneration. Bad scars are raised and lumpy due to excessive and disordered collagen production (e.g. keloid and hypertrophic scars). Excessive contraction and loss of function may also be an added complication (e.g. hypertrophic scars). Delay in healing on the other hand may occur with large wounds or impaired tissue viability and results in enhanced scarring.

1.4. The novel subject of Wound Pharmacology

Non-healing or chronic wounds take a great toll of diabetic and venous insufficiency patients in terms of morbidity and mortality, whilst incurring great healthcare expenditure (Harding and Boyce, 1998). The quest for wound healing agents is perhaps one of the oldest challenges for medical practice and various treatments have been long recorded in ancient writings (reviewed by Majno, 1975). In the late 20th century, we have moved from an era of simply removing noxious influences (e.g. infection, inflammation, necrotic tissue) and pain control, to one in which we visualise the healing process being positively fostered. Although much is known about tissue repair mechanisms, we still need a deeper understanding of such mechanisms in order to intervene at cellular or molecular level with the aim of improving adult wound healing or to make it more like foetal wound healing, which is so nearly perfect as to be considered scarless (Martin, 1997). Currently, foetal wound healing in adults is a Utopia, which may never be reached. The best we can hope for is to pharmacologically reduce healing time, the cost of care in certain cases, and to some extent modify the end result of function and cosmesis.

Since the discovery of the first growth factor (EGF; Cohen, 1962), it has been shown that cellular events in wounds could be potentially accelerated (Brown *et al.*, 1989). Such agents in experimental trials are usually delivered topically and therefore have different pharmacodynamics and kinetics from conventional, orally applied drugs. Unlike topical agents administered through transdermal devices, vulnerary agents are intended to localise and exert their effect in the wound only. Therefore, serum concentrations will not reflect their efficacy, but only indicate possible toxicity if systemic absorption were to take place.

The need to establish this new discipline of Wound Pharmacology has been recently recognised by the European Tissue Repair Society in 1995 (Arnold *et al.*, 1996), which encouraged new criteria for evaluating the safety and efficacy of topically applied wound healing agents. These require careful choices of suitable experimental systems and physiologically relevant bioassays. It is the aim of this thesis to examine one such vulnerary agent, PHT, from a Wound Pharmacology viewpoint.

1.4.1. General skin pharmacology

The intact epidermis, in particular the SC, presents a very effective barrier against percutaneous absorption of drugs and potentially toxic agents (Roberts, 1997). The SC can be visualised as a brick wall, with the corneocytes being the bricks and the lamellar lipids the mortar (Figure 1.6). The effectiveness of skin barrier function varies with racial differences (Kompaore *et al.*, 1993). As the SC is the rate-limiting barrier to the ingress of materials, delivery of agents for systemic therapy therefore requires the aid of penetration enhancers and transdermal devices. Topically applied dermatological formulations, on the other hand, gain direct access to the target area (Fig. 1.6).

In addition to presenting a hindrance to absorption of xenobiotics, skin cells are also capable of their metabolism, as an additional defence mechanism (Streinstrasser and Merkle, 1995). The activity of enzymes in the skin may even reach 80-90% of those in the liver (Barry, 1987). Immunohistochemistry of human skin has revealed that most of the cytochrome-P450 (CYP) isozymes are located in the epidermis, sebaceous glands, and the outer root-sheath of hair follicles. In particular, epoxide hydrolase and CYP-dependent monooxygenase were noted, both of which are essential in the metabolic conversion of PHT. Rat studies revealed that within the epidermis, they were found to be the highest in the differentiated layers (Guo *et al.*, 1990). Due to its thickness, however, the dermis exhibits greater metabolic capacity if the enzyme activity is expressed per unit area of skin (Finnen, 1987). Therefore, skin as a whole is an important extra-hepatic site for xenobiotic metabolism.

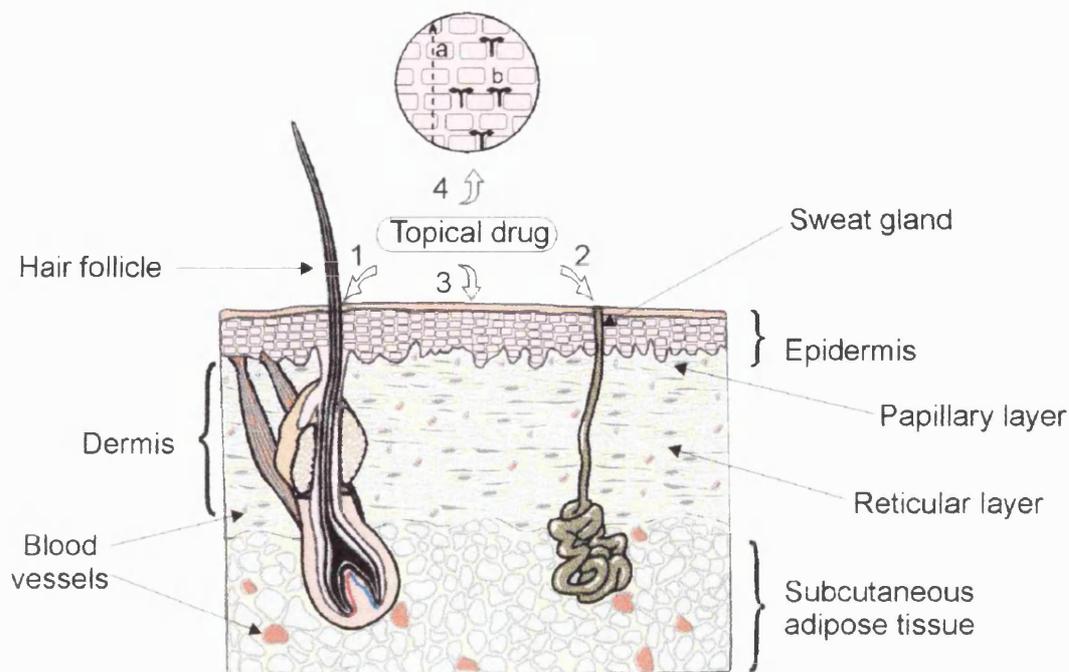


Figure 1.6. Diagrammatic representation of the absorption of substances through intact skin. Potential routes include transappendageal entry (1&2) and through the SC (3). Within the SC (4) the main route for transport is through the intercellular space (b). a = transcellular, b = intercellular passage within the SC.

1.4.2. Wound Pharmacology

In the case of topically applied vulnerary products, it is necessary that the medicament is released from the base vehicle, penetrates the skin at a suitable rate and is maintained at the target site in sufficient quantities to exert its therapeutic effect. It is thought that the permeability of the more lipophilic solutes is favoured from aqueous solutions for use on intact skin, whereas polar solute permeability is greatest from hydrocarbon-based vehicles (Roberts, 1997). If no epidermal barrier is present it is, however, desirable to manipulate delivery by controlling its release from the vehicle ensuring continuous local accumulation.

The amount of topical agent being absorbed in wounds is dependent upon many factors (Arnold *et al.*, 1996). Intact skin is an active barrier, particularly the dead cells of the SC, and many substances are impermeable, requiring the aid of penetration enhancers. Similarly, any eschar present in the

wound will prove to be a barrier and needs to be debrided. If the target site for delivery is the basal layer of the epidermis and the dermis, re-epithelialisation during healing will increasingly impede drug delivery with time, even though a fully differentiated SC is not yet present (Walker *et al.*, 1997). Other factors influencing absorption are: the physicochemical properties of the drug; the type, depth and inhomogeneity of the wound; the presence of granulation tissue; tissue oedema; vasculature and perfusion; type of dressings used; vehicle effects on skin permeability and local drug interactions. Furthermore, molecular modification of the xenobiotic during biotransformation may alter its solubility and penetration, but more importantly its activity.

Therefore Wound Pharmacology of a vulnerary agent should not only consider its absorption, distribution and elimination but also its metabolism during topical therapy. Experimental models should also take into account that well-known differences exist in enzyme activities and distribution between species, anatomical sites and processed tissues (Streinstrasser and Merkle, 1995). Considerable similarity exists concerning skin permeability properties for topical agents between human skin and species like miniature pigs, monkeys, and dogs, in that order. However, no such ranking has been established for the metabolic activity in skin. Moreover, the development of physiologically relevant bioassays and human, authentic, reproducible *in vivo* models of chronic wounds still remains the key objective of wound healing research.

1.5. Phenytoin and wound healing

PHT was introduced for the control of convulsive disorders in 1938 (Merritt and Putnam, 1938) and has been widely used ever since. With prolonged treatment, approximately 50% of epileptics develop fibrous overgrowth of the gingivae (Penarrocha-Diago *et al.*, 1990; Keith, 1978), commonly referred to as the "Dilantin gingival hyperplasia" (Esterberg and White, 1945; MacFarlane *et al.* 1942), although mild skin and skull thickening may also occur. This apparent stimulatory effect on connective tissue has inspired its use for wound healing.

1.5.1. Background - Clinical pharmacology (after systemic administration)

PHT is related to the barbiturates in chemical structure (but has a five-membered ring) and does not cause sedation or interfere with normal central function (Jones and Wimbish, 1985). Its primary site of action is in the motor cortex where the spread of seizure activity is inhibited by a membrane potential-dependent blockade of Na⁺ channels and perhaps presynaptic Ca²⁺ channels (Yaari *et al.*, 1986). Therapy is usually initiated with 300 mg/day and steady-state therapeutic levels are achieved after a minimum of 7 to 10 days (Barnhart, 1997). The plasma half-life after oral administration averages 22 hours, with a range of 7 to 42 hours. The clinically effective serum level is usually between 10-20µg/ml, and levels higher than 20µg/ml induce dose-related side effects (Jack, 1992). Serum blood level determinations are necessary for optimal dosage adjustments to provide maximum benefit and in most patients stable serum levels can be achieved. Individuals with unusually low serum levels may be hypermetabolisers of PHT; unusually high serum levels may be the result of liver disease, congenital enzyme deficiency or drug interactions (Ravel, 1995). PHT is 90% bound to serum proteins (Ravel, 1995), however free PHT levels may be altered in patients with abnormal protein binding characteristics.

The pharmacokinetics of oral PHT is well documented (Woodbury, 1989). PHT is absorbed predominantly in the duodenum, where the drug ionises and becomes considerably more soluble, maximally at 100µg/ml (pH=7.8, 37°C). In addition to the favourable pH, the surface area, vascularity and blood flow are higher here than in the stomach. PHT is relatively lipid soluble (log octanol/water partition coefficient=2.23) and is readily taken up by mucosal cells, therefore dissolution in the intestinal fluid is the rate limiting step in its absorption. However, the solubility of PHT in plasma is maximum 75µg/ml (pH=7.4, 37°C), therefore absorption is also dependent on the rate at which PHT is removed from the blood stream. PHT binds rapidly and reversibly to proteins and about 90% of it is linked to albumin in plasma. Unionised PHT then freely distributes by nonionic diffusion across cell membranes and in transcellular fluids.

Studies into its kinetics using 2-¹⁴C-PHT revealed that after oral administration PHT levels/gram tissue were in the following order:

liver>salivary gland>kidney>fat>muscle>brain>plasma (Noach *et al.*, 1958).

PHT can also be administered intramuscularly, but due to its poor water solubility a depot of crystals forms, whose complete absorption may take up to 5 days (Kostenbauder *et al.*, 1975). Therefore, im. PHT acts as a repository preparation and the parenteral administration (iv.) is preferred in emergencies.

PHT is metabolised in the liver by a saturable enzyme system as it exhibits non-linear pharmacokinetics in man (Richens, 1979). About 70% of the drug is metabolised in the liver and less than 5% is excreted unchanged through the kidneys (Ravel, 1995). The oxidative metabolism of PHT is summarised in Figure 1.7.

The principal metabolic pathways involve the production of p-HPPH and DHD. Together they account for 67-88% of the administered PHT and 7-11% of urinary metabolites (Browne and Chang, 1989; Dickinson *et al.*, 1985). The first step of these metabolic pathways is, however, the formation of an unstable intermediate, an "arene oxide" or epoxide (Claesen *et al.*, 1982). This is then spontaneously converted to p-HPPH and to DHD by the enzyme epoxide hydrolase and may also bind covalently to target tissues. Such compounds are therefore potentially carcinogenic, mutagenic and have hepatotoxic effects (Jerina and Daily, 1974). Substantial evidence points to the fact that anticonvulsant medications of the Hydantoin group cause malformations, referred to as Foetal Hydantoin Syndrome (Hanson, 1986). Studies into the teratogenic effects in mice (50 and 100 mg/kg PHT) suggest that there is a direct link between arene oxide production and the incidence of cleft lip and palate and embryo-lethality. Inhibition of epoxide hydroxylase with 1,2-epoxy-3,3,3,-trichloro-propane increased disfigurement, mortality of the foetus and doubled the covalent binding to gestational tissue (Martz *et al.*, 1977). The role of the embryotoxic epoxide intermediate (Fort and Bantle, 1990) and the bioactivation of embryonic CYP (Wells *et al.*, 1997) was later confirmed.

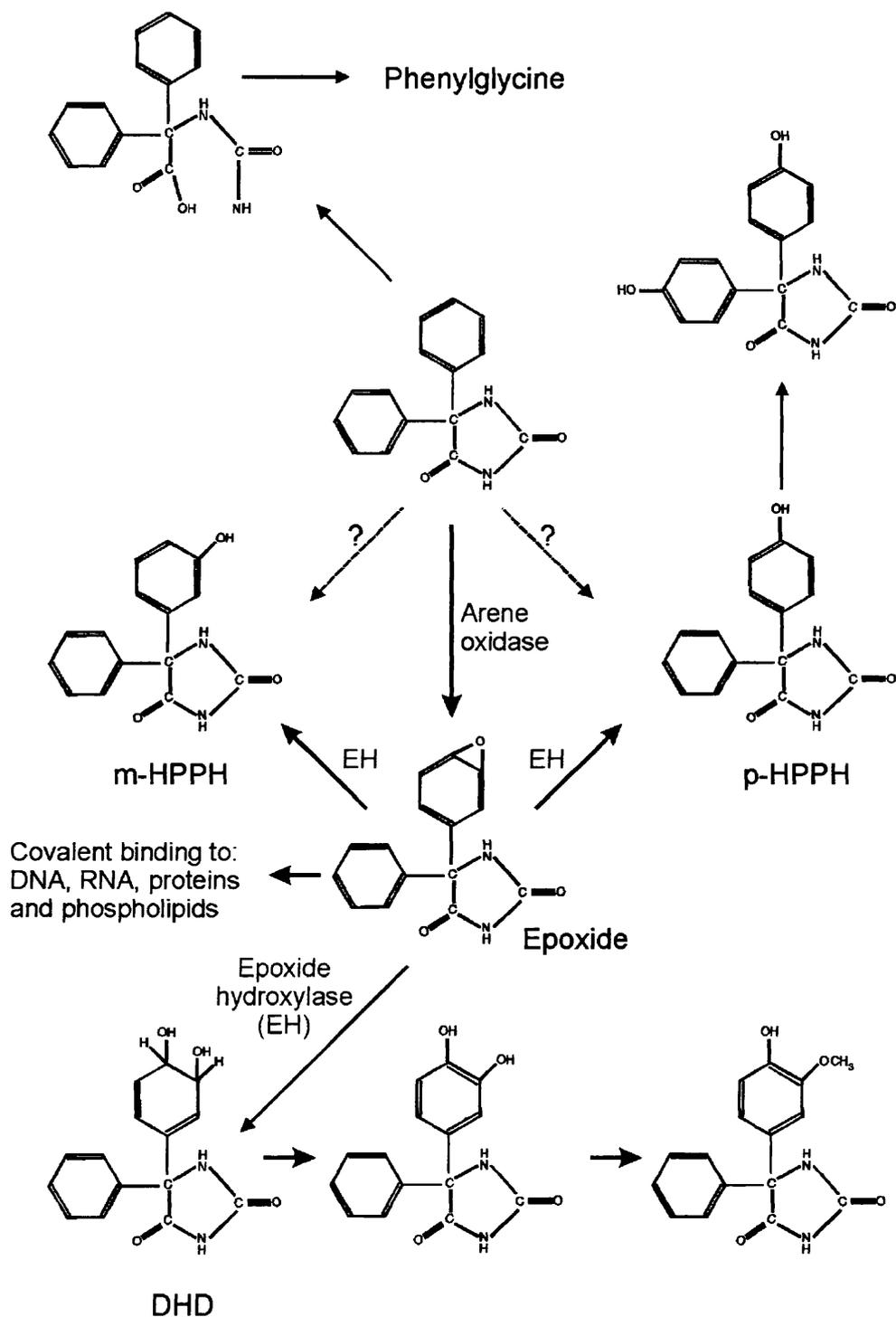


Figure 1.7. Summary of PHT metabolism in man (adapted from Browne and Chang, 1989; Blake and Martz, 1980). The major metabolic routes involve the formation of 5-(4-hydroxyphenyl)-5-phenylhydantoin (para-hydroxyphenyl-phenylhydantoin; p-HPPH), 5-(3,4-dihydroxy-1,5-cyclohexadien)-5-phenylhydantoin (dihydrodiol, DHD) and smaller amounts of 5-(3-hydroxyphenyl)-5-phenylhydantoin (meta-hydroxyphenyl-5-phenylhydantoin; m-HPPH).

To date, three members of the CYP2C sub-family (primarily CYP2C9, and to a lesser extent CYP2C18 and CYP2C19) were identified as being involved in the hydroxylation of PHT (Mamiya *et al.*, 1998a; Bajpai *et al.*, 1996; Krecic *et al.*, 1995). A small percentage of individuals treated with PHT were shown to metabolise the drug slowly (Vermeij *et al.*, 1988; Vasko *et al.*, 1979) and may go on to develop toxic serum concentrations with average doses (Glazko *et al.*, 1982; Kutt, 1982). This may be due to limited enzyme availability, lack of induction or mutations in isotypes of CYP2C (Mamiya *et al.*, 1998b). In particular, CYP2C19 [(S)-mephenytoin hydroxylase] has recently been found to exhibit genetic polymorphism, with approximately 5% of whites and 20% of Asians being poor metabolisers (Levy, 1995). In black Africans, on the other hand, Phenytoin is exclusively metabolised by CYP2C9 and CYP2C19 is associated only with mephenytoin (Horshmans *et al.*, 1996).

Because PHT is an asymmetric molecule, the carbon-5 of the hydantoin ring represents a prochiral centre. Therefore, stereoselective metabolism leads to the formation of enantiomers (Fig. 1.8). By careful isolation of the conversion products it was found that the majority of p-HPPH in man is formed through an (S)-stereospecific arene oxide step. However, a finite amount of (R)-p-HPPH and (R)-DHD was also found in urine (Maguire and McClanahan, 1986). In man the ratio of produced diastereoisomers is 3:1 (Maguire and Wilson, 1985; Maguire *et al.*, 1980). It is thought that (S)-hydroxylation by CYP2C9 is responsible for the disposition of PHT, whereas CYP2C19 metabolic polymorphism is associated with (R)-hydroxylation (Ieiri *et al.*, 1997).

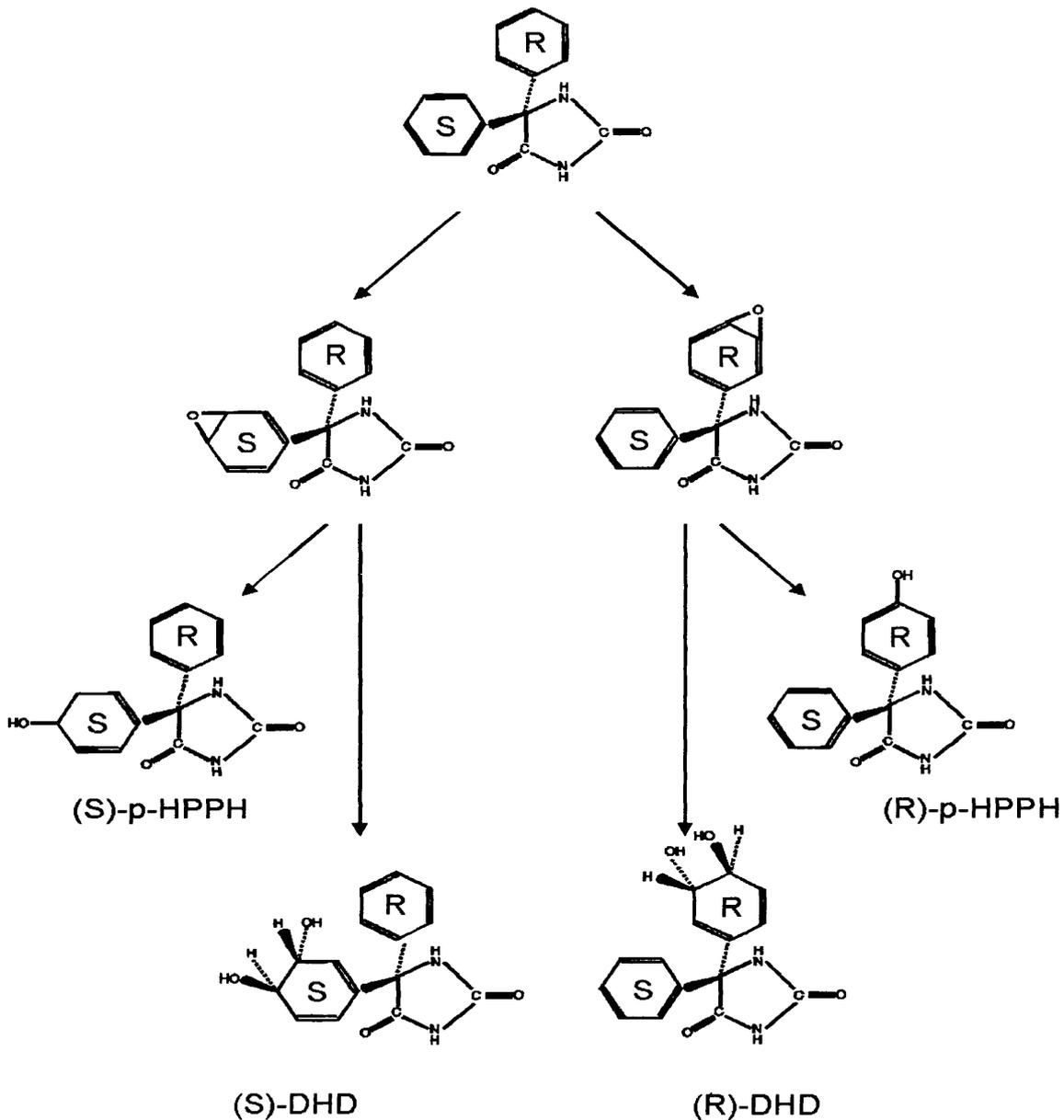


Figure 1.8. Stereoselective metabolic pathways of PHT (adapted from Maguire and McClanahan, 1986).

1.5.2. Phenytoin-induced gingival pathology

1.5.2.1. Phenytoin-induced gingival overgrowth (PIGO)

Interestingly, not all patients undergoing PHT therapy acquire gingival overgrowth and clearly many factors influence its development. The lesion is age and site specific (Fig. 1.9). It occurs mainly within the gingiva propria of young individuals (Hassell *et al.*, 1978) and is rarely seen in persons above 35-40 years of age (Aas, 1963).



Figure 1.9. Patient with PIGO (Courtesy of Prof. P. Speight, Eastman Dental Institute, London, UK). Note the enlarging interdental papillae between central and lateral incisors.

Gingival overgrowth develops within 6 months from the start of the therapy (Philstrom *et al.*, 1980) and regresses spontaneously upon termination of PHT treatment within about a year, provided that the teeth are kept free from bacterial plaque (Nuki and Cooper, 1972). Alternatively, the excess tissue may be excised, but the condition will return if PHT therapy is continued. Perlik and co-workers (Perlik *et al.*, 1995) showed a correlation between PIGO and PHT dose per unit body weight and duration of PHT administration.

Histologically, the lesion is characterised by epithelial acanthosis, with long, branched rete peg formation (Fig. 1.10). The main feature, however, is the excessive accumulation of collagen in the dermis. Gingival inflammation may also be present. A recent study by Dill and Iacopino (Dill and Iacopino, 1997) observed also the presence of myofibroblasts in human overgrown gingiva, which may play a role in its pathogenesis. Myofibroblasts are thought to be involved in the pathogenesis of fibrotic diseases such as hypertrophic scars and Dupuytren's disease but not keloids (Erlich *et al.*, 1994).

Considerable controversy persists as to whether PIGO should be classed as a hypertrophy, hyperplasia, or fibrosis. An extensive histopathological study by Hassell and co-workers (Hassell *et al.*, 1978) claimed that it merely represents uncontrolled growth with normal connective tissue composition. This implies that the lesion contains elevated number of cells, which in turn produce excessive gingival tissue. However, in line with previous investigators (Ishikawa and Glickman, 1961), the early, developing lesion may be classed as hyperplasia. The study of gingival overgrowth has been obscured by the fact that PHT-induced pathogenesis is species specific and only a limited number of animal models are available, together with the fact that not all animals metabolise PHT the same way.



Figure 1.10. High power view of H&E-stained section through severe PHT-induced gingival lesion. Keratinised oral epithelium exhibits long rete pegs penetrating into the subjacent connective tissue stroma. Massive accumulation of radiating large collagen fibres in the dermis within the gingiva propria.

1.5.2.2. Gingival metabolism of Phenytoin (local metabolism)

Although there are many proposed mechanisms put forward for PIGO, few theories explain why is the gingiva principally involved. Both periodontium (Bartold, 1995; Sempowski *et al.*, 1995) and healing wounds have been suggested as the most dynamic and metabolically active tissues in the adult human body. Perhaps this is why the effects of PHT are most prominent in these. Also, after oral administration the bioavailability of PHT is greater in gingival tissue than in normal skin or bone, hence the difference in the severity of the side effects at these locations.

Measurements of PHT and its metabolites in serum, gingiva and saliva suggested local metabolism in humans (Conard *et al.*, 1974). It is postulated that gingival tissues are exposed to PHT both from blood and saliva and additionally plaque may act as a reservoir for the drug. (Levels of unbound, therefore therapeutically active, PHT are similar in blood and saliva; Horning *et al.*, 1977; Conard *et al.*, 1974.)

Steinberg (1980) investigating the penetration of topically applied ¹⁴C-PHT in rabbit gingiva found that the gingival sulcus had a great capacity in the uptake of PHT. After two hours most of the radioactivity was found to be in the cerebral cortex and the least in serum. However, gingiva appeared to retain the label for the longest time. Interestingly, PHT also has higher affinity to gingival proteins *in vivo* than to extraoral tissues such as liver and kidneys, even though these organs are the prime sites for its metabolism and excretion (Rao and McLennon, 1977). As metabolic conversion is thought to be essential for covalent binding (Rao and Wortel, 1980), this has further instigated research into the metabolic capacity of the oral mucosa.

A breakthrough study by Zhou and co-workers in 1996, revealed that isozymes of CYP were present here in concentrations as much as fifty-fold greater than in hepatic microsomes (Zhou *et al.*, 1996), even though the number of microsomes found in oral mucosa was only about one-tenth. The activity of CYP1A1, CYP1A2, CYP2E1, CYP3A4, CYP2C9 was also measured but no CYP2B6 and CYP2D6 was detected. Microsomes obtained from gingiva showed significant PHT hydroxylase activity as determined by the production of p-HPPH (12.8-276.9pmol HPPH/min.mg microsomal protein).

leiri and co-workers (leiri *et al.*, 1995) have recently demonstrated that patients with gingival overgrowth have an unusually higher level of the (R)- rather than the (S)-enantiomer of the metabolite p-HPPH. They have also identified that *in vitro* (R)-p-HPPH, the least abundant metabolite, was responsible for increasing human dermal fibroblast growth as measured by ³H-thymidine incorporation after an 8-day incubation period. Therefore, their combined *in vivo* and *in vitro* results suggested that abnormal metabolism leading to the overproduction of (R)-p-HPPH may be responsible for gingival overgrowth. Importantly, this pioneering investigation is the first study ever to compare the pharmacologic properties of separate enantiomers, whereas all other studies used racemate of p-HPPH.

Interestingly, reduced (R)-enantiomer production can also be correlated with poor mephenytoin metabolism. As interethnic mephenytoin hydroxylation differences are well documented, leiri and co-workers have also suggested that considerable variations in PIGO may occur amongst populations of different origin. Based on this theory Caucasians are the most prone and East Asians the least.

As CYP dependent monooxygenase (Bickers *et al.*, 1985) and epoxide hydrolase (Baron *et al.*, 1983) are also documented in human skin, it appears that gingiva and skin are important extra-hepatic sites for xenobiotic metabolism. Unlike skin, however, oral mucosa is not as heavily cornified and the absorption of ingested agents is not so limited. This was confirmed *in vitro*, when the penetration of tritiated water and tritiated red tide toxin was tested in monkey buccal mucosa and skin (Mehta *et al.*, 1991).

1.5.2.3. Phenytoin and periodontal healing

The first controlled clinical trial for wound healing was carried out by Shapiro in 1958 treating periodontal patients with oral PHT prior to surgery resulting in accelerated healing of their gingiva, reduced inflammation and pain compared with control individuals (Shapiro, 1958). It was Shapiro who initially suggested that PHT might also be useful to increase the rate of wound healing in other areas of the body. Since then, further studies confirmed its beneficial effects when PHT was incorporated into gels and applied topically for the treatment of periodontal diseases (Ludewig and Otto, 1982; Otto *et al.*, 1977;

Swann *et al.*, 1975; Delaire *et al.*, 1974; Berenholc, 1972; Chikhani, 1972; Payen, 1972; Savini *et al.*, 1972) and in the promotion of healing of dental extraction sockets (Goebel, 1972). In the 1970s a topical PHT preparation (Pyoredol, Laboratoires Roussel, France) was available for use with patients suffering from periodontal disease and tissue loss. A decade later attempts have been made to evaluate the efficacy of a p-HPPH analogues in human gingival wound healing and para-chlorophenytoin (as 1% dentrifice) was claimed to accelerate the healing rate twice as fast as PHT (Savini *et al.*, 1980).

1.5.2.4. Putative mechanisms of action of Phenytoin in the pathogenesis of gingival overgrowth

The enigma of PIGO has occupied researchers for decades. Brown and co-workers (Brown *et al.*, 1991) have excellently reviewed the mechanistic details of drug-induced gingival hyperplasia recently. Briefly, the following factors have been suggested to play a key role in its induction based on this review together with recent advances:

- PHT pharmacokinetics and tissue binding,
- PHT local metabolism and metabolites (Zhou *et al.*, 1996; leiri *et al.*, 1995),
- Inflammation from bacterial plaque,
- Elevated number of Langerhans cells in gingiva (Kinane *et al.*, 1990),
- Immunoglobulin induction by PHT,
- PHT potentiates IL-1 induced prostaglandin biosynthesis by gingival fibroblasts (Modeer *et al.*, 1992ab),
- Gingival fibroblast phenotype changes,
- Presence of myofibroblasts (Dill and Iacopino, 1997),
- Perturbed collagen metabolism,
- Disruption of fibroblasts $\text{Na}^+/\text{Ca}^{2+}$ flux, increase in intracellular calcium in gingival fibroblasts,
- EGF receptor upregulation (Modeer *et al.*, 1990; Modeer and Andersson, 1990),
- 5α -dihydrotestosterone receptor upregulation,
- Inhibition of folic acid uptake,
- Combination theories (involving several of the above).

Clearly, these mechanisms are a complex cascade of events and some pathways are only relevant to gingiva. However, these studies may give clues as to how PHT may upregulate connective tissue activity in wound healing. In certain ways, the study of PHT's action on connective tissue has proven to be difficult. Contradictory results have been documented according to which PHT may either increase or decrease synthetic and proliferative activity or have no effect at all *in vitro*. The *in vivo* study of the cause of PIGO has also been obscured by the fact that PHT-induced pathogenesis is species specific and only a limited number of animal models are available. Moreover, not all animals metabolise PHT in the same way. Perhaps the production of reactive metabolites and the presence of responder cells together hold the key to the understanding of PHT's mechanism of action on connective tissue

1.5.3. Phenytoin and cutaneous wound healing

1.5.3.1. Clinical trials with Phenytoin in cutaneous wound healing

The conduction of double blind, randomised, placebo-controlled clinical trials with PHT presented many practical problems. In most cases PHT powder (of unknown quantity) was applied daily in a uniform layer over the wound area. The lack of suitable inert powder meant that the studies were not controlled or carried out in a double blind fashion. Many controlled studies, on the other hand, used agents which influenced the wound healing process and therefore cannot be regarded as true controls. Because of these difficulties the number of non-randomised, retrospective trials or anecdotal accounts are vast in number. However, the outcomes can still be compared with historical controls and a sense of efficacy still appreciated.

Favourable results were reported with topical PHT in the treatment of:

- trophic ulceration in leprosy (Bansal and Mukul, 1993; Menezes *et al.*, 1993; Malhotra and Amin, 1991; Bogaert *et al.*, 1990a),
- venous stasis ulcers (Rodriguez-Noriega *et al.*, 1983),
- decubitus ulcers (Anstead *et al.*, 1996, El-Zayat, 1989; Modaghegh *et al.*, 1989),
- diabetic foot ulcers (Muthukumarasamy *et al.*, 1991),
- ulcers of various aetiologies (Pendse *et al.*, 1993; Modaghegh *et al.*, 1989),

- large abscess cavities (Flanagan and Flanagan, 1992; Lodha *et al.*, 1991a),
- burns (Lodha, 1991b; Smith *et al.*, 1988; Mendiola-Gonzales *et al.*, 1983),
- clean surgical wounds (split-thickness skin autograft donor sites, Yadav *et al.*, 1993),
- Epidermolysis Bullosa (EB; Masgrau-Peya *et al.*, 1995).

1.5.3.2. Advantages of topical Phenytoin therapy

For the treatment of localised injuries, topical application gives direct access to the target site without undergoing classical metabolic pathways and there is a lower risk of causing systemic dose-related side effects (Cheng and Staple, 1972). Oral PHT has nevertheless been tested in diseases, which can involve the entire integument (EB, lichen planus, and discoid lupus erythematosus), with variable success (Rodriguez-Castellanos *et al.*, 1995; Caldwell-Brown *et al.*, 1992; Bogaert and Sanchez 1990b). These open trials should be interpreted with great caution as these conditions undergo variable disease progressions, and it is possible that the levels of PHT reaching the skin are below therapeutic levels for wound healing.

In vivo and clinical studies with topical PHT claim:

- Acceleration in healing and granulation tissue formation (Bansal and Mukul, 1993; Menezes *et al.*, 1993; Pendse *et al.*, 1993; Yadav, 1993; Flanagan and Flanagan, 1992; Lodha *et al.*, 1991ab; Malhotra and Amin, 1991; Muthukumarasamy, 1991; Bogaert *et al.*, 1990ab; El-Zayat, 1989; Modaghegh *et al.*, 1989; Smith *et al.*, 1988; Mendiola-Gonzales, 1983; Rodriguez-Noriega *et al.*, 1983).
- Reduction in oedema, inflammation, wound transudate and exudate (Flanagan and Flanagan, 1992; Lodha *et al.*, 1991ab; Malhotra and Amin, 1991; El-Zayat, 1989; Modaghegh *et al.*, 1989; Smith *et al.*, 1988).
- PHT decreased the bacterial load of wounds, therefore the need for antibiotic therapy. It was effective against *Staphylococcus Aureus*, *Escherichia Coli*, *Klebsiella spp.*, *Pseudomonas spp.* (coagulase positive) in clinical studies within 7-10 days. (Pendse *et al.*, 1993; Muthukumarasamy, 1991; El-Zayat, 1989; Modaghegh *et al.*, 1989). In a guinea pig model of

wound healing (Lodha *et al.*, 1991a) PHT cleared gram-negative bacteria more readily than gram-positive bacteria.

- Possible facilitation of nerve regeneration (Modaghegh *et al.*, 1989).
- Provision of rapid pain relief (Yadav, 1993; Lodha, 1991b; El-Zayat, 1989; Smith *et al.*, 1988; Mendiola-Gonzales, 1983; Rodriguez-Noriega *et al.*, 1983).
- High success rate in difficult, chronic cases unresponsive to traditional therapies.
- Safety: no adverse reactions were reported.
- Low cost and availability as opposed to expensive alternatives, such as a mixture of synthetic growth factors (Pierce and Mustoe, 1995; Bolton and Fattu, 1994; Knighton *et al.*, 1990).

1.5.3.3. *Phenytoin and Epidermolysis Bullosa*

EB is a collective term applied to a heterogeneous group of inherited diseases which are characterised by cutaneous and mucosal blister formation after minor trauma (Bruckner-Tuderman, 1993). According to the precise level of blister formation and clinical manifestations, three major varieties (Gedde-Dahl, 1971) are presented in Table 1.1. However, to date more than 23 subtypes have been reported.

The main discriminants in the precise diagnosis are clinical features, mode of inheritance together with ultrastructural (Electron Microscopy) and immunopathological investigations. Antibody probes have also been developed to confer some information about genetic pattern (Table 1.2) and diagnosis.

The type of EB investigated in this thesis, Recessive Dystrophic EB (RDEB; Hallopeau-Siemens variant) is a particularly debilitating condition, because cleavage occurs below the epidermal-dermal basement membrane and healing proceeds with atrophic scar formation (Lin *et al.*, 1993). This most severe, mutilating form of RDEB (Briggamann, 1992), is characterised by generalised mucocutaneous blistering, widespread scarring, flexural contractures and pseudo-webbing of the digits (Fig. 1.11).

Disease Group	Inheritance	Blister cleavage
EB Simplex	Autosomal dominant	Intraepidermal with clumped tonofilaments in EB herpetiformis
Junctional EB	Autosomal recessive	Intralaminal lucida with hypoplastic, reduced or absent hemidesmosomes and absent anchoring filaments in the "lethal" form
Dystrophic EB	Autosomal dominant, autosomal recessive	Sublamina densa with reduced anchoring fibrils in dominant Dystrophic EB and reduced or absent anchoring fibrils in recessive Dystrophic EB

Table 1.1. Classification of EB (from Bauer *et al.*, 1992).

Type of EB	Candidate genes
Simplex	Keratins and other cytoskeletal proteins, linking proteins, cell-cell adhesion molecules, β -1 integrins
Junctional	Hemidesmosomal proteins, α -6 / β -4 integrin, anchoring filament proteins (nicein, kalinin, epiligrin)
Dystrophic	Anchoring fibril proteins (type VII collagen), enzymes that degrade BMZ structural proteins (type I collagenase, stromelysin, type IV collagenase)

Table 1.2. Candidate genes in various forms of EB (from Bauer *et al.*, 1992).



Figure 1.11. Patients with RDEB. (Photographs reproduced from Champion *et al.*, 1998 and Callen *et al.*, 1993). Note that bullae are present at birth or appear in infancy (A&B). Repeated blistering with progressive scarring results in the fusion of adjacent digits. With time the digits then undergo progressive contractures and become encased in a cocoon of thin scar tissue (C) requiring surgical restoration (Greider and Flatt, 1988).

Although the exact pathogenesis of RDEB is not certain, there is evidence for two possible mechanisms:

- a.) defective or absent type VII collagen,
- b.) excessive matrix metalloproteinase activity.

The former is currently thought to be of greater importance.

Evidence from electron microscopy of both mechanobullous and non-traumatised RDEB skin showed weak or absent anchoring fibrils (Tidman and Eady, 1985; Briggamann and Wheeler, 1975). Immunostaining with antiserum to type VII collagen (Heagerty *et al.* 1986), the major component of anchoring fibrils (Sakai *et al.*, 1986), in some patients was negative (Bruckner-Tuderman *et al.*, 1989, 1988), while in the positive cases the staining pattern indicated its degradation or defective aggregation (Rushenko *et al.*, 1989). In line with these findings, a genetic link has been established between the collagen VII gene (COL7A1) mutations and RDEB (Hovnanian *et al.*, 1992; Uitto and Christiano, 1992) and this can be used as a prenatal diagnostic marker (Hovnanian *et al.*, 1995). However, incubation of healthy human skin culture with blister fluid from RDEB patients caused a subepidermal blister histologically similar to that of RDEB (Takamori *et al.*, 1985), indicating that excessive proteolytic degradation (Takamori *et al.*, 1983) could be responsible for blister formation. This has led to the suggestion that blistering is mediated by elevated collagenolytic activity through a number of enzyme activation pathways involving stromelysin (Unemori *et al.*, 1994; Sugawara *et al.*, 1993; Sawamura *et al.*, 1991), collagenase (Unemori *et al.*, 1994; Takamori *et al.*, 1983), or an altered form of collagenase (Bauer and Tabas, 1988; Bauer, 1982, Bauer *et al.*, 1977; Stricklin *et al.*, 1982). Furthermore it has been shown that interstitial collagenase and gelatinase are capable of cleaving type VII collagen (Seltzer *et al.*, 1989) *in vitro*, supporting the link between increased enzyme levels and the clinically observed diminution of anchoring fibrils.

Systemic PHT has also been tested for the treatment of patients having various forms of EB in numerous open trials (Table 1.3), based on its ability to inhibit collagenase (Bauer and Tabas, 1988; Fine, 1986; Moy *et al.*, 1985; Bauer and Cooper, 1981; Eisenberg *et al.*, 1978). It has been previously suggested that Dystrophic EB patients have a manifold increase in the collagenase activity in their skin (Eisenberg *et al.*, 1974; Eisen, 1969).

However, Wineberg and Gedde-Dahl (Wineberg and Gedde-Dahl; 1989) have reported that elevated collagenase production is not a marker for the entire disease, thus providing an explanation for non-response. Preliminary studies indicated that increased collagenolytic activity was also present in Junctional EB (Bauer *et al.*, 1977). Therapeutic trials with systemic PHT improved blistering in infants with generalised Junctional EB (Fine and Johnson, 1988), but the course of the disease still remained lethal (Rogers *et al.* 1983; Bergfeld and Orlowski, 1982). Incongruously, proteolytic enzyme activity was found to be dramatically reduced in some individuals with EB simplex, localised and generalised forms (Sanchez *et al.*, 1983), but a later study confirmed molecular heterogeneity amongst the affected individuals (Wineberg and Gedde-Dahl; 1986; Takamori *et al.*, 1983).

Type of EB	Response (as measured by reduced blistering)	Reference
Simplex	Beneficial*	Masgrau-Peya <i>et al.</i> , 1995
Simplex (Herpetiformis of Dowling-Meara)	Beneficial	Larregue <i>et al.</i> , 1990
Simplex (Letalis)	Beneficial	Bergfeld and Orlowski, 1982
Junctional	No effect (in Herlitz variant) Beneficial (in generalised atrophic benign EB)	Fine and Johnson, 1988
Junctional	Beneficial	Armoni <i>et al.</i> , 1985
Junctional	No effect	Oakley <i>et al.</i> , 1984
Junctional (Cicatricans)	Beneficial	Schnyder, 1983

Table 1.4. Clinical trials and case studies with oral PHT for the treatment of EB.

[*Topical PHT cream (exception).]

Type of EB	Response (as measured by reduced blistering)	Reference
Junctional	Beneficial	Rogers <i>et al.</i> , 1983
Junctional	Beneficial	Guill <i>et al.</i> , 1983
Recessive Dystrophic	Beneficial	Abahussein <i>et al.</i> , 1993
Recessive Dystrophic	Beneficial **	Abahussein <i>et al.</i> , 1992
Recessive Dystrophic	No effect***	Caldwell-Brown <i>et al.</i> , 1992
Recessive Dystrophic (inverse form)	Beneficial	Altomare <i>et al.</i> , 1990
Recessive Dystrophic	Beneficial	Yen <i>et al.</i> , 1990
Recessive Dystrophic	Beneficial	Seitz <i>et al.</i> , 1990
Dystrophic	Beneficial	Kern <i>et al.</i> , 1989
Recessive Dystrophic	No effect	Keefe <i>et al.</i> , 1988
Recessive Dystrophic (Inverse form)	Beneficial	Pearson <i>et al.</i> , 1988
Recessive Dystrophic (Vegetans form)	Beneficial	Pini <i>et al.</i> , 1986
Dystrophic	Beneficial	Weismann, 1985
Recessive Dystrophic	Beneficial	Weidauer <i>et al.</i> , 1985
Recessive Dystrophic	Beneficial	Kaluza <i>et al.</i> , 1985
Recessive Dystrophic	Beneficial	Kero <i>et al.</i> , 1984
Recessive Dystrophic	Beneficial	Feurle <i>et al.</i> , 1984
Recessive Dystrophic	Beneficial	Armoni <i>et al.</i> , 1984
Recessive Dystrophic	Beneficial	Cooper and Bauer, 1984

Table 1.4. continued. (**Controlled trial, ***Randomised, double-blind, placebo-controlled, crossover trial.)

Type of EB	Response (as measured by reduced blistering)	Reference
Recessive Dystrophic (Hallopeau-Siemens and Invers forms)	Beneficial	Wirth <i>et al.</i> , 1983
Recessive Dystrophic (Partim Inversa)	Beneficial	Bandmann and Perwein, 1982
Recessive Dystrophic	Beneficial	Bauer <i>et al.</i> , 1980
Dystrophic	Beneficial	Eisenberg <i>et al.</i> , 1978
EB Acquisita	No effect	Crow <i>et al.</i> , 1988

Table 1.4. continued.

The rationale behind systemic PHT therapy remains unclear, as collagenase is no longer believed to play a role in the etiopathogenesis of EB. A recent encouraging study by Masgrau-Peya *et al.* (Masgrau-Peya *et al.*, 1995) using a topical preparation may indicate that any beneficial effects, which aid wound healing, are exerted locally.

1.6. Aims and objectives of the thesis

The ultimate aim of any drug discovery process is to find agents that are suitable for use in humans. Under ideal circumstances the following objectives are achieved:

Firstly, a therapeutic rationale is drawn up with a particular mechanism of action in view and a clear target/cell/receptor for the agent. Secondly, the presence or involvement of the target/cell/receptor is proven in the clinical condition. Thirdly, model test systems are identified and used to indicate the drug's potential efficacy in humans.

In the case of PHT, this process of drug discovery was reversed. PHT already manifested its effect on the connective tissues of humans, as a side effect of anticonvulsant therapy. PHT was rapidly tested for wound healing

purposes. Despite its effects being known as long ago as the 1950's and the subsequent research that has followed, PHT remains till this present day an unlicensed drug for wound healing. Moreover, preclinical safety evaluations (safety and efficacy) had been bypassed for wound healing therapy. Therefore, it was the aims of this thesis to examine the role of PHT from a wound pharmacological point of view establishing a retrospective therapeutic rationale. Having identified its mechanism of action, the second aim was to select novel compounds structurally related to PHT with potentially improved safety and efficacy profiles.

1.6.1. Primary aim: identification of the mechanism of action of Phenytoin

Review of the literature of PIGO and clinical trials of PHT in wound healing has indicated some clues as to the stimulatory action of PHT on connective tissue. In order to rationalise its therapeutic use during the different stages of wound healing, the objectives were to find relevant bioassays reflecting the various aspects of wound healing.

For this purpose, the following models had been investigated:

- Proliferation assays were used to test whether low concentrations of PHT were stimulatory and higher concentrations to establish its toxicity.
- Migration assays were used to investigate its effect on cell locomotion.
- Effects on cell contraction were observed using two model systems, the traditional Fibroblast Populated Collagen Gels, and a novel device called the Culture Force Monitor.
- Immunocytochemistry was carried out to observe any cytoskeletal changes in normal fibroblasts treated with PHT.
- PHT (in various formulations) was also tested *in vivo* in a porcine full thickness wound model, because of its similarity to human skin (Monteiro-Riviere, 1986; Meyer et al., 1978).

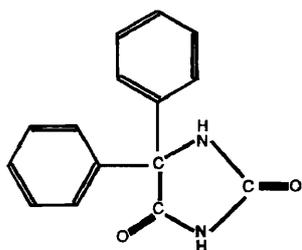
Having developed the relevant assay systems, the next objective was to hypothesise about the fate of topical PHT in wounds, analyse its wound pharmacology, and suggest formulations for its successful delivery to wounds.

1.6.2. Secondary aim: identification of a new vulnerary agent related to Phenytoin

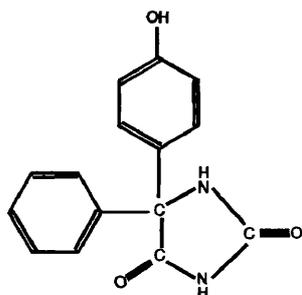
Once the relevant bioassays were identified for PHT activity, metabolites of PHT (Fig. 1.12) and other compounds structurally related to Hydantoin (Fig. 1.13) were tested for their ability to alter fibroblast migration, fibroblast-mediated gel contraction and for their effects on the cytoskeleton. Additionally, the effects of known cytoskeletal poisons were also examined for comparison (Fig. 1.14.).

1.6.2.1. Background – Metabolites of Phenytoin used in this thesis

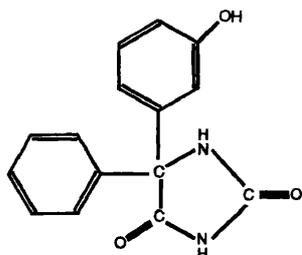
Two commercially available metabolites of PHT were analysed for their activities (Fig. 1.12). Although both compounds were the products of hydroxylation [See section 1.5.1. 'Background - Clinical pharmacology (after systemic administration)], they differed in the position of their substituents and were both racemic mixtures. A recent report by Ieiri and co-workers particularly implicated the role of (R)-p-HPPH in the pathogenesis of PIGO and it was also reported to be mitotic to human dermal fibroblasts (Ieiri *et al.*, 1995). Contrary to this, Modeer and co-workers found that cell number and rate of DNA synthesis of gingival fibroblasts were not affected by racemate of p-HPPH (Modeer *et al.*, 1982). To date, no information is available on the effects of m-HPPH on either gingival or dermal cells. Both p-HPPH and m-HPPH were however much less developmentally toxic than PHT (Fort and Bantle, 1990).



Phenytoin,
PHT



5-(4-hydroxyphenyl)-5-phenylhydantoin,
p-HPPH



5-(3-hydroxyphenyl)-5-phenylhydantoin,
m-HPPH

Figure 1.12. The structure of PHT and its metabolites investigated in this thesis.

1.6.2.2. Background – Hydantoin derivatives used in this thesis

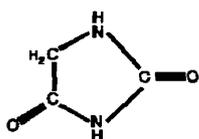
Historically, in seeking an explanation for PHT-induced chronic side effects, it was attempted to evaluate the action of PHT and related compounds *in vitro* on gingival fibroblast proliferation (Shafer, 1961a). Later on, further compounds were investigated *in vivo* for their ability to alter dermal composition (Nakamura and Masura, 1965; Houck, 1963) and to increase the tensile strength of rat experimental wounds (Kelln and Gorlin, 1960). All these studies have reported conflicting results, and no structure-activity relationship could be drawn up to identify the active component of the PHT molecule which is responsible for either the pathogenesis of PIGO or the enhancement of wound healing.

In this thesis, compounds were systematically investigated using *in vitro* in assay systems, which are considered to be relevant to wound healing. Firstly, HYD, which represents the basic structure of PHT without substituents, together with HYDAC (a compound resulting from the ring opening of HYD), were investigated. Although a previous report by Shafer reported that HYD had no effect on the proliferation of fibroblasts derived from a patient with fibromatosis gingivae (Shafer, 1961a) it was reported to be able to induce gingival hyperplasia (Bonnaure-Malet *et al.*, 1995). Secondly, the importance of the phenyl rings in the structure of PHT was determined by testing compounds with various substituents at the 5 position [ALLAN, HYDACAC, Me-PH and (MePh)-PH; Fig. 1.13].

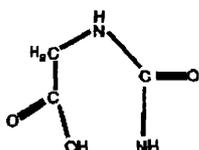
ALLAN is a naturally occurring substance present in comfrey, and has been reported to have had medicinal uses for wound healing as early as 200 A.D (reviewed by Mecca, 1955). However, the presence of ALLAN is not restricted to plants (such as wheat germ, rice polishings, sycamore tree leaves, and the bark of horse chestnut tree) but is also found in human urine and foetal allantoic fluid. Although in recent years the effectiveness of maggot therapy was attributed to their ability to cleanse necrotic tissue (Stoddard *et al.*, 1995), Robinson in the 1930s identified ALLAN crystals in maggot excrement (Robinson, 1942, 1935), which may also be a contributory factor in aiding chronic wound healing. Since then, synthetic ALLAN has been incorporated into a variety of skincare items and toiletries (Mecca, 1963). These currently include products from Boots, Tesco, Sainsbury's, Safeway and other High Street shops' own brands (personal correspondence with Mr. P.E. Glodkowski, 1999). However, the concentration of ALLAN is so low (1-2%) in these products that therapeutic effects for wound healing are debatable. In clinical studies ALLAN has been reported to possess soothing abilities, stimulate granulation tissue formation and chemically debride necrotic tissue and facilitate re-epithelialisation (Mecca, 1978; Bartolet, 1970; Robinson, 1935). It has been tested for the treatment of various skin conditions and surgical wounds in combination with other agents (Willital and Heine, 1994; Harrington, 1989; Kloucek-Popova *et al.*, 1982), leg ulcers (Margraf and Covey, 1977), burns

(Sheker *et al.*, 1972) and chronic wounds of various aetiologies (Fischer, 1969; Dorre, 1966; Hariri, 1966).

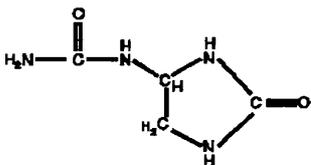
No information is currently available on the activities of HYDACAC and (MePh)-PH on gingival or dermal fibroblasts. Me-PH, on the other hand, was reported to increase proliferation of gingival fibroblasts derived from a patient with fibromatosis gingivae (Shafer, 1961a).



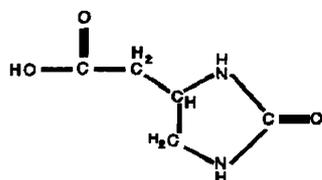
Hydantoin,
HYD



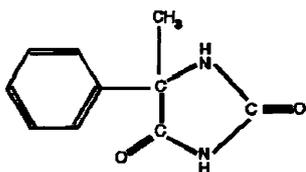
Hydantoic acid,
HYDAC



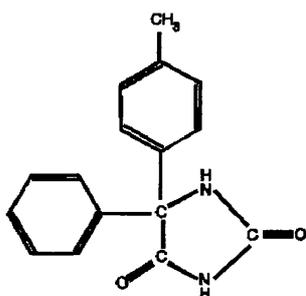
Allantoin,
ALLAN



5-Hydantoinacetic acid,
HYDACAC



5-Methyl-5-phenylhydantoin,
Me-PH



5-(4-Methylphenyl)-5-phenylhydantoin,
(MePh)-PH

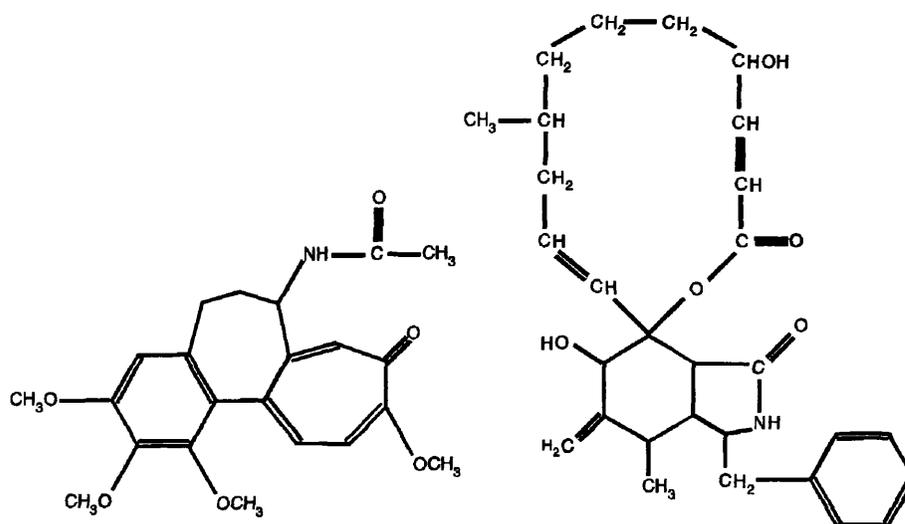
Figure 1.13. Other HYD-related compounds investigated in this thesis.

1.6.2.3. Background – Cytoskeletal poisons used in this thesis

Colchicine (Fig. 1.14), an alkaloid extracted from the meadow saffron, was used to treat gout by the ancient Egyptians (reviewed by Alberts *et al.*, 1994). Colchicine exerts its effects by binding to tubulin heterodimers, thus preventing microtubule polymerisation (Hastie, 1991; Wilson *et al.*, 1974). However, Colchicine does not directly cause the disassembly of microtubules. Blockage of tubulin polymerisation results in a net loss of microtubules and the accumulation of free tubulin. As tubulin synthesis is autoregulated, the latter will lead to the inhibition of tubulin synthesis. In accordance with this, cytoskeletal staining of fibroblasts grown on glass slides confirmed their disruption at the concentrations used in this thesis.

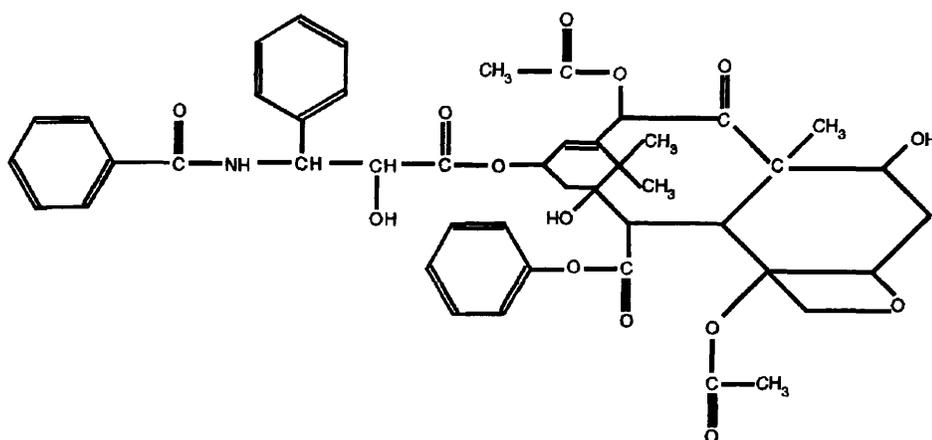
Taxol (Fig. 1.14), the second drug studied which influences the cytoskeleton, is an extract obtained from the bark of yew trees and is potent anti-cancer drug (reviewed by Alberts *et al.*, 1998). Although Taxol also arrests dividing cells in mitosis, it has the opposite effect to Colchicine. It binds and stabilises microtubules and causes all the free tubulin to assemble into microtubules (Derry *et al.*, 1997).

Cytochalasin B (Fig. 1.14) belongs to a group of fungal products (reviewed by Alberts *et al.*, 1998). These compounds have been reported to depolymerise actin filament bundles by binding to the plus end, which results in a rounder cell shape. In cultured fibroblasts the actin filaments appear in stress fibres, which play an important role in cell motility and attachment. Cytochalasins therefore also hinder cell movement by inhibiting the formation of the cell-surface membrane projections necessary for locomotion (Tomasek and Hay, 1984).



Colchicine, COL

Cytochalasin B, CYTO



Taxol, TAX

Figure 1.14. The structure of cytoskeletal poisons used in this thesis.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials used and their sources

The sources of materials (listed in alphabetical order) used in this thesis are as follows:

Agar Scientific Ltd., Stanstead, UK: gluteraldehyde.

Biogenesis Ltd., Poole, UK: mouse anti-human urokinase-type plasminogen activator (7441-0634); rabbit anti-PHT antibody (7339-9955).

First Link, West Midlands, UK: collagen (type I, rat tail); dispase; epidermal growth factor; foetal calf serum; Hydrocortisone.

Gibco/BRL Life Technologies, Paisley, UK: Amphotericine B; Dulbecco's Modified Eagle's Medium (x1 and x10); Ham's F12; Leibovitz-15; L-glutamine; Streptomycin/Penicillin.

Merck Ltd., Lutterworth, Leicestreshire, UK: acetone; Aquamount; ethanol; Formaldehyde; Harris' Haematoxylin; methanol; sodium hydroxide; Toluidine blue.

Oxoid Ltd., Basingstoke, Hampshire, UK: phosphate-buffered saline tablets.

Sigma Chemicals, Dorset, UK: Adenine; bovine serum albumin; Cholera toxin; dimethyl sulfoxide; goat anti-mouse FITC-conjugated antibody (F5262); goat anti-mouse TRITC-conjugated antibody (T6653); goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (F9887); Hoechst-3325; Keratinocyte Medium Kit (KMK-2); methyl umbelliferyl heptanoate; mouse anti-human β -tubulin antibody (T4026); mouse anti-human vimentin antibody (V5255); mouse anti-human vinculin antibody (V9131); Neutral Red; Phalloidin-tetramethylrhodamine B isothiocyanate (TRITC)-labelled (P1951); propidium iodide; propylene glycol; sodium ascorbate; sodium bicarbonate; Transferrin; Tri-iodothyronine; trypsin/ethyldiaminetetraacetic acid (x10).

University of Kent at Canterbury, Canterbury, UK: Citifluor.

All tissue culture plasticware was purchased from Bibby Sterilin, Stone, Staffordshire, UK. Multiwell spot-slides were bought from C.A. Hendley (Essex) Ltd., Loughton, Essex, UK.

2.2. Drugs used and their formulations

The sources of drugs (in alphabetical order) used in this thesis are as follows:

Aldrich Chemicals, Dorset, UK.

(+/-) 5-(4-hydroxyphenyl)-5-phenylhydantoin;

(+/-) 5-(3-hydroxyphenyl)-5-phenylhydantoin; (+/-) 5-methyl-5-phenylhydantoin;

(+/-) 5-(4-methylphenyl)-5-phenylhydantoin and hydantoin acetic acid.

Laboratorios Rubio, Barcelona, Spain:

phenytoin* (Fenitoin; 5,5-diphenylhydantoin, sodium salt).

Sigma Chemicals, Dorset, UK:

allantoin, colchicine, cytochalasin B, hydantoin, hydantoic acid, phenytoin* (5,5-diphenylhydantoin, sodium salt), Taxol.

*Source indicated in text.

PHT was either dissolved in a special 'vehicle' or dimethylsulphoxide (DMSO) at 10mg/ml concentrations as indicated. The specialised 'vehicle' solution consisted of 1:1:1 volume/volume ratio of ethanol, propylene glycol, and 0.01% sodium hydroxide. If required, this 'vehicle' stock solution was then further diluted ten times on the day of the experiment and added to the final media in the necessary amounts. In experiments, when PHT was compared with other HYD derivatives (Fig. 1.12-13) or cytoskeletal poisons (Fig. 1.14), all compounds were dissolved in DMSO at 10mg/ml concentrations and further diluted with media as required.

2.3. Methods (*in vitro* studies)

2.3.1. Sources of cells

Normal dermal fibroblasts (Table 2.1) were grown from redundant skin obtained from routine operations carried out at UCL-Middlesex, St. George's and Queen Mary University Hospitals, London.

Cell line number	Patient's initials	Age (years)	Sex	Type of operation
nf-1	EH	23	Female	Mammoplasty
nf-2	LB	15	Female	Mammoplasty
nf-3	JQ	45	Female	Mammoplasty
nf-4	SR	36	Female	Abdomino-plasty
nf-5	DV	28	Female	Mammoplasty
nf-6	SJ	35	Female	Mammoplasty
nf-7	PN	20	Female	Mammoplasty
nf-8	JW	38	Female	Mammoplasty
nf-9	NN	26	Female	Abdomino-plasty
nf-10	WS	28	Female	Mammoplasty
nf-11	WM	31	Female	Mammoplasty
nf-12	DP	23	Female	Mammoplasty

Table 2.1. Details of patients used to obtain normal dermal fibroblasts.

EB fibroblasts (Table 2.2) were cultured from patients diagnosed with Recessive Dystrophic EB skin (Hallopeau-Siemens variant) at Great Ormond Street and St. Thomas' Hospitals, London. Cells were propagated from redundant donor skin fragments (taken from the thigh) used for the correction of mitten deformity of the hands.

Cell line number	Patient's initials	Age (years)	Sex	Type of operation
ebf-1	DS	13	Male	Release of mitten deformity of the hand
ebf-2	OR	14	Female	As above
ebf-3	TE	8	Female	As above
ebf-4	HI	16	Male	As above
ebf-5	DB	6	Female	As above

Table 2.2. Details of patients used to obtain EB fibroblasts.

Normal keratinocytes (Table 2.3) were grown from redundant skin obtained from routine operations carried out at UCL-Middlesex, St. George's and Queen Mary University Hospitals, London.

Cell line number	Patient's initials	Age (years)	Sex	Type of operation
k-1	SN	41	Male	Gynaecomasty
k-2	BL	17	Female	Mammoplasty
k-3	DV	28	Female	Mammoplasty
k-4	MZ	61	Female	Autografting for burns*

Table 2.3. Details of patients used to obtain normal keratinocytes.

(*Donor skin was obtained from the thighs.)

2.3.2. Preparation of fibroblast cultures

Human, dermal fibroblasts were cultured from explants, as described previously (Burt and McGrouther, 1994). 2-4mm cubes of normal or EB skin were plated into 25cm² culture flasks, with 5ml of complete Dulbecco's Modified Eagle's Medium (DMEM) and fibroblasts were grown in monolayer cultures in the presence of 5% CO₂ at 37°C. Complete DMEM consisted of DMEM supplemented with 10% foetal calf serum (FCS), Streptomycin/Penicillin (500 units/ml Penicillin, 500µg/ml Streptomycin), L-glutamine (2µM/ml) and sodium ascorbate (50µg/ml). Fibroblasts were routinely fed twice a week and passaged (1:3) when confluent. Fibroblasts were used up to passage 12. However, in drug studies, cells of the same passage number were always compared.

2.3.3. Preparation of keratinocyte cultures

Keratinocytes were grown without the presence of a feeder layer and with the help of specialised media (Green *et al.*, 1979). Firstly, full thickness skin was washed in phosphate-buffered saline (PBS), and then the fat was scraped off. It was further scored with a scalpel at about 1cm intervals. The epidermis was separated from the dermis enzymically, using dispase (0.2% w/v) in Leibovitz-15 medium (1:1) overnight in a refrigerator. The epidermis was stripped off with forceps and washed with PBS. Then the cells were harvested by placing the strips in trypsin/ethyldiaminetetraacetic acid (2.5mg/ml in PBS) in an incubator at 37°C for 10 minutes with slight agitation. The resulting cell suspension was diluted with equal amounts of complete DMEM (10% FCS) and centrifuged at 1000 rpm for 10 minutes. The cell pellet was resuspended in keratinocyte primary (KP) growth medium and the viable cells, stained with Neutral Red (3.3mg/ml in PBS), were counted on a haemocytometer. Keratinocytes were then inoculated into 75cm³ culture flasks at 4-5x10⁶ cell density in 10ml of KP media and gassed with 10% CO₂. KP media consisted of DMEM/Ham's F12 mixture medium supplemented with 10% FCS, Streptomycin/Penicillin (1%), L-glutamine (2µM/ml), Transferrin (5µg/ml), Tri-iodothyronine (6ng/ml), Adenine (50ng/ml), Insulin (5µg/ml), Hydrocortisone (0.4 µg/ml), Cholera toxin (8ng/ml), EGF 10ng/ml).

After 24 hours the cells were cultured in a media made up from Sigma Keratinocyte Medium Kit (KMK-2). The cells were grown to subconfluency and used at passage 1.

2.3.4. Proliferation studies

Cell proliferation was measured using a fluorimetric method (Stadler *et al.*, 1995; Dotsika and Sanderson, 1987). This highly sensitive method measured cell viability based on their ability to hydrolyse a fluorogenic substrate (4-methyl umbelliferyl heptanoate) by cell esterases.

96 well plates were seeded with 10^4 cells/well and the cells were allowed to attach for 24 hours in complete DMEM (10% FCS), in a humidified incubator at 37°C with 5% CO₂. They were then washed three times with PBS and incubated with serum-free DMEM for a further 36 hours to arrest cell division (Khan *et al.*, 1998). Cells were then incubated with complete DMEM (2% FCS) containing PHT (Laboratorios Rubio) in 'vehicle' (final concentration 5-100µg/ml; 12 wells/dose). For keratinocyte growth studies, the serum deprivation step was omitted and the plates were incubated with PHT in DMEM containing 2% FCS immediately after 24 hours. In a separate experiment the effect of 10µl/ml 'vehicle' (equivalent to the amount found in the media containing 100µg/ml PHT) was tested on all three cell types (nf-2, ebf-2, k-2) after 72 and 120 hours.

Cell number measurements were made at 72 and 120 hours post dosing. Also, the medium was changed at 72 hours for the 120 hour test plates. At each time point the plates were washed three times with PBS and incubated with MUH (100µl/well) for one hour at 37°C, in 5% CO₂. MUH was stored as a stock solution (10mg/ml in DMSO) at -20°C and diluted 30-fold immediately before use in PBS. Fluorescence was determined as absolute fluorescence units (LS50B Luminescence Spectrometer, Perkin Elmer, Beaconsfield, Buckinghamshire) at 368nm excitation and at 446nm emission. Readings were then converted to % of control fluorescence (control set as 100%). Statistical analysis was carried out using Minitab v.11 software (Minitab, State College, PA, USA).

2.3.5. Cell migration assays

2.3.5.1. Chemotaxis assays

Migration assays were performed using a 48 well Micro Chemotaxis Chamber (Neuro Probe Inc. Cabin John, Maryland, USA) adapting the method of Peacock and co-workers (1992). Briefly, the Chamber consists of two compartments separated by a filter and migration was measured as the number of nuclei that pass through the membrane (12µm pore size; Nuclepore, Oxshott, Surrey). Polycarbonate membranes, were collagen-coated soaking in type I, rat tail collagen (1mg/ml) for 48 hours, then washed for 2 hours in distilled water and air dried. Cells to be assayed were 70-80% confluent in monolayers and were fed 24 hours prior to the experiment to achieve optimum mobility.

The Chamber was assembled containing the test material in serum-free DMEM in the lower compartments (27µl/well) with a collagen-coated membrane on top, followed by a silicone gasket and the upper compartment containing the cell suspension in serum-free DMEM (50µl/well; 15×10^{-3} cell/well).

The Chamber was then incubated at 37°C in 5% CO₂ for 6 hours. After the migration period, the membrane was recovered, and mechanically scraped, gently washed three times with PBS on the topside to remove unmigrated cells. The membrane was then immersed in ice-cold methanol for 5 minutes and stained with Harris' Haemotoxylin and mounted with Aquamount. The migrated cells were counted under oil immersion at 100x magnification and the sum of 10 random fields per well was taken. Serum-free DMEM was used as the negative control and human plasma FN (Cotton and Brown, 1985; 2.5µg/ml, The Bio-Products Laboratory, Elstree, Hertfordshire, UK) as the positive control.

The activity of laboratory-produced FN (5-50µg/ml) was also compared with that from a commercially available source (Sigma Chemicals, Dorset, UK). Phenytoin (Laboratorios Rubio) concentrations ranging between 5-50µg/ml in serum-free DMEM were tested for each cell line. Each chemoattractant was tested in six identical wells and standardised as percentage of the positive control, FN (taken as 100 %). The activity of the 'vehicle' (5µl/ml) alone was also tested in one cell line from each group of cells (nf-1, ebf-2, k-2).

2.3.5.2. Checkerboard analysis

In order to test whether the observed movement of the fibroblasts (nf-4) was due to chemotaxis or chemokinesis, a “checkerboard” analysis was performed (Peacock *et al.*, 1992). Varying concentrations of PHT (Laboratorios Rubio) were placed in both the lower and the upper chamber of the Micro Chemotaxis Chamber in such combinations that, in some cases, there was no gradient, and in others the gradient was variable. PHT concentrations used ranged between 0-20µg/ml in both the upper and lower chambers. Each combination was tested in three identical wells. This experiment was performed three times and the data shown are representative of the typical results.

2.3.5.3. Comparison of Hydantoin-related compounds as chemoattractants

Migration assays were performed as described previously (see section 2.3.5.1. ‘Chemotaxis assays’), but this time a range of HYD-related compounds were used as chemoattractants at 3.64×10^{-5} M concentrations (equivalent to 10µg/ml PHT) made up from a stock solution in DMSO. Each compound was tested in 6 identical wells and two Micro Chemotaxis Chambers were required to accommodate all the test drugs. This set of experiments was performed three times. Data shown are representative of the typical results obtained from two Micro Chemotaxis Chambers. Values were expressed as percentages of the positive control, FN (100%).

2.3.6. Immunohistochemical evaluation of the effect of PHT on urokinase type plasminogen activator expression

Normal or EB fibroblasts were plated into pre-sterilised multiwell spot-slides at a density of 10^4 cells/well in complete DMEM (2% FCS, 150µl/well) within square Petri dishes. The cells were allowed to settle for two hours in a humidified incubator in the presence of 5% CO₂ at 37°C. The slides were then flooded with complete DMEM (2% FCS, 30ml/Petri dish). After 24 hours, half the study slides were incubated with PHT (Sigma, 10µg/ml) in complete DMEM (2% FCS), while the other half received media plus the equivalent ‘vehicle’ alone. The media was regularly changed every second day. After 7 days, the cells were fixed in methanol at -10°C for 10 minutes. The slides were then stored in PBS at 4°C until staining.

U-PA activator expression was visualised by indirect fluorescence staining. All antibody dilutions were made up in PBS (pH=7.35) containing 2% BSA. Firstly, mouse anti-human u-PA primary antibody (1:50) was applied for 60 minutes at 37°C. The slides were washed three times for 5 minutes in PBS and incubated with goat anti-mouse FITC -conjugated antibody (1:50) for 60 minutes. After washing, the slides were counterstained with 0.01% propidium iodide for 10 minutes at room temperature. The slides were washed again and mounted with Citifluor. Immunofluorescent images were visualised using a Leitz Laborlux S microscope (Wild Leitz GmbH, Wetzlar, Germany) and photographs were taken on Kodak P1600 ASA Ektachrome professional film. This experiment was performed in quadruplicate wells and the pictures shown are representative of the typical results.

2.3.7. Immunohistochemical evaluation of Phenytoin uptake

Normal fibroblasts (nf-4) were plated to pre-sterilised multiwell spot-slides at a density of 5×10^3 cells/well in complete DMEM (10% FCS, 150 μ l/well) in square Petri dishes. The cells were allowed to settle for two hours in a humidified incubator in the presence of 5% CO₂ at 37°C. The slides were then flooded with complete DMEM (10% FCS, 30ml/Petri dish). After 24 hours half the slides were incubated with PHT (Laboratorios Rubio, 20 μ g/ml) in complete DMEM (10% FCS), while the other half received media plus 'vehicle' (2 μ l/ml). After 24 hours the slides were fixed with methanol (-10°C) for 5 minutes followed by acetone (-10°C) for 30 seconds. The slides were then stored in PBS at 4°C until staining.

Indirect immunofluorescence staining was used to visualise PHT within the cells. All antibody dilutions were made up in PBS (pH=7.35) containing 2% BSA. Firstly, rabbit anti-Phenytoin primary antibody (1:50, 1:250, and 1:500) was applied for 60 minutes at 37°C. The slides were washed three times for 5 minutes in PBS and incubated with goat anti-rabbit FITC -conjugated antibody (1:200) for 60 minutes. After washing, the slides were mounted with Citifluor. The best antibody titre was found to be 1:250 for the primary antibody.

A charge-coupled device camera was used for visualisation of intracellular PHT distribution. An inverted microscope (Olympus IMT-2) with epifluorescence and phase-contrast attachments was used for microscopy.

The FITC fluorescence was excited by a filtered Xenon-arc lamp (460±20nm) coupled to a liquid light guide with the beam directed into the dichroic mirror housing the epifluorescence studies. The exposure time was approximately 10 seconds, which did not bleach the specimen. Fluorescence was detected between 510 and 540nm using a combination of band-pass and long-pass filters. The fluorescence was imaged quantitatively using a highly sensitive cryogenically cooled CCD camera (600x400 pixels; 0.5µm resolution per pixel) fitted to the microscope. The signal was processed by an IBM personal computer into a falsely colour-coded microscopic image of the cells depicting the mean counts per pixel. This experiment was performed in quadruplicate wells and the pictures shown are representative of the typical results.

2.3.8. The effect of PHT on fibroblast mediated collagen gel contraction

2.3.8.1. Measurements using untethered fibroblast-populated collagen lattices

A collagen gel was prepared by mixing of native, acid soluble type I rat tail collagen (12ml of 2.28mg/ml solution) with 10x strength DMEM (1.5ml) containing sodium bicarbonate and neutralised by the dropwise addition of 1M sodium hydroxide. Cell suspensions of normal and EB fibroblasts were prepared by trypsinisation of monolayer cultures and added immediately to the gel, so that the final concentration was 0.25×10^6 cells per ml of gel. Using 12 well plates, 1ml of gel was cast per well, detached after setting and allowed to free float in complete DMEM (10% FCS, 2ml) with/without PHT (Sigma) in 'vehicle'. The final concentration of PHT in the wells was either 10 or 20µg/ml.

FPCL area measurements were carried out daily for seven days after casting. The well plates were placed on top of a light box and photographs were taken of the illuminated gels. FPCL areas were digitised using a Kurta 1212 board (Kurta, Phoenix, AZ) from the photographs into an IBM personal computer. The areas from these digitised images were then calculated using Sigmascan software (Jandel Scientific, Corte Madera, CA). FPCL areas were expressed as the mean of 12 well readings for each day ± SD.

2.3.8.2. Measurements using the Culture Force Monitor (a tethered fibroblast-populated collagen lattice model)

The Culture Force Monitor (CFM) is a device (Fig. 2.1) used for the accurate measurement of forces exerted by cells in a tethered collagen lattice (Eastwood *et al.*, 1994). Collagen lattices for CFM experiments were prepared by mixing native, acid soluble type I rat tail collagen (12ml of 2.28mg/ml solution; First Link) with x10 strength DMEM (0.5ml) containing sodium bicarbonate and neutralised by the dropwise addition of 1M NaOH to approximately pH=7.6. Cell suspensions of normal or EB fibroblasts (in 0.5ml complete DMEM, 10% FCS) were then added to the gel, so that the final cell density inside the gels ranged between $0.6-1.0 \times 10^6$ cells per ml. The collagen/cell suspension was poured into the culture well (75mm x 25mm x 15mm) of the CFM, and allowed to set in a humidified CO₂ incubator at 37°C for ten minutes. The polymerised gel was gently released from the edges of the silicone mould and the gel was floated by the addition of complete DMEM (10% FCS, 15ml), containing Amphotericine B (2.5µg/ml). Measurements of force generation were started immediately over a period of 24 hours. Data capture from the CFM was at the rate of 1 reading per second, the computer software averaged 600 readings to produce 1 graphical data point.

Drugs were applied to the gels via the Drug Administration Device (DAD) by pumping the DMEM-drug solution (0.5ml) with a syringe through a tube from outside the incubator (Fig. 2.2). This represented minimal disturbance to the measurements. The tube ran over the full length of the gel and was perforated five times to ensure equal dosing.

Drugs were tested in 7.3×10^{-5} M or 10^{-4} M concentrations as indicated. Also, the effect of pre-treatment with PHT (Sigma, 20µg/ml, 7.3×10^{-5} M, for 5 days) on cell contraction was investigated. Each experiment was performed at least twice and the data shown are representative of the typical results.

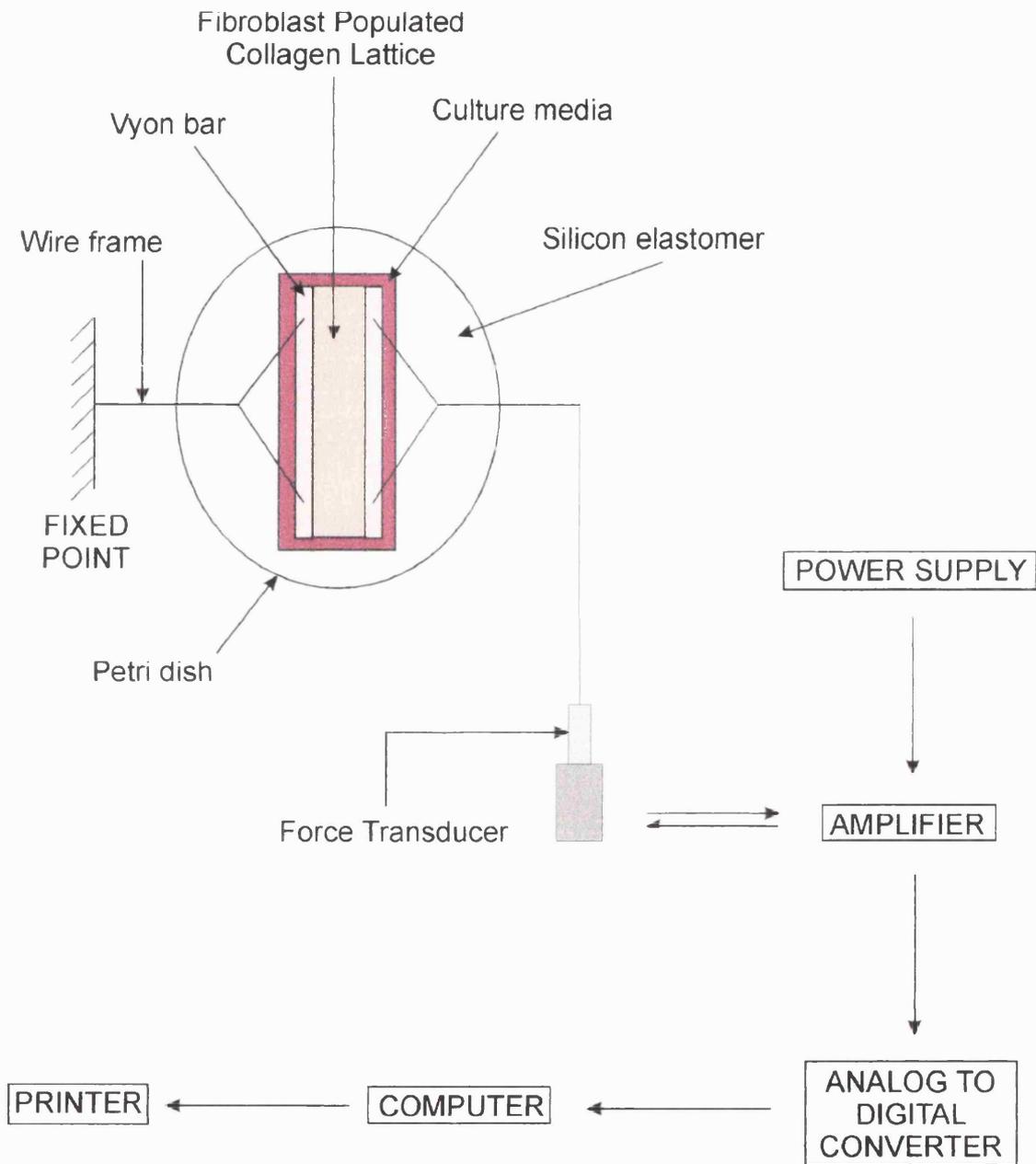


Figure 2.1. Schematic diagram of the CFM.

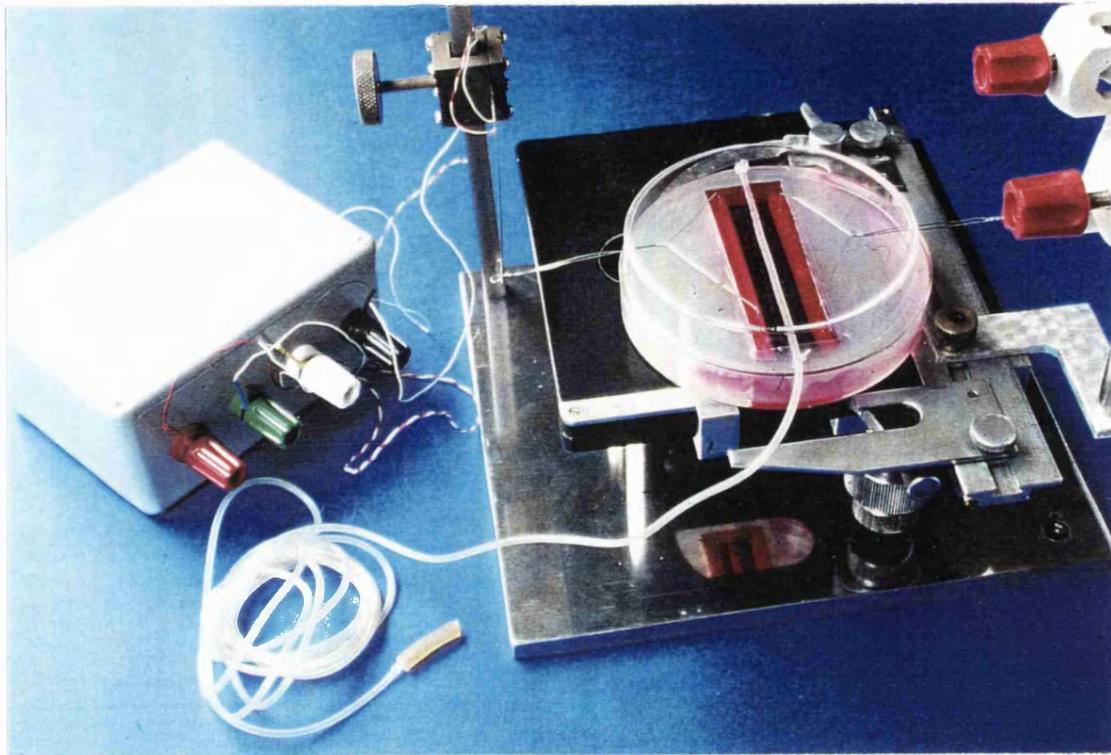


Figure 2.2. Photograph of DAD.

2.3.8.3. Morphological examination of tethered fibroblast-populated collagen lattices

Tethered gels for timed morphological examination were prepared as before for the CFM [see section 2.3.8.2. 'Measurements using the Culture Force Monitor (a tethered fibroblast-populated collagen lattice model)'], but poured into 4 well plates (1ml/well). After setting, 0.5ml of complete DMEM (10% FCS) with/without PHT (Sigma) in 'vehicle' was added at 0 hours so that the final concentration was 20 μ g/ml PHT or 20 μ l/ml 'vehicle'. (These gels remained attached to the culture wells throughout the experiment.) After increasing contraction periods (0, 1, 4, 8, 24 hours) the gels were fixed by the addition of 2.5% gluteraldehyde in 0.1M phosphate buffer (pH=7.5) at 4°C for 1 hour followed by washing in phosphate buffer. The gels were then stained with 1% Toluidine blue, destained with distilled water, for stereomicroscopic examination (Greenberg *et al.*, 1993) on an Edge High Definition Stereo Light Microscope (Edge Scientific Instrument Corporation, LA, USA).

2.3.9. Comparison of the effects of Colchicine and Phenytoin on cell morphology and cytoskeleton

Normal fibroblasts were plated onto 7 pre-sterilised multiwell spot-slides at a density of 2×10^3 cells/well in complete DMEM (10% FCS, 150 μ l) within 7 square Petri dishes. The cells were allowed to settle for two hours in a humidified incubator in the presence of 5% CO₂ at 37°C. The slides were flooded with complete DMEM (10% FCS, 30ml/Petri dish) for 24 hours and were then incubated as follows:

- Slide 1: Cells were grown in complete DMEM only for 6 days.
- Slide 2: Cells were grown in complete DMEM for 5 days then treated with COL (7.3×10^{-5} M) for 6 hours.
- Slide 3: Cells were incubated with PHT (Sigma, 20 μ g/ml, 7.3×10^{-5} M) for 6 days.
- Slide 4: Cells were incubated with PHT (Sigma, 50 μ g/ml, 1.825×10^{-4} M) for 6 days.
- Slide 5: Cells were incubated with PHT (Sigma, 100 μ g/ml, 3.6×10^{-4} M) for 6 days.
- Slide 6: Cells were incubated with PHT (Sigma, 20 μ g/ml, 7.3×10^{-5} M) for 5 days, then treated with COL (7.3×10^{-5} M) for 6 hours with PHT (Sigma, 20 μ g/ml, 7.3×10^{-5} M) still present in the media.
- Slide 7: Cells were incubated with PHT (Sigma, 20 μ g/ml, 7.3×10^{-5} M) for 5 days, then treated with COL (7.3×10^{-5} M) for 6 hours without PHT present in the media.

The cells were then fixed in 4% formaldehyde at room temperature and rinsed twice with PBS. The slides were fixed by immersing first in methanol (-10°C) for 10 minutes, followed by 30 seconds in acetone (-10°C) and air-dried. Indirect immunofluorescence staining was used to visualise β -tubulin within the cells. All antibody dilutions were made up in PBS (pH=7.35) containing 2% BSA. Firstly, mouse anti-human β -tubulin primary antibody (1:100) was applied for 60 minutes at 37°C. The slides were washed three times for 5 minutes in PBS and incubated with goat anti-mouse FITC-conjugated antibody (1:130) for 60 minutes. After washing, the slides were with Citifluor.

Immunofluorescent images were visualised using a Leitz Laborlux S microscope (Wild Leitz GmbH, Wetzlar, Germany) and photographs were taken on Kodak P1600 ASA Ektachrome professional film.

2.3.10. Comparison of the effects of Phenytoin and Hydantoin derivatives on cell morphology and cytoskeleton

Normal fibroblasts were plated onto pre-sterilised multiwell spot-slides at a density of 2×10^3 cells/well in complete DMEM (10% FCS, 150 μ l) within 7 square Petri dishes. The cells were allowed to settle for two hours in a humidified incubator in the presence of 5% CO₂ at 37°C. The slides were flooded with complete DMEM (10% FCS, 30ml/Petri dish) for 24 hours and were then incubated with HYD derivatives at 7.3×10^{-5} M concentrations.

To stain for cytoskeletal components within fibroblasts, firstly one spot on each slide was incubated with TRITC-labelled Phalloidin (1:500) in PBS. The slides were washed three times with PBS for 5 minutes. Then, separate spots were incubated with either mouse anti-human β -tubulin or vinculin or vimentin in such a way, that β -tubulin staining overlapped with Phalloidin. After washing in PBS, each spot was incubated with goat anti-human FITC-conjugated antibody (1:130), except for the one stained for vimentin, which was incubated with goat anti-human TRITC antibody (1:50). All antibody dilutions were made up in PBS (pH=7.35) containing 2% BSA. After washing, the cells were counterstained with Hoechst-3325 (1 μ g/ml in PBS) nuclear stain. The slides were washed again in PBS and mounted in Citifluor.

The labelling was visualised using a fluorescence microscope (Zeiss Axiophot; Carl Zeiss Ltd., Welwyn Garden City, Herts, UK) and images were captured using a cooled CCD camera (Photonic Science; Millham, East Sussex, UK.) and the Image Proplus (Media Cybernetics) software program. Images were saved according the following colour code: FITC-green, TRITC-red and Hoechst nuclear stain as blue. The images were then overlaid using the Adobe Photoshop program (Adobe UK Ltd., Edinburgh, Scotland, UK) to yield a 2 or 3 colour image.

2.4. Methods (*in vivo* studies) - The effect of Phenytoin on porcine wound healing

2.4.1. Surgical procedures

A total of 4, middle white female domestic pigs (6 to 8 weeks old, approximately 30-40kg) were used in the study. The operations were carried out by Dr R. A. Brown (holder of a Home Office licence), Mr G. Batchelor acting as anaesthetist and Miss R. A. Porter and Mrs. G. Talas assisting.

The pigs were injected with Stresnil (Azaperone, Janssen Pharma Ltd., Oxford, UK) and anaesthetised with halothane/air (Rhone Merriex, Harlow, Essex, UK). An intravenous cannula (20G, Millplege, Germany) was also inserted into the ear vein allowing the withdrawal of blood into vacutainer blood sampling tubes containing citrate buffer. Then the central dorsal skin was cleaned with Savlon disinfectant and shaved with clippers and razors. All operative procedures were performed using routine aseptic techniques. On each pig, 16 full-thickness excision wounds (12mm x 8mm) were cut using a custom made template (Fig. 2.3).

All wounds were approximately identical in anatomical depth down to the deep fascia overlying the body wall muscle layer. Wounds were located in a pattern of 8 on each side of the pig, in two rows of four, several centimetres away from the midline (spine). Dosing with PHT powder +/- Fibrin Sealant (FS) was carried out immediately after wounding followed by sealing with Opsite dressing (Safety First Aid, London, UK). Blood samples were taken at the start of the experiment, 15, 45 minutes and 24 hours post-dosing.

Pigs 1-2 were sacrificed at 3 weeks, while pigs 4-5 were two weeks post-operative. The pigs were anaesthetised as before, then the wounds were photographed and widely excised for histologic study. Animals were culled by injecting with Euthanol under anaesthesia.

2.4.2. Wound treatments

Figures 2.4 and 2.5 describe the topical dosage regimes of each pig treated. Pigs 1&2 received a total of 600mg/pig of topical PHT powder (Sigma). Pigs 3&4 on the other hand received 240mg/pig of PHT (Laboratorios Rubio, Spain) either as powder or in combination with FS.



Figure 2.3. Photograph of the wounding procedure using a template (A) and the resulting wound (B). Wounds were created diagonally to coincide with the stretchlines of the skin.

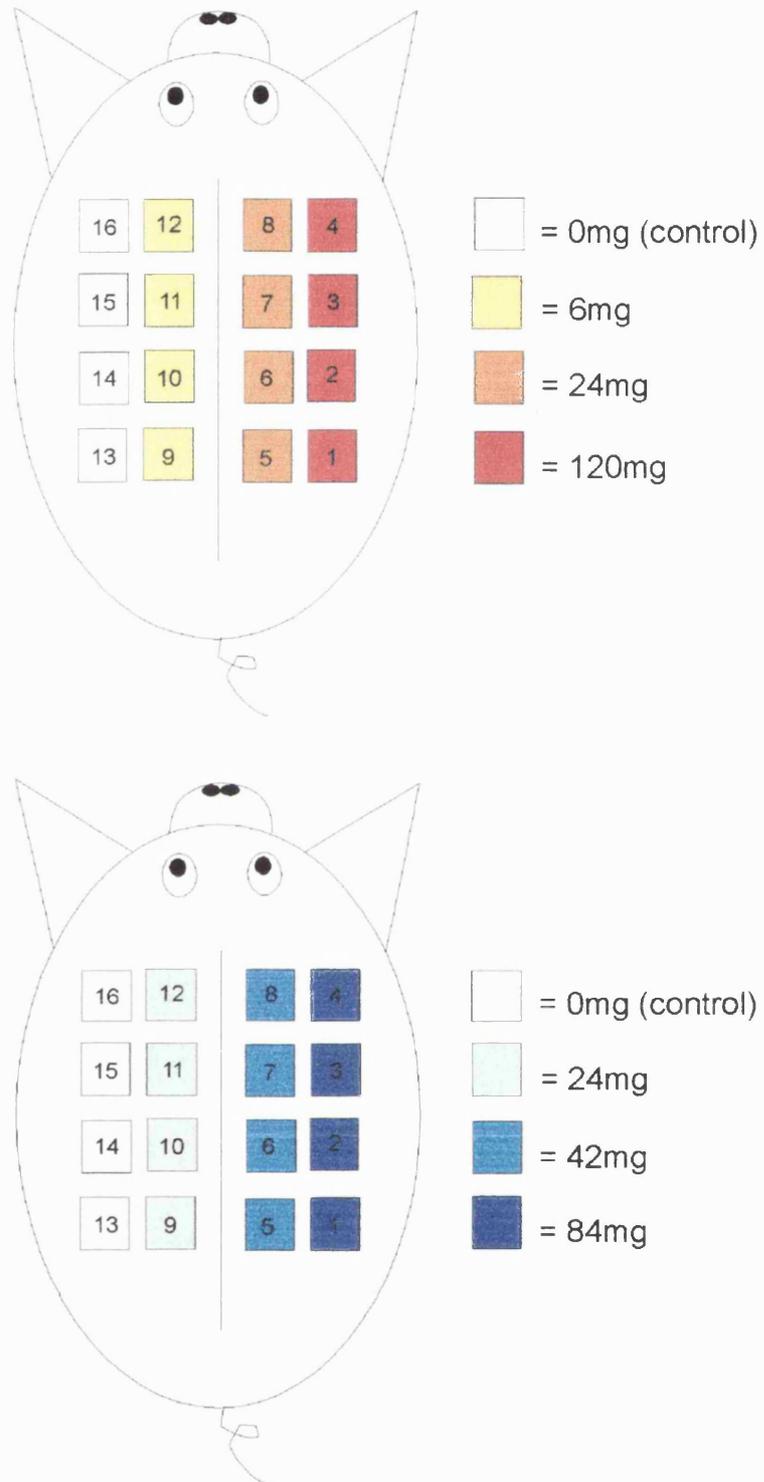
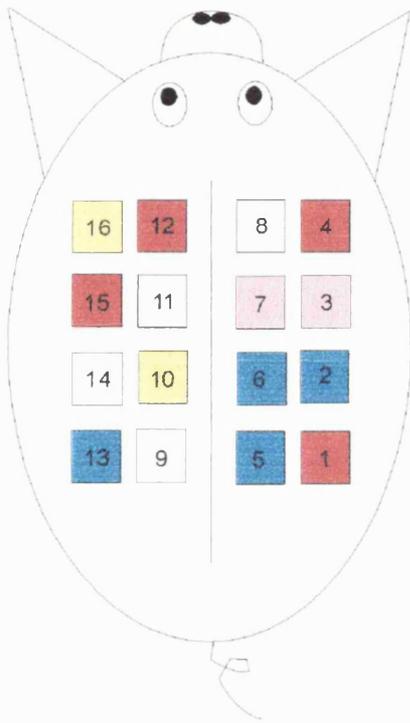
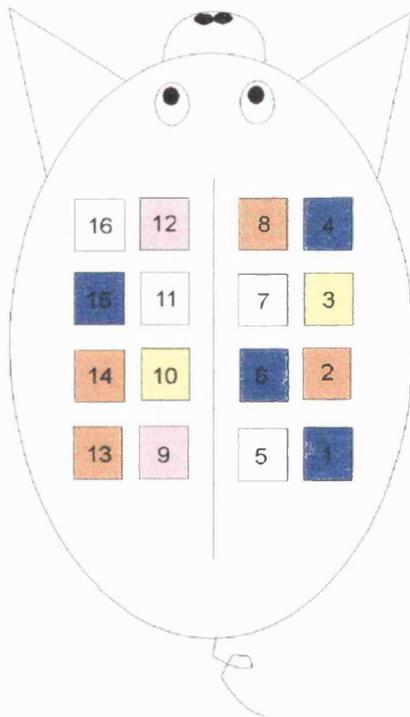


Figure 2.4. Schematic diagram of the wound treatments received by pigs 1&2.



- = 0mg (control)
- = 42mg PHT
- = Fibrin sealant
- = 6mg PHT+FS
- = 24mg PHT+FS



- = 0mg (control)
- = 42mg PHT+FS
- = Fibrin sealant
- = 6mg PHT
- = 24mg PHT+FS

Figure 2.5. Schematic diagram of the wound treatments received by pigs 3&4.

FS was prepared using a Fibrin Sealant Kit (Scottish National Blood Transfusion Service, Edinburgh, Scotland). A separate FS was prepared for each wound by mixing human Fibrinogen (200 μ l) and human Thrombin (200 μ l) and after clotting it was transferred and moulded into the wound (Fig. 2.6). In order to incorporate PHT into the Sealant, the required amount of PHT was mixed with the Fibrinogen first and clotting was achieved by mixing with Thrombin.

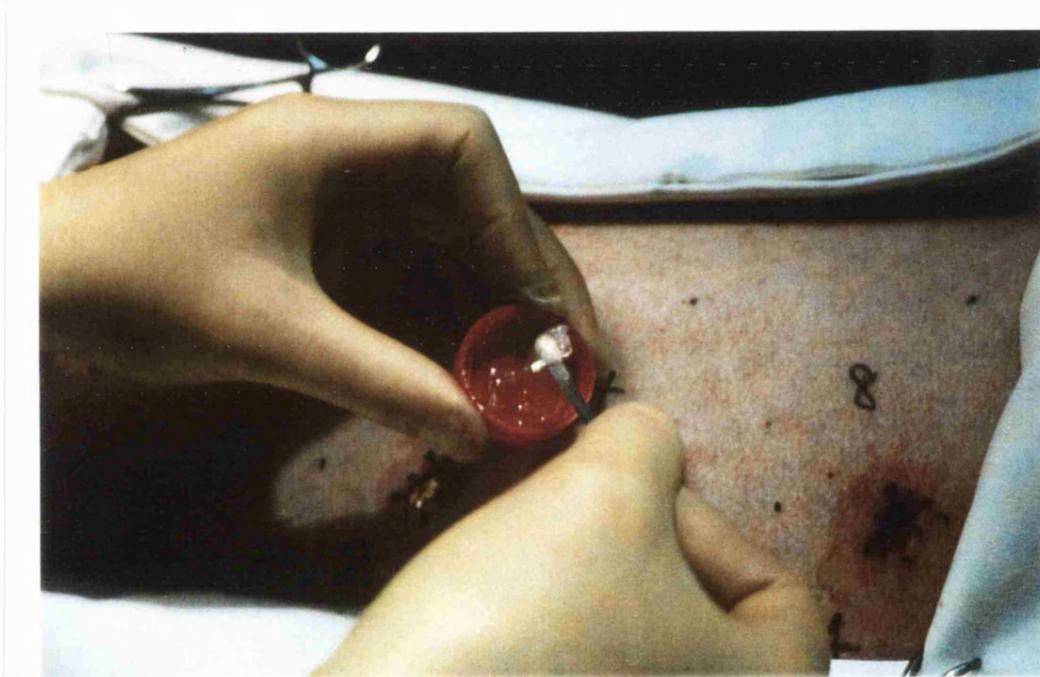


Figure 2.6. Photograph of the FS prior to placement into the wound.

2.4.3. Image analysis of wounds

Wound areas were digitised using a Kurta 1212 board (Kurta, Phoenix, AZ) from the photographs into an IBM personal computer. Each wound was scanned three times. The areas from these digitised images were then calculated using Sigmascan software (Jandel Scientific, Corte Madera, CA). Statistical analysis was carried out using Minitab v.11 software (Minitab, State College, PA, USA).

2.4.4. Structural analysis of wound tissues

Each wound sample was fixed in 10% Formalin, processed for histology and embedded in wax. Slides were routinely stained with Haematoxylin and Eosin to observe the presence of specific cell types, while Picro-Sirius red staining was performed to locate newly synthesised collagen in the wound area. The latter was also examined under polarised light for birefringence of collagenous structures.

2.4.5. Measurement of Phenytoin in blood

Blood samples (10ml) were taken from pigs 1&2 at the following intervals: 0 time (at the start of the operation), then 30, 60, 90 120, 240 minutes after the application of PHT powder. Finally, further samples were taken after 24 hours and 3 weeks post operatively. Routine HPLC (analysis was carried out by V. Taylor (sandwich student from the University of Greenwich) according to the method of Kabra and co-workers (Kabra *et al.*, 1976). The HPLC equipment consisted of a LKB 2150 HPLC pump (Pharmacia, St. Albans, UK), Rheodyne 7125 manual injector (Rheodyne Inc., Cotati, California, USA), HPLC column (PLRP-S, 8mm, 150 x 4.6mm I.D; Polymer Laboratories, Church Stretton, Shropshire, UK), UV detector (LKB 2238 Uvicord SII , Pharmacia) with a 226nm filter. A Spectra Physics SP4270 integrator (obtained from LC Services, Bedfordshire, UK) was used for data acquisition. The mobile phase consisted of 60% phosphate buffer, 21.5% acetonitrile and 18.5% methanol.

Whole blood was centrifuged at 3000rpm for 15 mins and the plasma layer retained and stored at 4°C. The plasma was then mixed with an equal volume of internal standard [(MePh)-PH; 250 µg/ml in methanol]. To 0.5ml of this sample plasma was then added 3.5 ml distilled water and 0.75 µl of glacial acetic acid and the whole was Vortex mixed for 20 seconds. 5.5ml chloroform was added and the sample mixed for 5-10 min on a Spiramix. The sample was then centrifuged at 3000rpm for 10 min and the lower layer containing chloroform retained. The sample was evaporated using a rotary evaporator (70°C water bath, liquid N₂ trap) and then reconstituted with 100µl ethanol. 20µl samples were analysed on the HPLC. The lowest possible measurable concentration of PHT or its metabolites was 100ng in one 20µl sample (0.005mg/ml).

CHAPTER 3. RESULTS

3.1. The effect of Phenytoin on cell proliferation

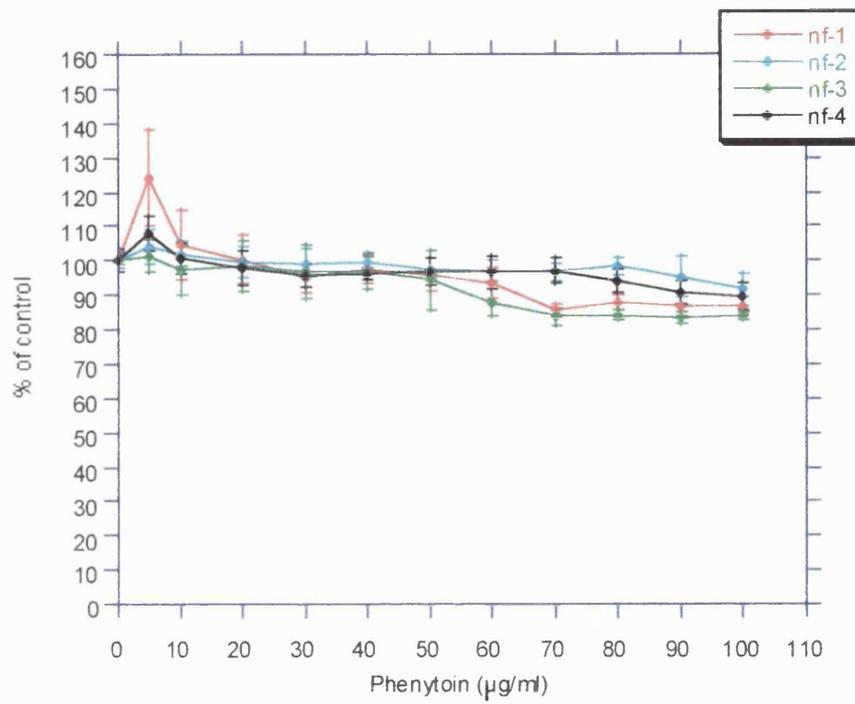
The effects of PHT were investigated on 4 normal, 4 EB fibroblast and 4 keratinocyte cell lines at two time points over a range of concentrations. Data below compare collective responses according to cell types with time and concentrations of PHT. Importantly, separate cell line responses were also compared to test for heterogeneity within a group.

Firstly, the 'vehicle' alone (10 μ l/ml, equivalent to the amount found in the media containing 100 μ g/ml PHT) had no statistically significant effects on cell proliferation (nf-2, ebf-2 and k-2) after 72 or 120 hours.

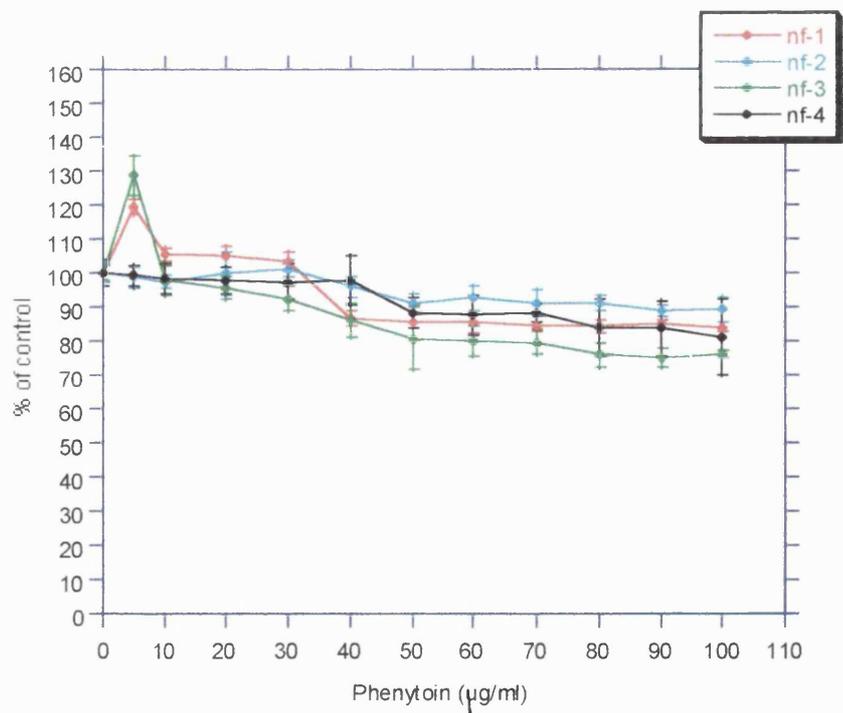
3.1.1. The effect of Phenytoin on normal fibroblast proliferation

When the data was pooled from the four cell lines, it was found that after 72 hours 5 μ g/ml PHT was stimulatory to a small extent (by 9%, t-test, $P < 0.00001$), 10-20 μ g/ml had no effect and concentrations equal to or higher than 30 μ g/ml reduced proliferation maximally by 12% (at 100 μ g/ml, t-test, $P < 0.00001$). After 120 hours 5 μ g/ml remained stimulatory (by 12%, t-test, $P < 0.00001$), whereas 10-30 μ g/ml had no effect and concentrations of 40 μ g/ml or higher reduced it maximally by 17% (at 100 μ g/ml, t-test, $P < 0.00001$).

Results of individual cell lines were also compared to assess population heterogeneity within normal fibroblasts (Fig. 3.1). In all cases 5 μ g/ml PHT was found to be the optimal concentration for the stimulation of cell proliferation. Interestingly, in nf-1 there was a significant increase after both 72 and 120 hours (t-tests; 24%, $p=0.0001$ and 20%, $P < 0.0001$, respectively) indicating that this early response was maintained. In nf-3 this response was latent, reaching significance only at 120 hours (28%, t-test, $P < 0.00001$). In the remaining two cell lines (nf-2 and nf-4), while there was some small increase in proliferation observed after 72 hours (t-tests; 4%, $P = 0.011$ and 8%, $P = 0.0001$, respectively), it diminished completely after 120 hours.



a.



b.

Figure 3.1. The effect of PHT on normal fibroblast proliferation after 72 hours (a) and after 120 hours (b). Growth is expressed as a percentage of the control, which was taken as 100%.

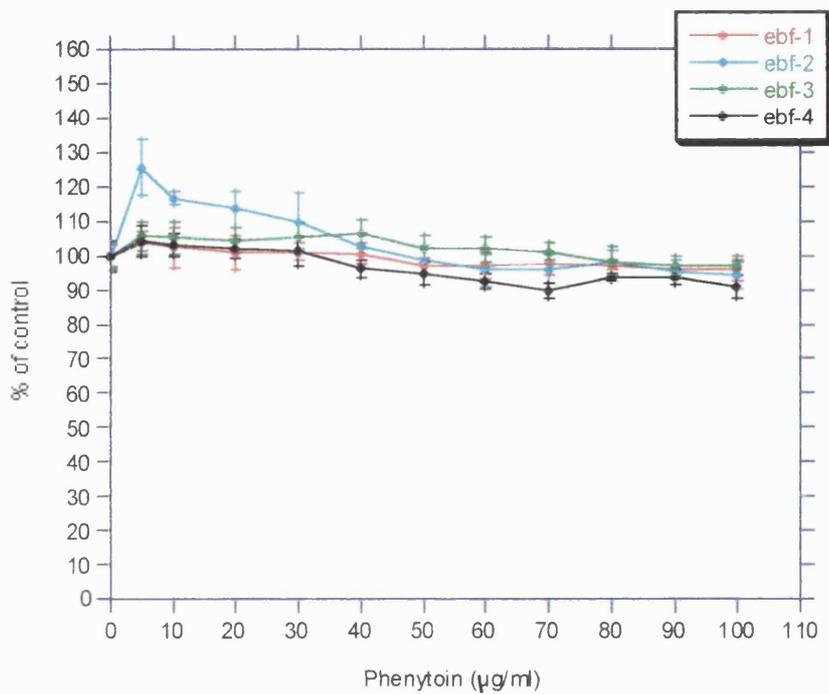
3.1.2. The effect of Phenytoin on Epidermolysis Bullosa fibroblast proliferation

Pooling the data from the four cell lines revealed that after 72 hours 5-30 μ g/ml PHT were all stimulatory, maximally by 10% with 5 μ g/ml (t-test, $P < 0.00001$). 40 μ g/ml had no effect and concentrations equal to or higher than 50 μ g/ml reduced proliferation maximally by 6% (at 100 μ g/ml, t-test, $P < 0.00001$). After 120 hours 5 μ g/ml was slightly stimulatory (2.4%, t-test, $P = 0.0031$), whereas 10-20 μ g/ml had no effect and concentrations equal to or higher than 30 μ g/ml reduced it maximally by 10% (at 100 μ g/ml, t-test, $P < 0.00001$).

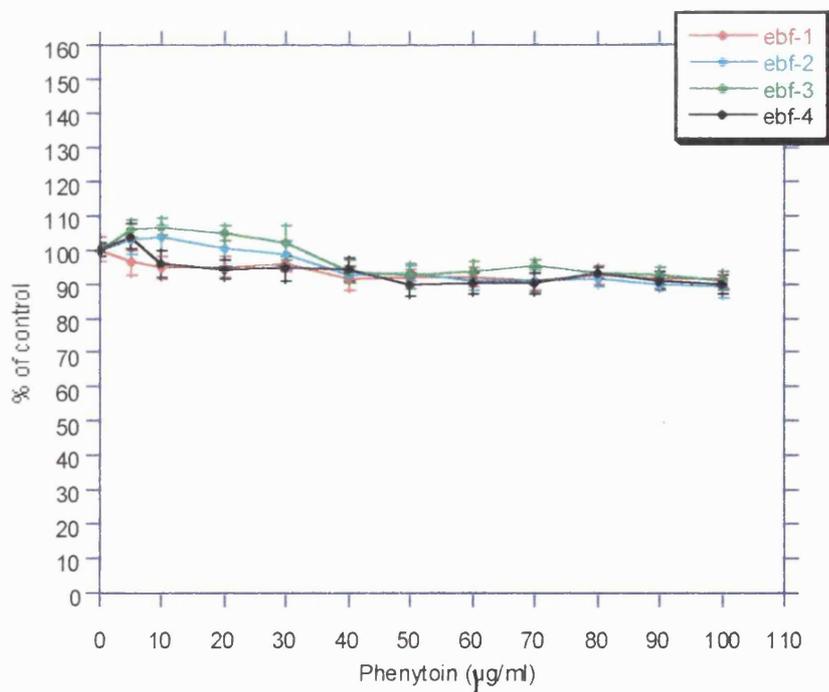
Results of individual cell lines were also compared to assess population heterogeneity within EB fibroblasts (Fig. 3.2). The most prominent effect was seen in ebf-2 after 72 hours (26% increase, t-test, $P < 0.00001$). However, it was transient, and was not found to be significant after 120 hours. In cell lines ebf-3 and ebf-4, 5 μ g/ml PHT induced a mild increase in growth after 72 hours (t-tests, 6%, $P = 0.0013$ and 4%, $P = 0.012$, respectively), which was maintained to the same degree after 120 hours (t-tests, 6%, $P = 0.0004$ and 4%, $P = 0.0038$, respectively). Although in ebf-1, 5 μ g/ml PHT slightly increased cell proliferation, after 120 hours even such low concentration reduced it by 4% (t-test, $P = 0.025$).

3.1.3. Comparison of the effect of Phenytoin on cell proliferation between normal and Epidermolysis Bullosa fibroblasts

The effect of PHT on the proliferation of normal and EB fibroblasts was compared. Analysis of variance (balanced design) of the pooled data determined that there was a statistically significant difference in their response to PHT with respect to cell type ($F=185.98$, $P < 0.0001$), PHT concentrations ($F=181.5$, $P < 0.0001$) and time ($F=303.83$, $P < 0.0001$). Although in both normal and EB fibroblasts 5 μ g/ml PHT elicited the greatest response (9% and 10% respectively), in EB fibroblasts concentrations up to 30 μ g/ml PHT were stimulatory after 72 hours. After 120 hours, however, the magnitude of the PHT-induced effect was reduced in EB fibroblasts (2.4%) compared to normal fibroblasts (12%). EB fibroblasts, on the other hand, were more tolerant of PHT at higher concentrations than normal fibroblasts at both time points.



a.



b.

Figure 3.2. The effect of PHT on EB fibroblast proliferation after 72 hours (a) and after 120 hours (b). Growth is expressed as a percentage of the control, which was taken as 100%.

3.1.4. The effect of Phenytoin on keratinocyte proliferation

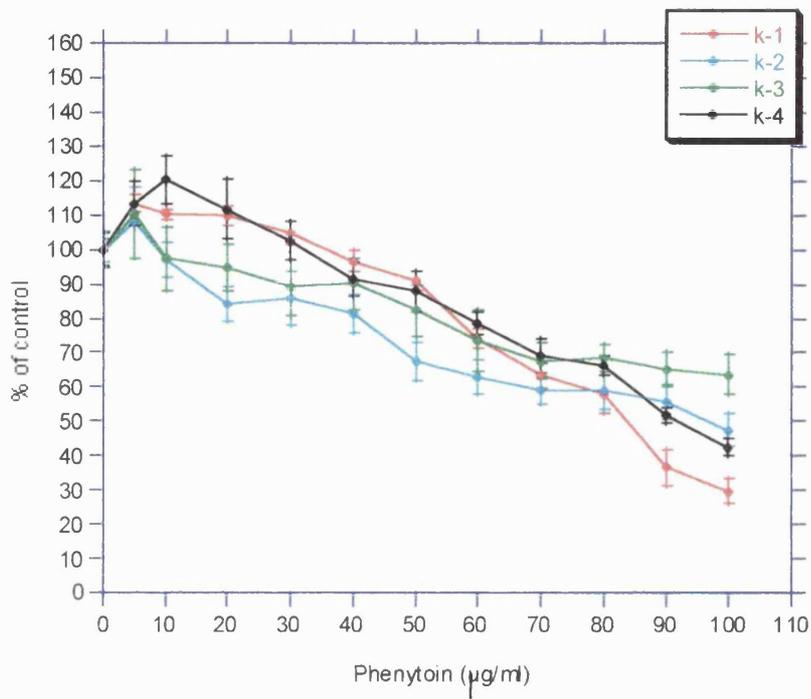
The data was pooled from the four cell lines and it was shown that after 72 hours 5-10 μ g/ml PHT were all stimulatory, maximally by 11% with 5 μ g/ml ($P < 0.00001$). 20 μ g/ml had no effect and concentrations equal to or higher than 30 μ g/ml reduced proliferation maximally by 54% (at 100 μ g/ml, t-test, $P < 0.00001$). After 120 hours 5-10 μ g/ml remained to be stimulatory (maximally 19% with 5 μ g/ml, t-test, $P < 0.00001$), and concentrations equal to or higher than 20 μ g/ml reduced it. Maximum inhibition of proliferation was seen with 100 μ g/ml PHT (t-test, $P < 0.00001$), when only 38% of cells remained alive.

Results of individual cell lines were also compared to assess population heterogeneity within keratinocytes (Figure 3.3). After 72 hours 5 μ g/ml PHT was found to stimulate cell proliferation in all four cell lines. However, in one case (k-4) the maximum increase was noted to be with 10 μ g/ml PHT (20%, t-test, $P < 0.00001$). Interestingly, after 120 hours the optimum concentration was, however, 5 μ g/ml PHT (8% increase, t-test, $P = 0.0014$) for the same cell line. After 120 hours cell line k-1 displayed the greatest response to PHT at both 5 μ g/ml PHT (38%, t-test, $P < 0.00001$) and 10 μ g/ml PHT (40%, t-test, $P < 0.00001$). (The responses were not significantly different between these concentrations, t-test, $P = 0.47$.) Similarly, in another cell line, k-2, maximum effects were seen after 120 hours with 5 μ g/ml PHT (22% increase, t-test, $P < 0.00001$). Although these cell lines (k-1 and k-2) displayed the greatest response to low concentrations of PHT, they were also the most inhibited with high concentrations of PHT.

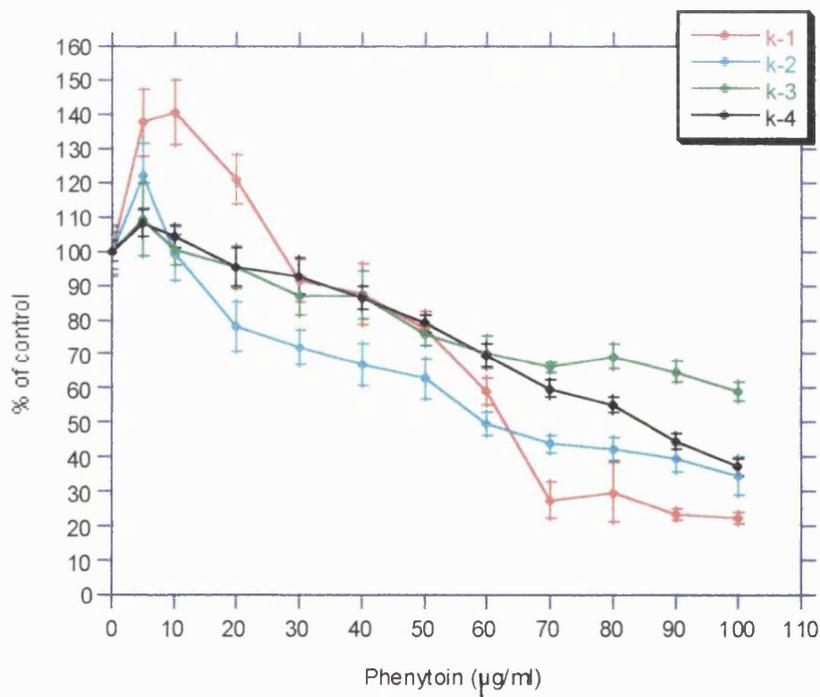
3.1.5. Comparison of the effect of Phenytoin on cell proliferation between normal fibroblasts and keratinocytes

The effect of PHT on the growth of normal fibroblasts and keratinocytes was also compared. Analysis of variance (balanced design) of the pooled data showed that there was a statistically significant difference in their response to PHT with respect to cell type ($F=800.72$, $P < 0.0001$), PHT concentrations ($F=293.2$, $P < 0.0001$) and time ($F=79.52$, $P < 0.0001$). Overall, keratinocytes displayed a latent maximal response (120 hours, 19%) to 5 μ g/ml PHT compared with normal fibroblasts (12%). However, keratinocytes were also very

sensitive to high concentrations of PHT compared to fibroblasts at both time points.



a.



b.

Figure 3.3. The effect of PHT on keratinocyte proliferation after 72 hours (a) and after 120 hours (b). Growth is expressed as a percentage of the control, which was taken as 100%.

3.2. The effect of Phenytoin on cell migration

Firstly, FN was chosen to be the positive control for these experiments for all cell types, as it is a chemoattractant for both fibroblasts (Kirschberg *et al.*, 1995; Mensing *et al.*, 1983; Postlethwaite *et al.*, 1981; Gauss-Muller *et al.*, 1980) and keratinocytes (Nickoloff *et al.*, 1988; Sugita *et al.*, 1987). Although various concentrations of FN have been reported to trigger the chemotactic response *in vitro*, 2.5µg/ml was established as the optimal FN concentration for human adult dermal fibroblasts derived from abdominal skin for this study (Fig.3.4). All of the other studies cited differed in their experimental methodologies, sources of FN, types of fibroblasts and therefore are not comparable.

Secondly, the chemotactic activity of purified plasma FN (BPL) was compared with that from a commercial source (Sigma), and they were found to be similar at all concentrations by Analysis of Variance (balanced design, $F=0.26$, $P=0.615$).

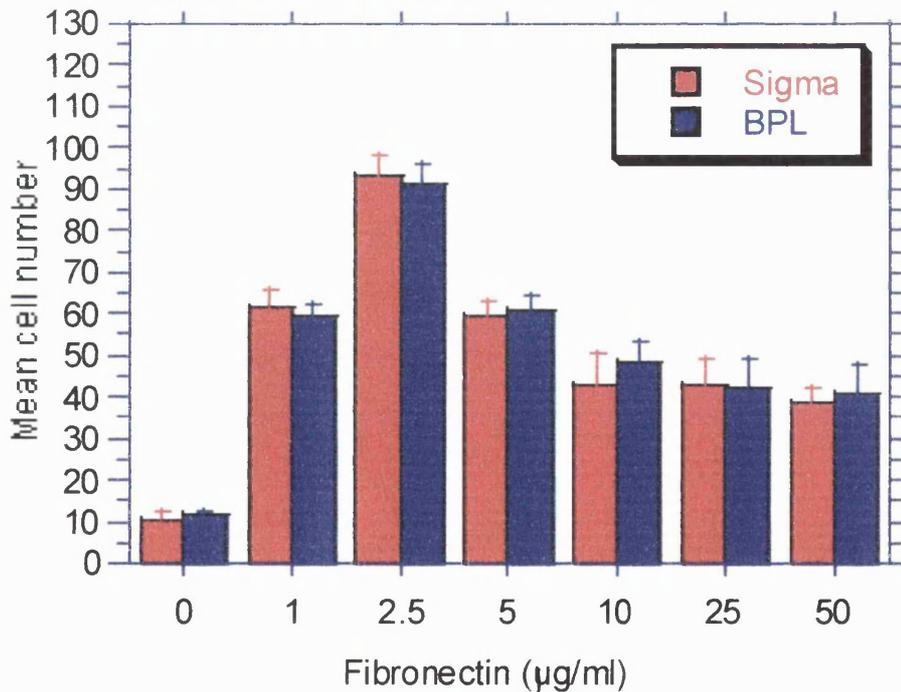


Figure 3.4. Chemotactic response of normal dermal fibroblasts towards increasing concentrations of FN from various sources (Sigma and BPL). Histograms represent the mean cell number \pm SD obtained from three identical wells per chemoattractant.

In all test cases, there was minimum migration in serum-free medium alone (maximum 12% of FN or less). The effects of PHT were investigated on the migration of 5 normal, 5 EB fibroblast and 4 keratinocyte cell lines over a range of concentrations. Again, collective responses according to cell type and individual cell line responses were compared to test for heterogeneity within a group. 'Vehicle' alone (5µl/ml) did not have any significant effects on cell migration as determined in nf-1, ebf-2 and k-2 cell lines.

3.2.1. The effect of Phenytoin on the migration of normal fibroblasts

When the data was pooled from the five cell lines (Fig. 3.5), it was determined that 5µg/ml PHT was optimal (111.32±15.45%, mean±SD) and induced ten times greater migration than the control media (serum-free DMEM; 10.878±1.88%, mean±SD). This was found to be statistically significant by Mann-Whitney test ($P < 0.00001$). Moreover, concentrations higher than 5µg/ml PHT remained to stimulate migration significantly. However, the magnitude of the stimulus fell with increasing PHT levels. This was perhaps due to either desensitisation or toxicity at higher PHT concentrations.

There was little heterogeneity observed between individual cell line responses, except for cell line nf-1, in which 10µg/ml PHT brought about the maximum response (117±7.56%, mean±SD, t-test, $P < 0.00001$).

3.2.2. The effect of Phenytoin on the migration of Epidermolysis Bullosa fibroblasts

When the data was pooled from the five cell lines (Fig. 3.6), it was determined that 10 µg/ml PHT was optimal (106.76±19.63%, mean±SD) and induced 10.6 times greater migration than the control media (serum-free DMEM; 10.083±3.256%, mean±SD). This was found to be statistically significant by Mann-Whitney test ($P < 0.00001$). Concentrations higher than 5µg/ml PHT remained to stimulate migration significantly. However, the magnitude of the stimulus fell with increasing PHT levels. This was perhaps due to either desensitisation or toxicity at higher PHT concentrations.

It must be also noted that there was significant heterogeneity observed between individual cell line responses. In two cell lines (ebf-2, ebf-3) the optimum PHT concentration was found to be 20 μ g/ml (97.3 \pm 18.6% and 145.8 \pm 11.85%, mean \pm -SD respectively) whereas 5 μ g/ml caused maximal stimulation in ebf-4 (135.74 \pm 13.56%, mean \pm -SD).

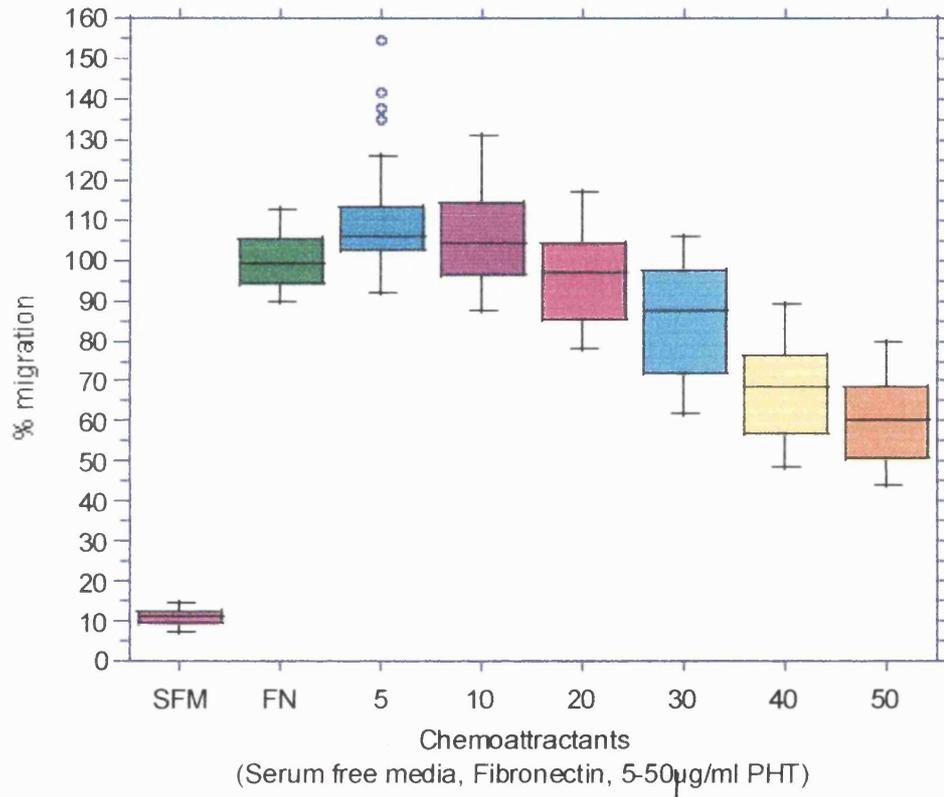


Figure 3.5. Boxplot representation of the effect of PHT on normal fibroblast migration (nf-1-5) in the Micro Chemotaxis Chamber. Values are expressed as percentages of the positive control, FN (100%). The horizontal lines within the boxes (25th to 75th percentiles) represent the median. Error bars denote 5th to 95th percentiles and unfilled circles indicate results outside of these ranges.

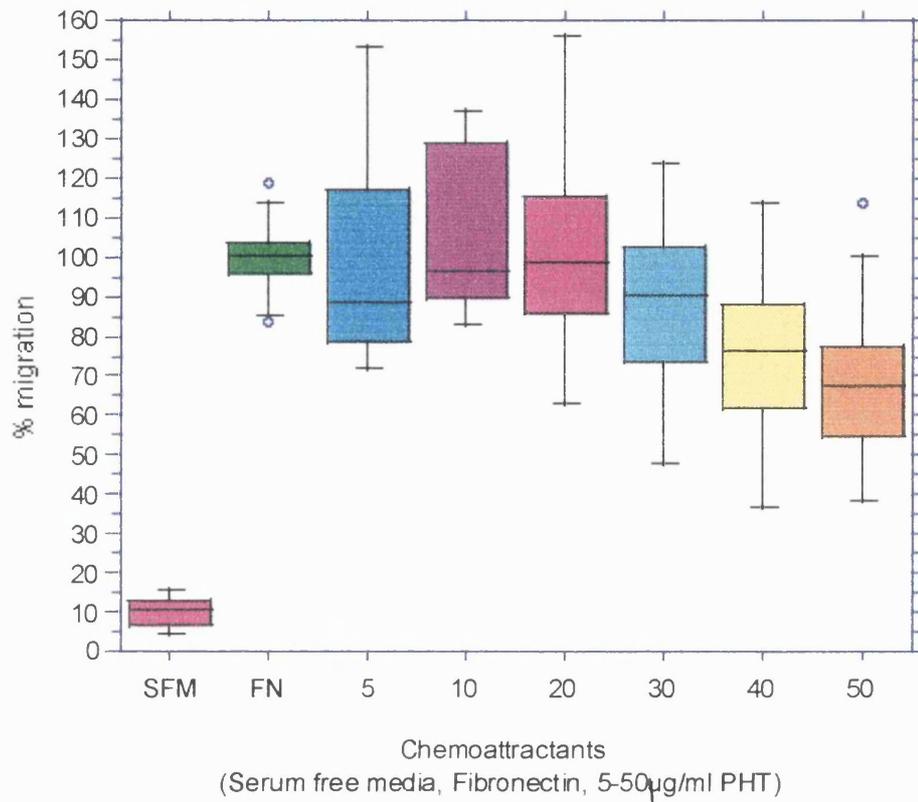


Figure 3.6. Boxplot presentation of the effect of PHT on EB fibroblast migration (ebf-1-5) in the Micro Chemotaxis Chamber. Values are expressed as percentages of the positive control, FN (100%). The horizontal lines within the boxes (25th to 75th percentiles) represent the median. Error bars denote 5th to 95th percentiles and unfilled circles indicate results outside of these ranges.

3.2.3. Comparison of the effect of Phenytoin on cell migration between normal and Epidermolysis Bullosa fibroblasts

Analysis of Variance (balanced designs) of normal and EB fibroblasts showed that there was a statistically significant difference in their response to PHT with respect to PHT concentrations ($F=233.29$, $P < 0.0001$), but not cell type ($F=0.98$, $P = 0.322$). The optimal PHT concentration for EB fibroblasts was higher (10µg/ml) than for normal fibroblasts (5µg/ml) and there was greater heterogeneity within EB cell line responses.

3.2.4. The Effect of Phenytoin on the migration of keratinocytes

Pooled data from the four cell lines (Fig. 3.7) revealed that 5 μ g/ml PHT was optimal (107.93 \pm 8.63%, mean \pm SD) and induced 17.6 times greater migration, than the control media (serum-free DMEM; 6.127 \pm 2.416%, mean \pm SD). This was found to be statistically significant by Mann-Whitney test ($P < 0.00001$). Concentrations higher than 5 μ g/ml PHT remained to stimulate migration significantly. However, the magnitude of the stimulus fell dramatically with increasing PHT levels. This was perhaps due to cell toxicity rather than desensitisation at higher PHT concentrations (Fig. 3.3). There was little heterogeneity observed between individual cell line responses and 5 μ g/ml PHT was the optimum concentration in all cases.

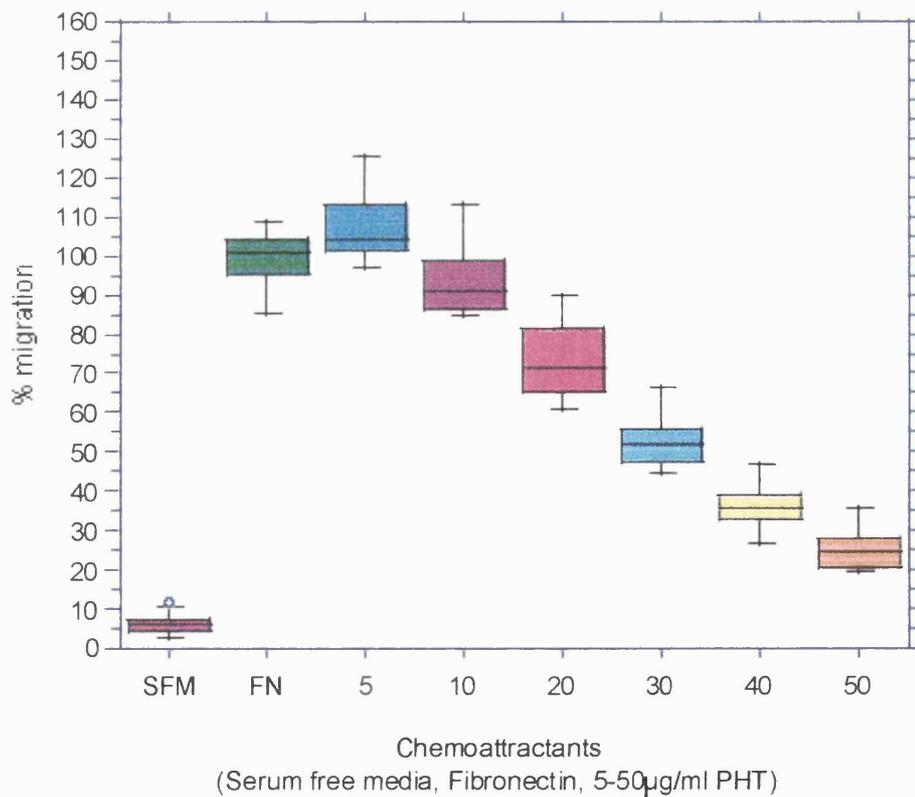


Figure 3.7. Boxplot presentation of the effect of PHT on keratinocyte migration (k-1-4) in the Micro Chemotaxis Chamber. Values are expressed as percentages of the positive control, FN (100%). The horizontal lines within the boxes (25th to 75th percentiles) represent the median. Error bars denote 5th to 95th percentiles and unfilled circles indicate results outside of these ranges.

3.2.5. Comparison of the effect of Phenytoin on cell migration between normal fibroblasts and keratinocytes

Analysis of Covariance (orthogonal designs) of fibroblasts and keratinocytes revealed that there was a statistically significant difference in their response to PHT with respect to cell type ($F=289.39$, $P < 0.0001$) and PHT concentrations ($F=489.96$, $P < 0.0001$). Although keratinocytes displayed a greater response to the mutually optimal concentration ($5\mu\text{g/ml}$ PHT), they were also more sensitive to higher concentrations of PHT and their migratory capacity was greatly reduced.

3.2.6. Checkerboard analysis

Checkerboard analysis (Table 3.1.) indicated that directional migration (chemotaxis) was stimulated to a greater extent than random motility (chemokinesis). PHT elicited movement of normal fibroblasts (nf-4) against a concentration gradient with a maximal chemotactic response of 145 ± 6.24 cells (mean \pm SD) when at a concentration of $5\mu\text{g/ml}$ in the lower compartment, compared with a migratory response of 96 ± 5.29 (mean \pm SD) when this concentration was present in both compartments. Maximal chemokinetic response of 91.33 ± 6.03 cells (mean \pm SD) was established when at a concentration of $5\mu\text{g/ml}$ in the upper compartment. Therefore, the magnitude of the maximal chemokinetic response was approximately 63% of the maximal chemotactic response.

UPPER LOWER	0	5	10	20
0	9.3 +/- 1.5	91.33 +/- 6.03	42.67 +/- 5.03	34.33 +/- 5.51
5	145 +/- 6.24	96 +/- 5.29	54.67 +/- 3.06	47.33 +/- 8.02
10	85.67 +/- 5.51	73 +/- 5	49.67 +/- 2.08	42.67 +/- 4.04
20	67.67 +/- 6.51	55 +/- 5.57	34.33 +/- 9.71	32.67 +/- 3.21

Table 3.1. Checkerboard analysis of PHT stimulatory effect on normal fibroblast migration (nf-4). Values are means +/- SD of three identical wells for each combination. Grey square = migration to serum-free DMEM only; Yellow squares = chemokinetic responses; Blue squares = chemotactic responses; Green squares = combined responses.

3.3. Immunohistochemical evaluation of the effect of Phenytoin on urokinase-type plasminogen activator expression

The transition of a stationary cell to a motile cell is associated with the expression of enzymes responsible for the degradation of the extracellular matrix. In order to investigate the effect of PHT on the expression of one such protease, u-PA was visualised using indirect immunofluorescence. Cultures of both normal (nf-7) and EB fibroblasts (ebf-2) stained positive for u-PA only after PHT treatment (Fig. 3.8.C, D and F). When cells of subconfluent areas were observed, it was found that this antigen was associated with the cell membranes (Fig. 3.8.D&F) and not all cells expressed u-PA to the same extent.

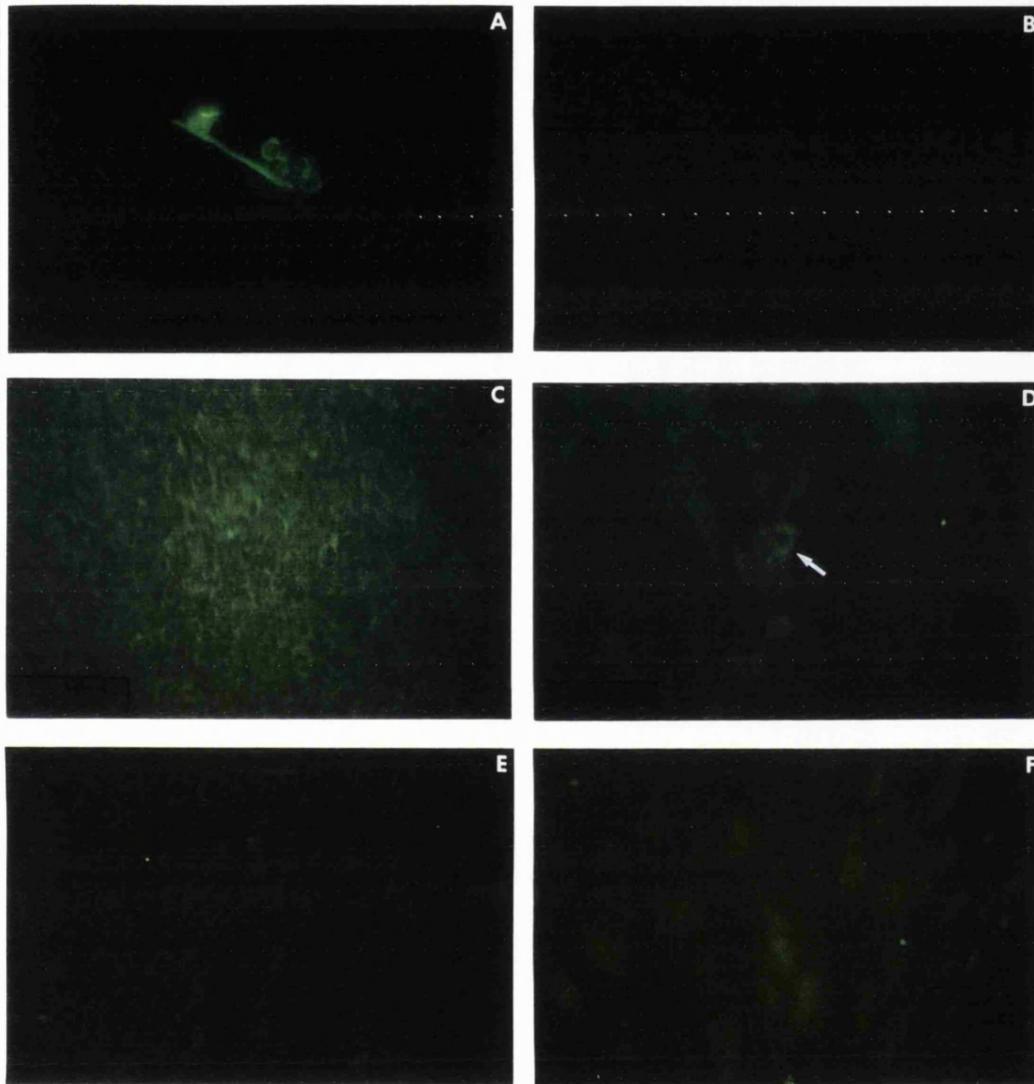


Figure 3.8. The effect of PHT (20 μ g/ml, for 7 days) u-PA expression in normal (nf-7) and EB (ebf-2) fibroblasts. A = positive control (human kidney fragment); B = normal fibroblasts (nf-7) grown without PHT; C = normal fibroblasts (nf-7, confluent area) grown with PHT; D = normal fibroblasts (nf-7, subconfluent area) grown with PHT; E = EB fibroblasts (ebf-2) grown without PHT; F = EB fibroblasts (ebf-2, subconfluent area) grown with PHT.

3.4. Immunohistochemical evaluation of Phenytoin uptake by normal fibroblasts

The mechanism by which PHT upregulates connective tissue activity may be initiated by the formation of reactive metabolites. During metabolic conversion a small amount of drug (Fig. 1.7) may bind to cell components (e.g. DNA, RNA, proteins and phospholipids). In order to evaluate this hypothesis, indirect immunofluorescence staining was carried out on normal fibroblasts (nf-4) which were incubated with and without PHT (20 μ g/ml). After 24 hours, PHT was found to be in all treated fibroblasts to a variable extent (Fig. 3.9). This suggests that either not all cells take up, bind or metabolise PHT the same way, or perhaps these processes are cell cycle-dependent. In cells where PHT was found in abundance, it was bound around the nucleus.

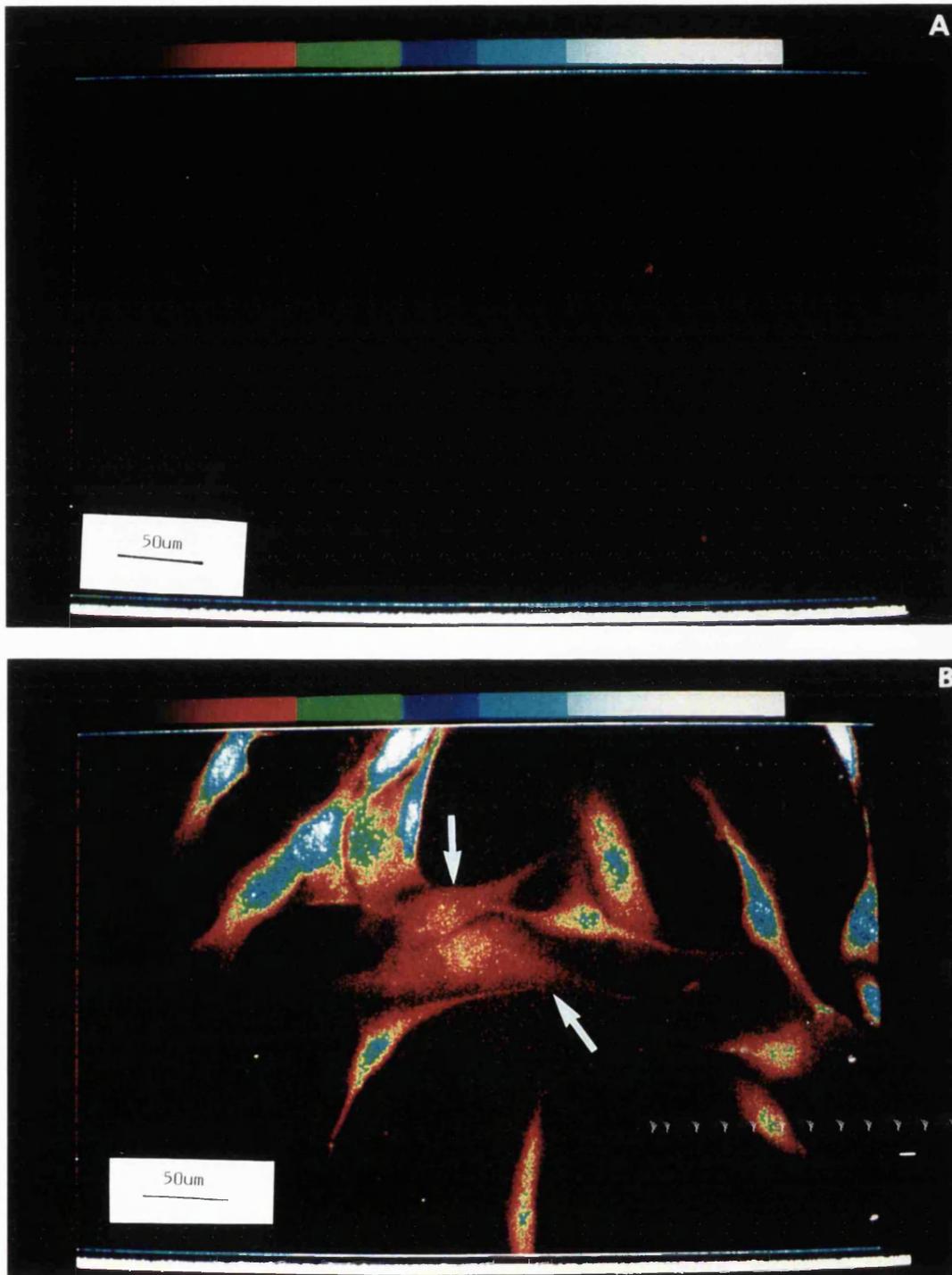


Figure 3.9. The differential uptake, metabolism and binding of PHT by human dermal fibroblasts. A = control, normal fibroblasts (nf-4) grown without PHT; B = normal fibroblasts (nf-4) grown in the presence of PHT (20µg/ml, for 24 hours). Only cells treated with PHT stained positive. The bar at the top of the picture indicates the intensity range (red being the least and white the most intense) of the falsely colour-coded microscopic image depicting the mean counts per pixel. Fibroblasts indicated with the white arrow contained the least amounts of PHT.

3.5. The effect of Phenytoin on fibroblast-mediated collagen gel contraction

3.5.1. Measurements using untethered fibroblast-populated collagen lattices

Experiments using untethered FPCLs enabled the comparison of contractile profiles of normal and EB fibroblasts over a period of seven days and the investigation of the long-term effects of PHT treatment on contraction.

3.5.1.1. Comparison of contraction of a normal and an Epidermolysis Bullosa fibroblast cell line

This pilot study was carried out to compare the contractile profiles of a normal and an EB fibroblast cell line. Both normal (nf-7) and EB (ebf-2) fibroblasts were able to contract collagen lattices by exertion of contractile forces within the gel. Figure 3.10. shows the reduction of surface area of FPCL gels recorded over 7 days. Cell-free gels did not undergo significant contraction. EB fibroblasts contracted significantly more than normal fibroblasts at all time points (t-test, all $P < 0.00001$). Within 24 hours normal fibroblasts reduced the gel area to 59% of its original size, and EB fibroblasts to 42%. At the end of the experiment, normal FPCLs contracted to 37% of their original area and EB FPCLs to 14%, which represented an additional contraction of 23%.

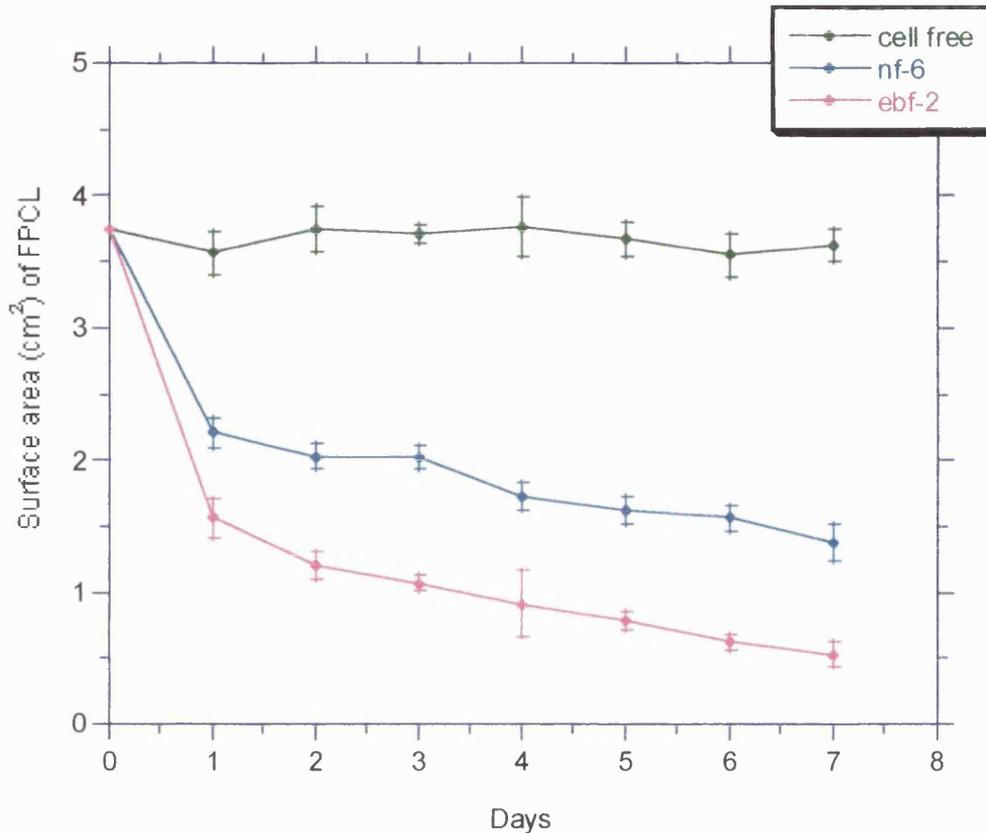


Figure 3.10. Comparison of contraction of one normal dermal (nf-7) and one EB (ebf-2) cell line in untethered FPCLs. The contraction of gels without cells is represented in green. (Error bars indicate the means of 12 well readings +/-SD.)

3.5.1.2. The effect of Phenytoin on the contraction of a normal fibroblast cell line

The effects of two concentrations of PHT on normal fibroblasts (nf-7) are represented in Figure 3.11. Whilst 10 μ g/ml PHT produced no significant change in contraction, 20 μ g/ml significantly reduced contraction between days 1 and 7 (t-test, all $P < 0.0002$), decreasing the contraction of normal cells maximally by 15% (day 7).

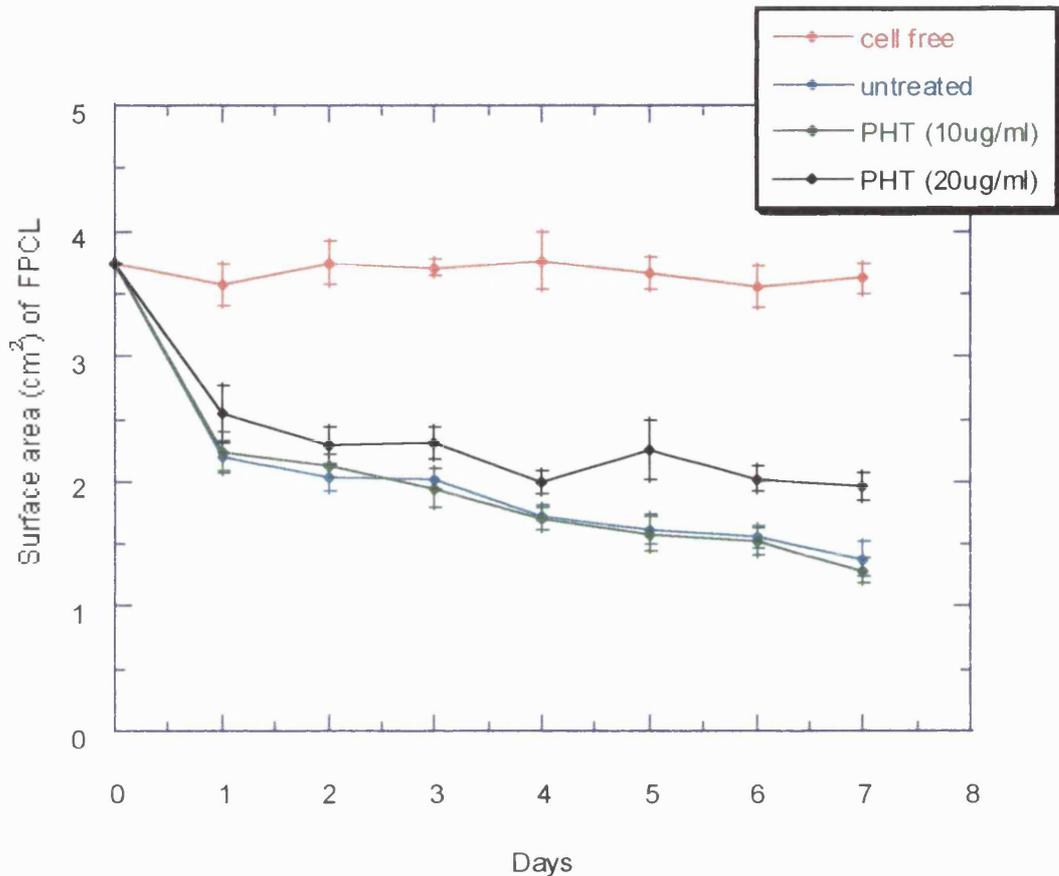


Figure 3.11. The effect of PHT on the contraction of a normal dermal cell line (nf-7) in untethered FPCLs. Immediately after casting the FPCLs, the gels were incubated with medium containing 10 μ g/ml (green) or 20 μ g/ml PHT (black). FPCLs without PHT and gels without cells are represented by blue and red lines respectively. (Error bars indicate the means of 12 readings \pm SD.)

3.5.1.3. The effect of Phenytoin on the contraction of an Epidermolysis Bullosa fibroblast cell line

When applied to EB cells (ebf-2), PHT (20 μ g/ml) produced a significant decrease in contraction to levels similar to normal fibroblasts (nf-7), as seen on Figure 3.12. Hence, between days 1 and 7, contraction of EB cells with PHT was higher than normal, but significantly lower than that of untreated EB fibroblasts (t-test, all $P < 0.0002$). Maximal reduction of contraction was seen after 7 days amounting to a difference of 16%.

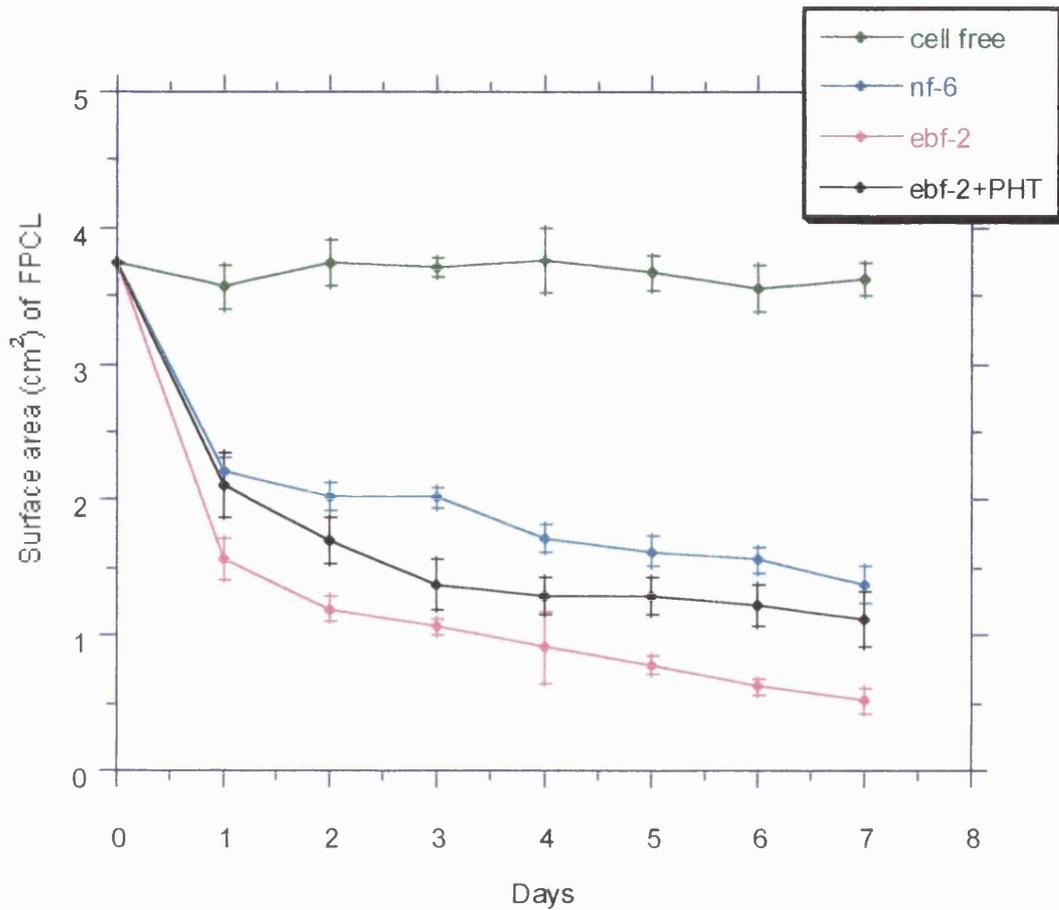


Figure 3.12. The effect of PHT on the contraction of an EB cell line in untethered FPCLs. The difference in contractile behaviour of normal FPCLs (nf-7, blue) and EB FPCLs (ebf-2, red) was reduced upon incubation of EB fibroblasts with 20 μ g/ml PHT (black) immediately after the gels were cast. The contraction of cell-free gels is represented in green. (Error bars indicate the means of 12 well readings \pm SD.)

3.5.2. Measurements using the Culture Force Monitor (a tethered fibroblast-populated collagen lattice model)

The CFM provides an accurate, computerised measurement of the direct force exerted by fibroblasts in a tethered, type I collagen matrix over 24 hours. The graphs presented in this study show the net contractile force generated. Thermal drift of the force transducer and contractile force of the collagen gel itself were deducted and the results were standardised to Force per 10^6 cells.

Figure 3.13. shows the characteristic contraction pattern of a normal dermal fibroblast cell line (nf-4) as measured in the CFM.

Forces (10^{-5} Newtons/million cells) generated by the cells were monitored over two stages: primary phase (I), from the start of the contraction, until peak levels were reached by 6-10 hours) followed by a secondary phase (ii). Previous work with the CFM has shown that the contraction of the primary phase corresponds closely with cell attachment and extension of cell processes into the collagen matrix (Eastwood *et al.*, 1996). In the secondary phase, cells achieve and maintain tensional homeostasis against external loading exerted by the surrounding matrix.

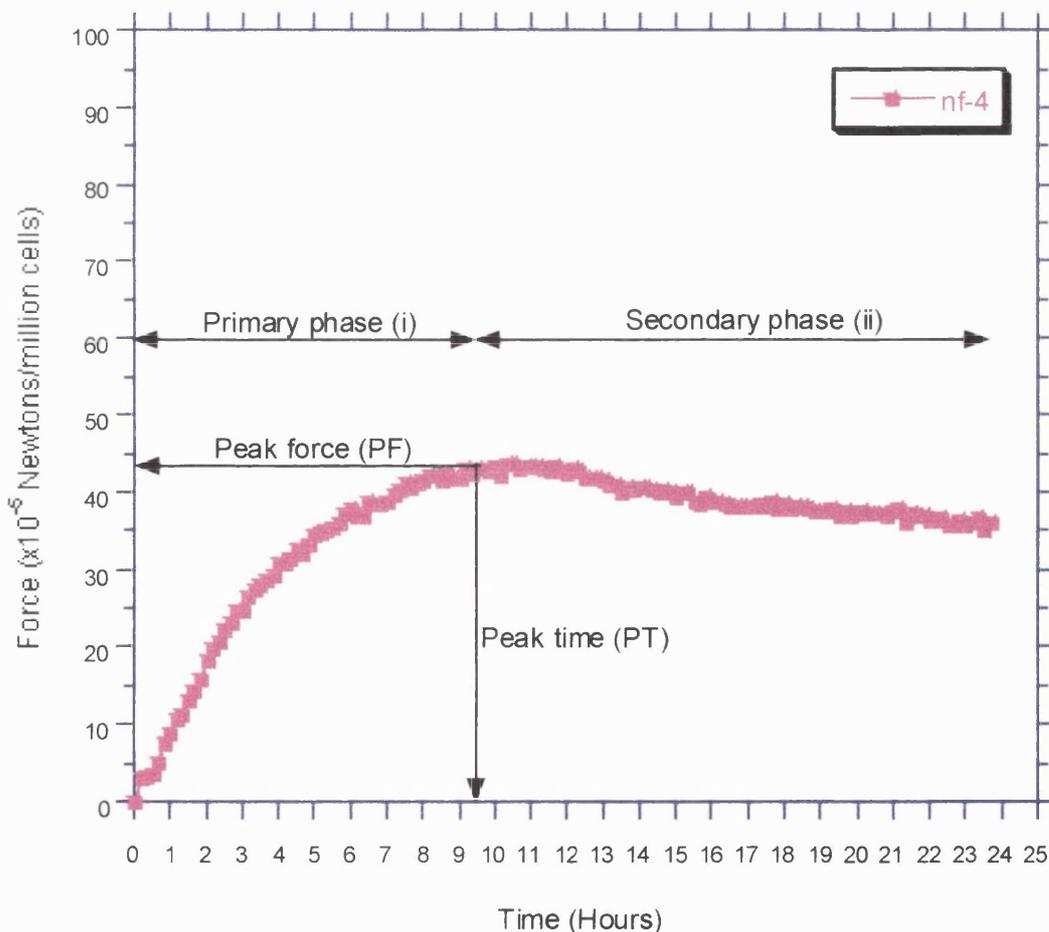


Figure 3.13. Contraction analysis of a normal fibroblast cell line (nf-4) in the Culture Force Monitor. The characteristic contraction pattern is composed of a primary phase (i), until maximum force is achieved (PF), which is followed by a secondary phase (ii) in which force levels reach equilibrium and tensional

homeostasis is achieved. In this case the $PF = 43.1 \times 10^{-5}$ Newtons/million cells, $PT = 9.66$ hours. Each data point is an average of 600 readings per 10 minutes.

3.5.2.1. Comparison of contraction of normal and Epidermolysis Bullosa fibroblasts

Fibroblasts from EB skin produced significantly greater contractile forces than normal fibroblasts. Figure 3.14. shows the contraction patterns of one normal (nf-4) and one EB (ebf-2) cell lines.

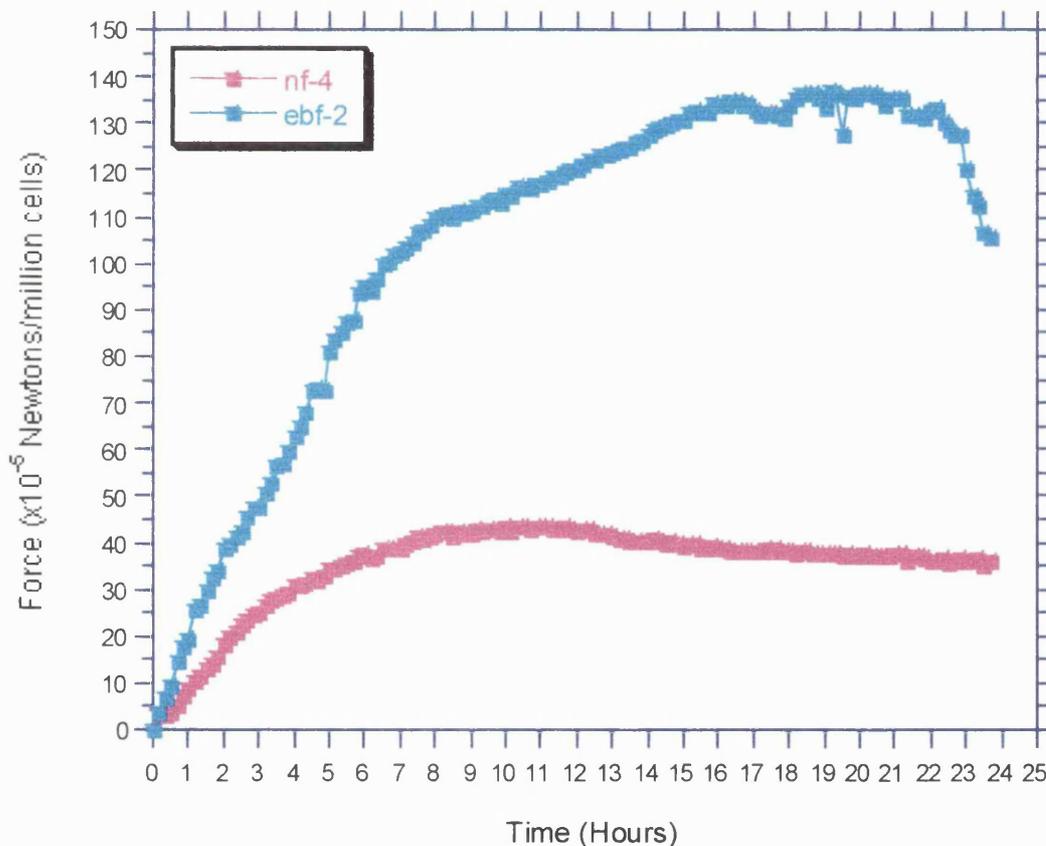


Figure 3.14. Comparison of contraction of normal dermal and EB fibroblasts in the Culture Force Monitor. EB cells (ebf-2) displayed a much greater force exertion than normal fibroblasts (nf-4). Moreover, this EB cell line produced an additional contraction during this secondary phase, a phenomenon not observed in any of the 6 normal cell lines investigated. Each data point is an average of 600 readings per 10 minutes.

Analysis of the CFM data measurements (as described previously in section 3.5.2.) produced measures of peak force, time to reach peak force (end of the primary contraction) and also the initial gradient of contraction (between 1 and 4 hours) was also calculated. Table 3.2. summarises the analysis of 6 normal cell lines (nf-4, 6, 7, 10, 11 and 12) and 3 EB cell lines (ebf-2,3 and 5).

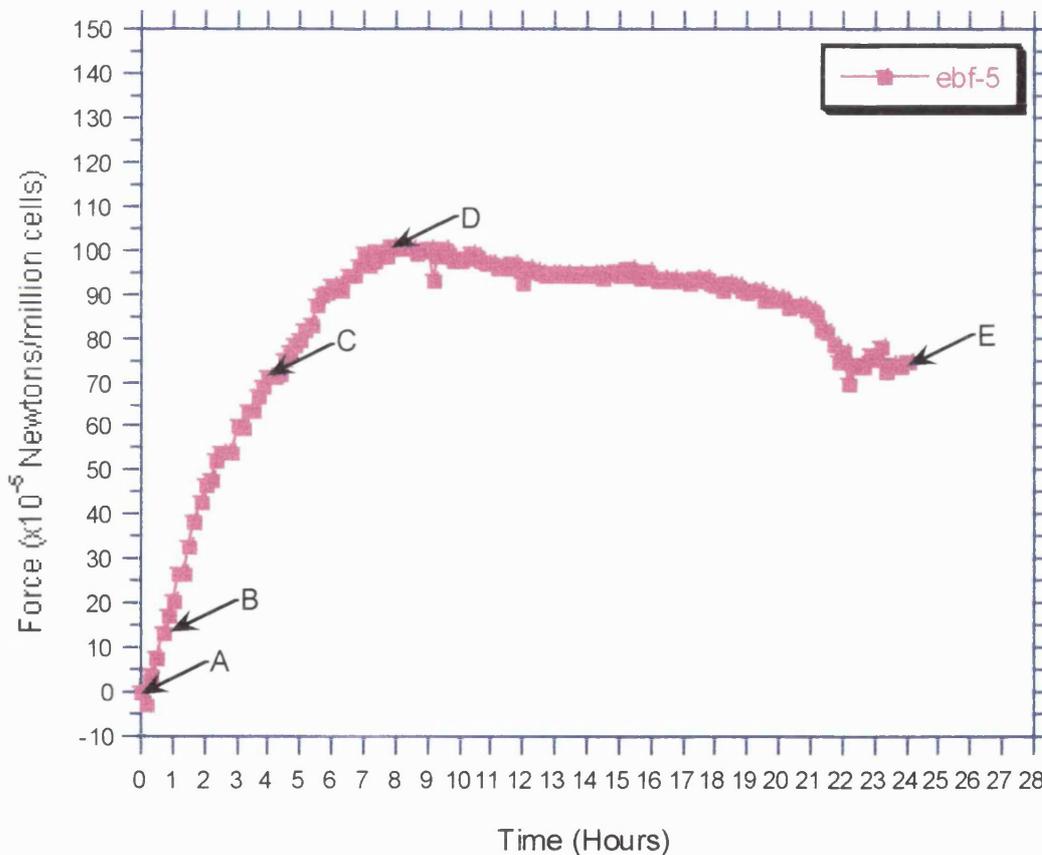
Cell type	Number of cell lines tested (n)	Mean peak time (hr +/-SD)	Mean peak force ($\times 10^{-5}$ N +/-SD)	Mean initial gradient between 1 and 4 hrs ($\times 10^{-5}$ N/hr +/-SD)
Normal	6	7.92+/-1.8	40.12+/-15.55	7.04+/-3.28
EB	3	8.22+/-0.01	98.13+/-16.83	15.39+/-1.31

Table 3.2. Contraction analysis of 6 normal and 3 EB fibroblast cell lines.

EB cells exhibited approximately 2.5 times stronger significant mean peak contraction than normal fibroblasts (Fisher-Behrens test, $P < 0.01$). However, both cell lines took the same time to reach their primary peaks. This was also reflected in the gradient measure, in which normal cells had a significantly slower rate of contraction (2.2 times) than EB cells (Fisher-Behrens test, $P = 0.001$).

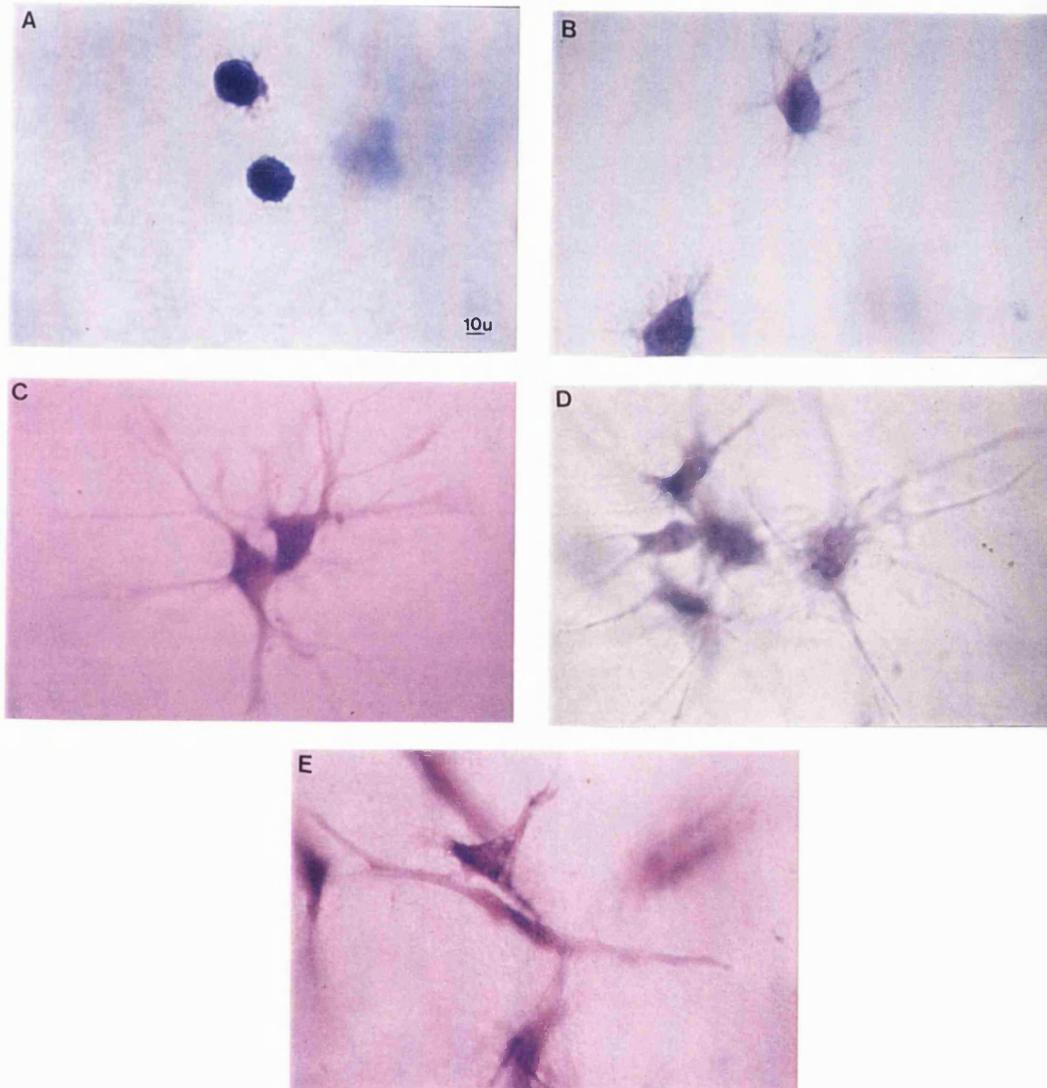
The individual contractile profiles of nf-7 and ebf-2 fibroblast cell lines were also compared as measured in the CFM and untethered FPCLs. Although both test systems demonstrated that EB cells were hypercontractile, the magnitude of the difference seen compared with normal fibroblasts was strikingly different. In the CFM, the direct force of the contraction displayed by EB cells (102.4×10^{-5} Newtons/million cells) was measured to be 6.4 times greater normal cells (15.9×10^{-5} Newtons/million cells), whereas in the untethered system gel surface area measurements suggested a mere 1.3 times difference (Fig.3.10).

EB fibroblast (ebf-5) morphology was also observed by stereo microscopy at various time points during contraction of the collagen lattice (Fig. 3.15). Cells were round and with few processes and no filapodia were present at 0 hours. By 1 hour initial elongation of the processes had occurred; these were further extended after 4 hours and the characteristic stellate cell shape was seen by 8 hours. At 24 hours the majority of cells were bipolar. The same pattern of shape change was seen as for normal cells (Eastwood *et al.*, 1996), indicating that there were no gross alterations in cell behaviour in EB fibroblasts.



a.

Figure 3.15. Stereo microscopic examination of EB fibroblasts (Fig. 3.15.b) fixed during the various stages of contraction in the CFM (Fig. 3.15.a) at A = 0 hr, B = 1 hr, C = 4 hr, D = 8 hr and E = 24 hr. Note that EB fibroblasts (ebf-5) were capable of extending cell processes in the same way as normal fibroblasts over 24 hours. Scale bar = 10 μ m for pictures A – E. (Figure 3.15 is continued on the next page.)



b.

Figure 3.15 (continued). Stereo microscopic examination of EB fibroblasts (Fig. 3.15.b) fixed during the various stages of contraction in the CFM (Fig. 3.15.a) at A = 0 hr, B = 1 hr, C = 4 hr, D = 8 hr and E = 24 hr. Note that EB fibroblasts (ebf-5) were capable of extending cell processes in the same way as normal fibroblasts over 24 hours. (Scale bar = 10 μ m for pictures A - E)

3.5.2.2. The effect of Phenytoin (single dose) on cell contraction

Addition of the drug solvent media alone (125 μ l DMSO + 375 μ l complete DMEM, equivalent to the maximum amount of DMSO added as a drug solvent) was shown to have a transient relaxing effect on gel contraction (Fig. 3.16), approximately -5.5×10^{-5} Newtons/million cells. After full recovery, the peak force of both treated and untreated cells was similar at 24 hours. Likewise, the 'vehicle' alone [40 μ l 'vehicle' + 460 μ l complete DMEM (10% FCS)], equivalent to the amount added to achieve 20 μ l/ml PHT in the CFM) at 20 hours had minimal effects on contraction (-3.85×10^{-5} Newtons/million cells, with total recovery to tensional homeostasis within 2.5 hours) in another cell line (nf-9, not shown).

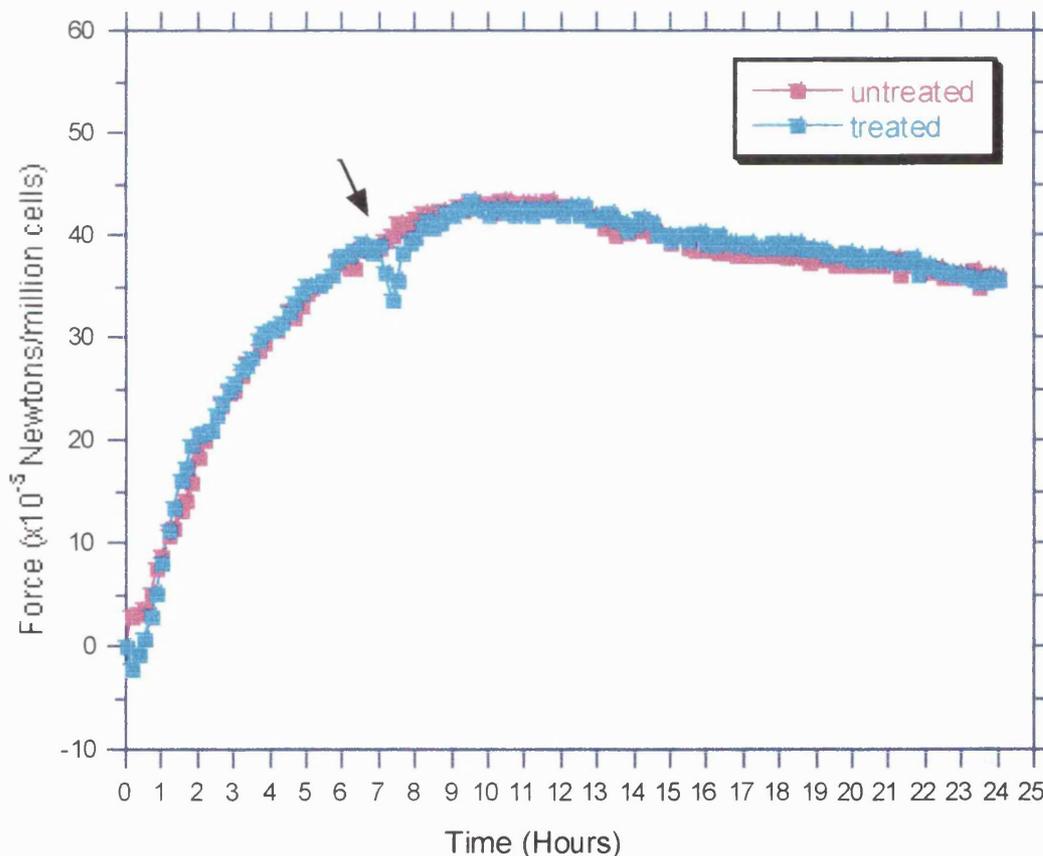


Figure 3.16. The effect of a single administration of drug solvent media (125 μ l DMSO + 375 μ l complete DMEM, represented by the arrow) on the contraction of normal fibroblasts (nf-4). Note the near perfect reproduction of the contractile profile and the restoration of the tensional homeostatic level in less than 3 hours.

Addition of a single dose of PHT (20 μ g/ml) around the end of the primary contraction phase induced a further peak of contraction in normal fibroblasts (Fig. 3.17). This effect of PHT (single administration to previously untreated cells) and other drugs was evaluated as follows:

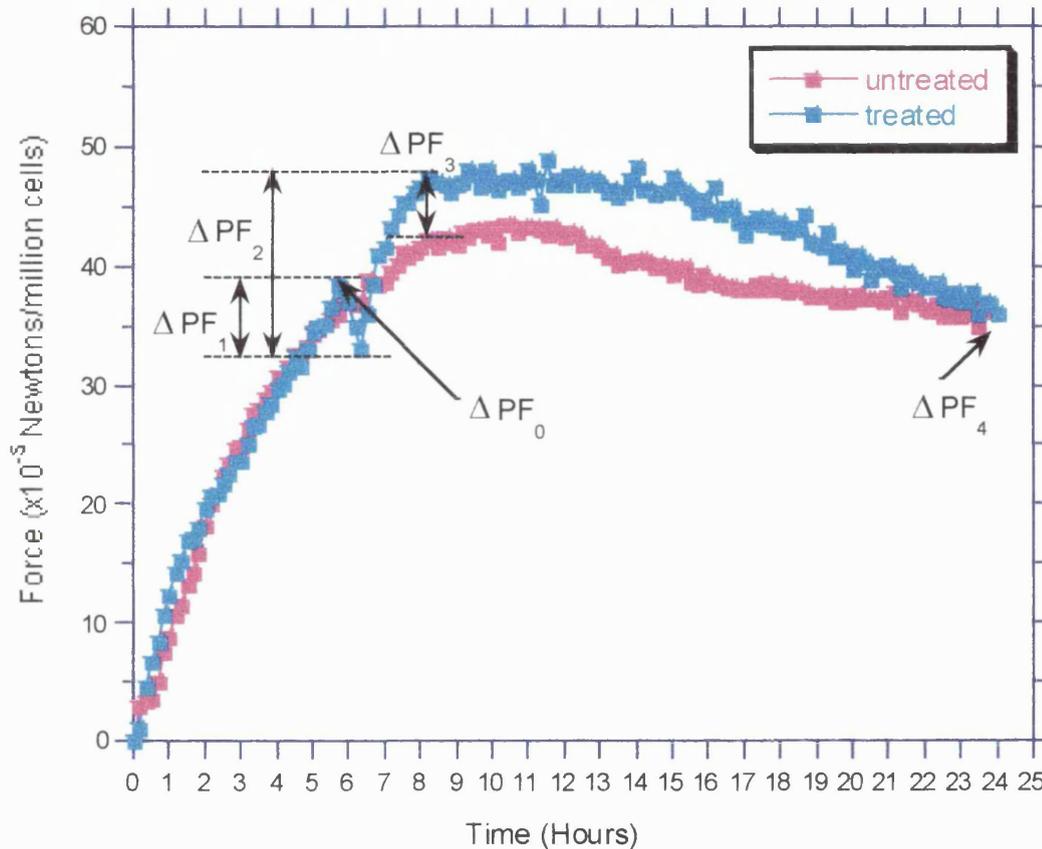


Figure 3.17. The effect of a single administration of PHT (20 μ g/ml or 7.3x10⁻⁵ M PHT made up in 'vehicle') at 5.83 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +0.66 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -5.02 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_1 = 0.5 \text{ hours};$$

$$\Delta PF_2 = +12.49 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_2 = 1.17 \text{ hours};$$

$$\Delta PF_3 = +4.32 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = +0.03 \times 10^{-5} \text{ Newtons/million cells}.$$

Firstly, the difference in peak force (ΔPF_0) between the control and the experimental contraction curve was calculated at the time of drug administration. This was indicative of the precision of the experimental set-up. Then, the change in peak force (ΔPF_1) was determined after drug addition to evaluate whether it was similar to that caused by the addition of drug solvent media alone. In this case $\Delta PF_1 = -5.02 \times 10^{-5}$ Newtons/million cells, indicating that it could be associated with the addition of media only. The time to reach this dip was calculated to be $\Delta T_1 = 0.5$ hours. PHT induced a further contraction ($\Delta PF_2 = +12.49 \times 10^{-5}$ Newtons/million cells), which was achieved within $\Delta T_2 = 1.17$ hours. This peak was greater ($\Delta PF_3 = +4.32 \times 10^{-5}$ Newtons/million cells) than the maximum primary contraction force of the fibroblasts. Interestingly, the contraction force exerted by both untreated and treated fibroblasts was determined to be the same after 24 hours ($\Delta PF_4 = +0.03 \times 10^{-5}$ Newtons/million cells).

As numerous reports claimed that PHT inhibits tubulin polymerisation (thereby inducing metaphase arrest) and that PHT has Colchicine-like activity in various cell types (Estus and Blumer 1989; MacKinney *et al.*, 1984, 1980, 1978), the effects of this drug were also investigated in the CFM. The contraction peak produced by the addition of PHT was compared with that of COL (a known microtubule poison) at equimolar concentration (Fig. 3.18) in the same cell line. Although the magnitude of the extra peak (ΔPF_2) produced by COL was twice that induced by PHT, the difference in force displayed between treated and untreated cells (ΔPF_3) was 5.5 times greater with COL.

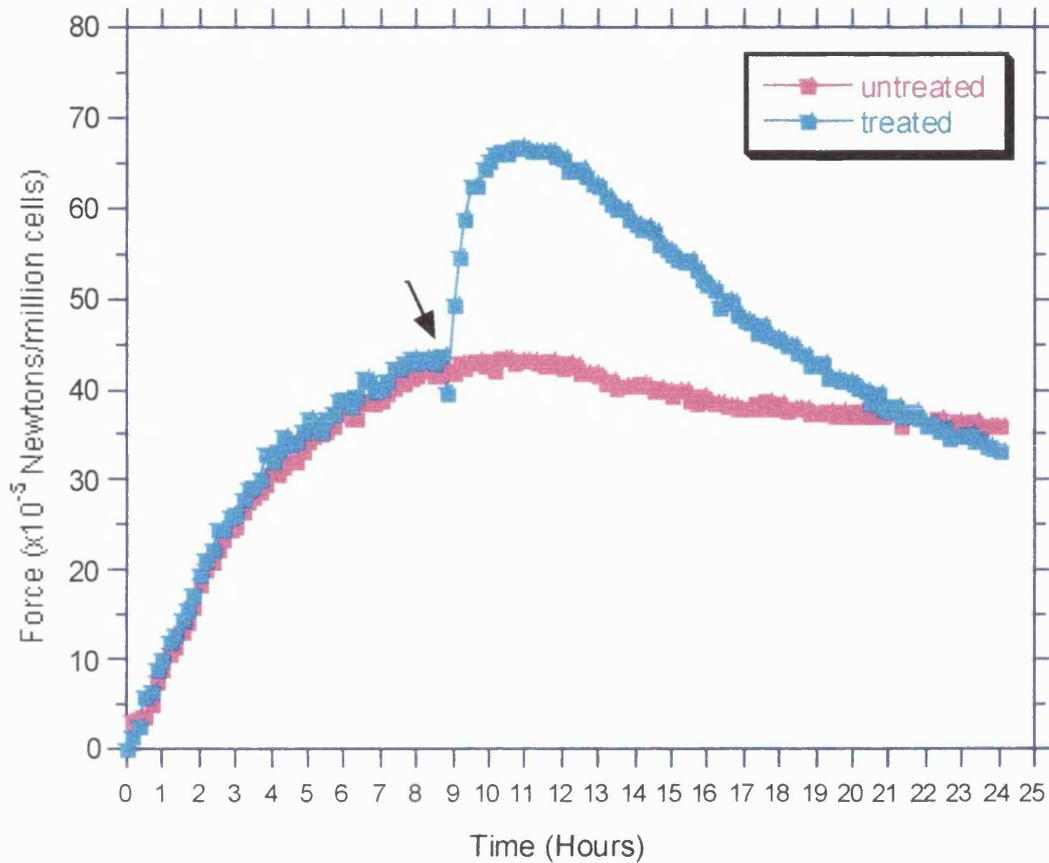


Figure 3.18. The effect of a single administration of COL (7.3×10^{-5} M, represented by the arrow) at 8.67 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +1.26 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -4.11 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 0.16 \text{ hours;}$$

$$\Delta PF_2 = +27.3 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 2.0 \text{ hours;}$$

$$\Delta PF_3 = +23.64 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -2.7 \times 10^{-5} \text{ Newtons/million cells.}$$

The acute effects of PHT (20µg/ml or 7.3x10⁻⁵ M PHT made up in 'vehicle') were also tested in 3 EB cell lines (Table 3.3) around the end of the primary contraction phase. As for normal fibroblasts (Fig. 3.17), PHT induced a further contraction in all cases. Although there were some deviations between individual responses, this peak was greater in EB fibroblasts (based on the larger ΔPF₂ and ΔPF₃ values) and was achieved during a longer time period (ΔT₂) than in normal fibroblasts.

Cell line	Δ PF ₀	Δ PF ₁	Δ T ₁	Δ PF ₂	Δ T ₂	Δ PF ₃	Δ PF ₄
ebf-2	+3.1	-1.42	0.5	+37.45	4.33	+9.33	-1.69
ebf-3	-0.91	-5.96	1.5	+16.07	3.83	+10.79	+3.55
ebf-5	-2.38	-5.34	1.0	+37.8	2.33	+8.73	-2.48

Table 3.3. Contraction analysis of the effect of single administration of PHT (20 µg/ml or 7.3x10⁻⁵ M PHT made up in 'vehicle'), around the end of the contraction phase in three EB cell lines. (ΔPF values = x10⁻⁵ Newtons/million cells, ΔT values = hours)

3.5.2.3. The effect of Phenytoin pre-treatment on cell contraction

A marked difference in contraction was seen when normal fibroblasts were pre-treated for five days with PHT (20 µg/ml or 7.3x10⁻⁵ M) as seen on Figure 3.19. Although the Peak Force value was reduced by 52%, there was little difference in contraction (ΔPF) at the end of the experiment at 24 hours.

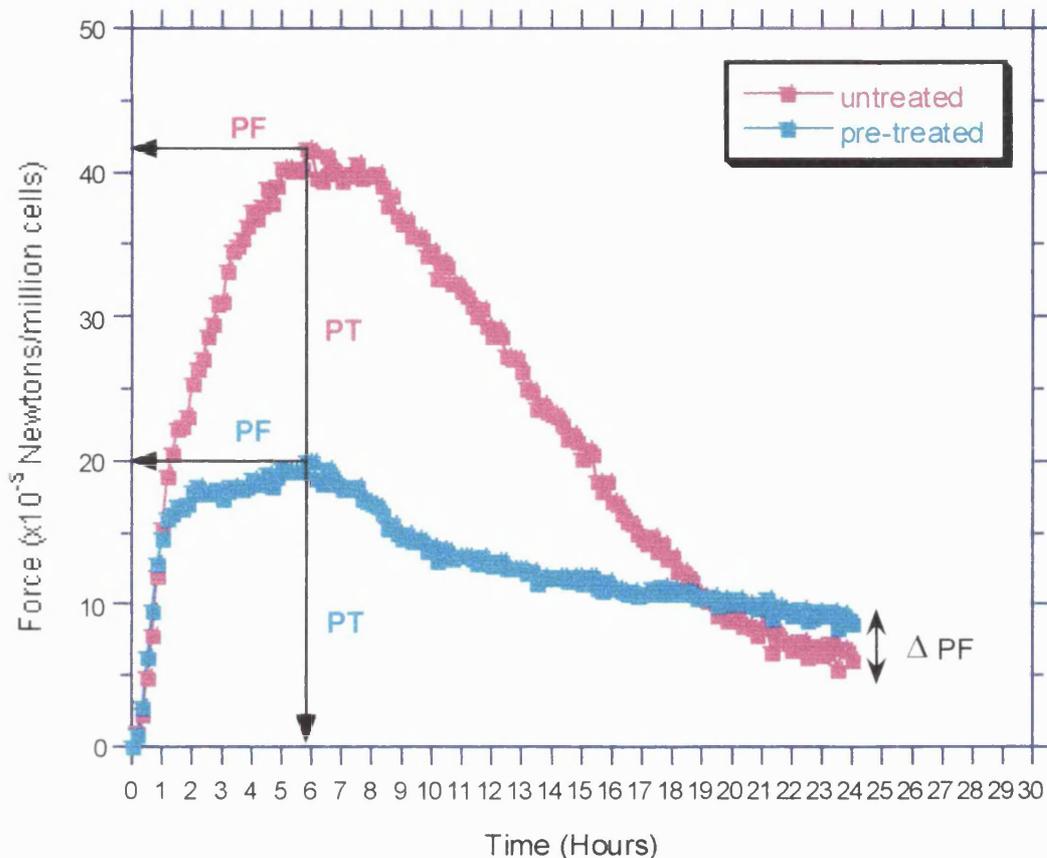


Figure 3.19. The effect of PHT pre-treatment (20 $\mu\text{g/ml}$ or 7.3×10^{-5} M PHT made up in 'vehicle', for 5 days) on the contraction of normal fibroblasts (nf-8). PHT was also added at this concentration to the culture media of the CFM. Contraction analysis: PF = 41.59×10^{-5} Newtons/million cells, PT = 5.83 hours (untreated); PF = 20.02×10^{-5} Newtons/million cells, PT = 5.83 hours (treated); $\Delta\text{PF} = +2.64 \times 10^{-5}$ Newtons/million cells.

Similarly, as seen in normal fibroblasts, chronic PHT treatment also reduced contraction significantly in EB fibroblasts (Table 3.4). Although the magnitude of this effect was variable according to cell line, it was maintained (ebf-5) or increased (ebf-2 and 3) at the end of the experiment at 24 hours.

Cell line	Treatment	PT	PF	Peak force reduction (%)	Δ PF
ebf-2	Control	8.5	110.64	28	-37.36
	PHT	8.33	79.36		
ebf-3	Control	8.33	80.02	23	-34.7
	PHT	7.66	61.45		
ebf-5	Control	8.33	101.09	63	-53.12
	PHT	6	37.38		

Table 3.4. The effect of PHT pre-treatment (20 µg/ml or 7.3×10^{-5} M PHT made up in 'vehicle', for 5 days), on the contraction of EB fibroblasts. PHT was also added at this concentration to the culture media of the CFM. (PF and ΔPF values = $\times 10^{-5}$ Newtons/million cells, PT values = hours).

3.5.2.4. Investigation of the effects of Phenytoin and Colchicine on cell contraction and microtubule structure

These experiments were carried out to further elaborate on any similarities between the activities of PHT and COL. Firstly, the efficacy of COL was investigated on PHT pre-treated cells. Secondly, parallel with this, the tubulin structure of cells grown on glass sides undergoing similar drug treatments was observed by indirect immunostaining for β-tubulin.

As before, PHT pre-treatment (for five days with 20 µg/ml or 7.3×10^{-5} M PHT) induced a significant change in contraction, and did not depend on PHT being present in the media during the CFM measurement (Fig. 3.20). Although the Peak Forces (PF) were equivalent in the pre-treated cells with and without PHT in the culture media, Peak Times (PT) were remarkably different. Moreover, the peak associated with COL was greatly reduced in PHT pre-treated cells. Addition of extra COL to these cells failed to elicit further responses.

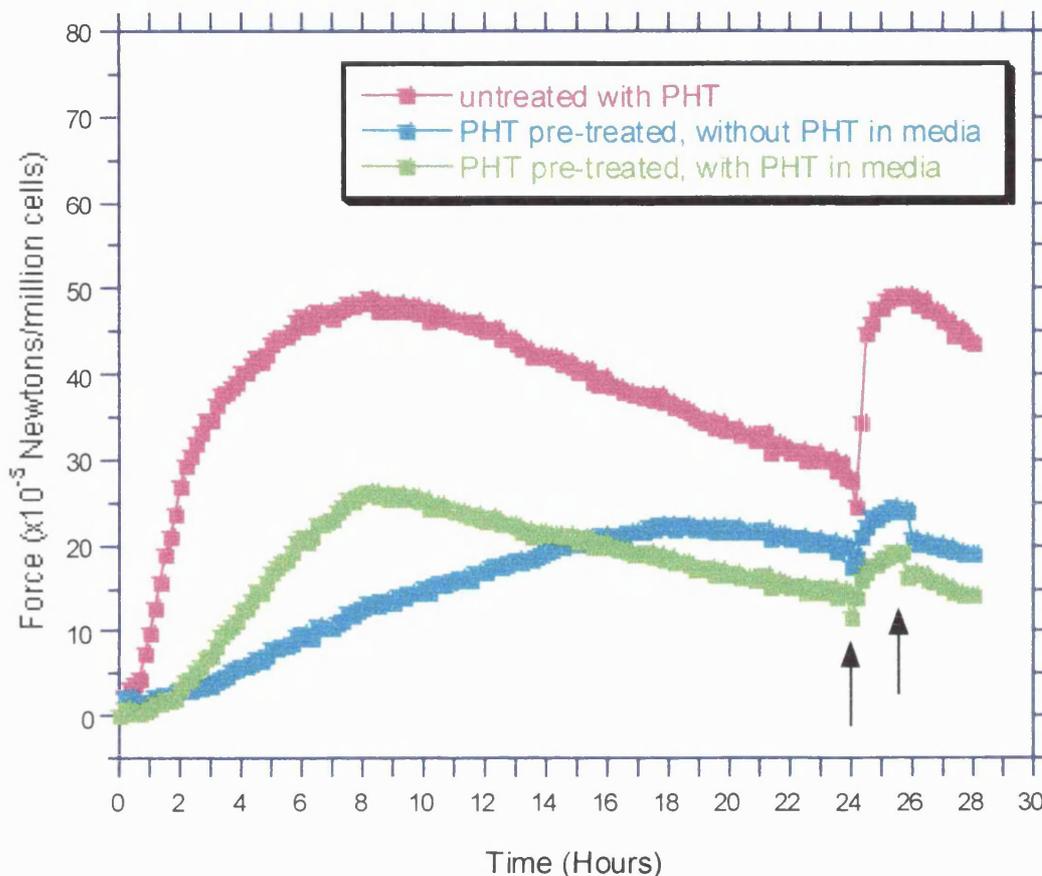


Figure 3.20. The effect of PHT pre-treatment treatment ($20 \mu\text{g/ml}$ or 7.3×10^{-5} M PHT made up in 'vehicle', for 5 days), on the contraction of normal fibroblasts (nf-4) and their response to COL.

Analysis of the effect of PHT pre-treatment on contraction: $\text{PF} = 48.92 \times 10^{-5}$ Newtons/million cells, $\text{PT} = 8.17$ hours (control); $\text{PF} = 22.47 \times 10^{-5}$ Newtons/million cells, $\text{PT} = 18$ hours (pre-treated -PHT in medium); $\text{PF} = 26.49 \times 10^{-5}$ Newtons/million cells, $\text{PT} = 8.17$ hours (pre-treated +PHT in medium).

Analysis of the effect of PHT pre-treatment on the contraction response to COL (equimolar amount): the initial reduction of contraction associated with drug addition was less than 3×10^{-5} Newtons/million cells in all cases. COL induced Peak Force values: $+24.54 \times 10^{-5}$ Newtons/million cells (control) with a Peak Time of 1.16 hours; $+7.14 \times 10^{-5}$ Newtons/million cells (pre-treated -PHT in medium) with a Peak Time of 1.33 hours; $+7.933 \times 10^{-5}$ Newtons/million cells (pre-treated +PHT in medium) with a Peak Time of 1.33 hours. Further addition of COL (7.3×10^{-5} M) produced no significant changes.

As both PHT and COL induced a contraction peak in untreated cells in the CFM (Fig 3.17&18, respectively), their effects on the integrity of microtubules were also examined at 7.3×10^{-5} M concentrations. Cells grown on glass slides received the same drug treatment as described above (Fig. 3.20). However, COL was applied only once (Figure 3.21). PHT alone did not affect microtubule structure, even when applied at high concentrations (Figure 3.22) in a separate experiment. COL, on the other hand, induced the total disruption of microtubules, as expected. PHT pre-treatment of cells ($20 \mu\text{g/ml}$ or 7.3×10^{-5} M PHT for 6 days) did not however affect the ability of COL to disrupt microtubules (with or without PHT in the culture medium).

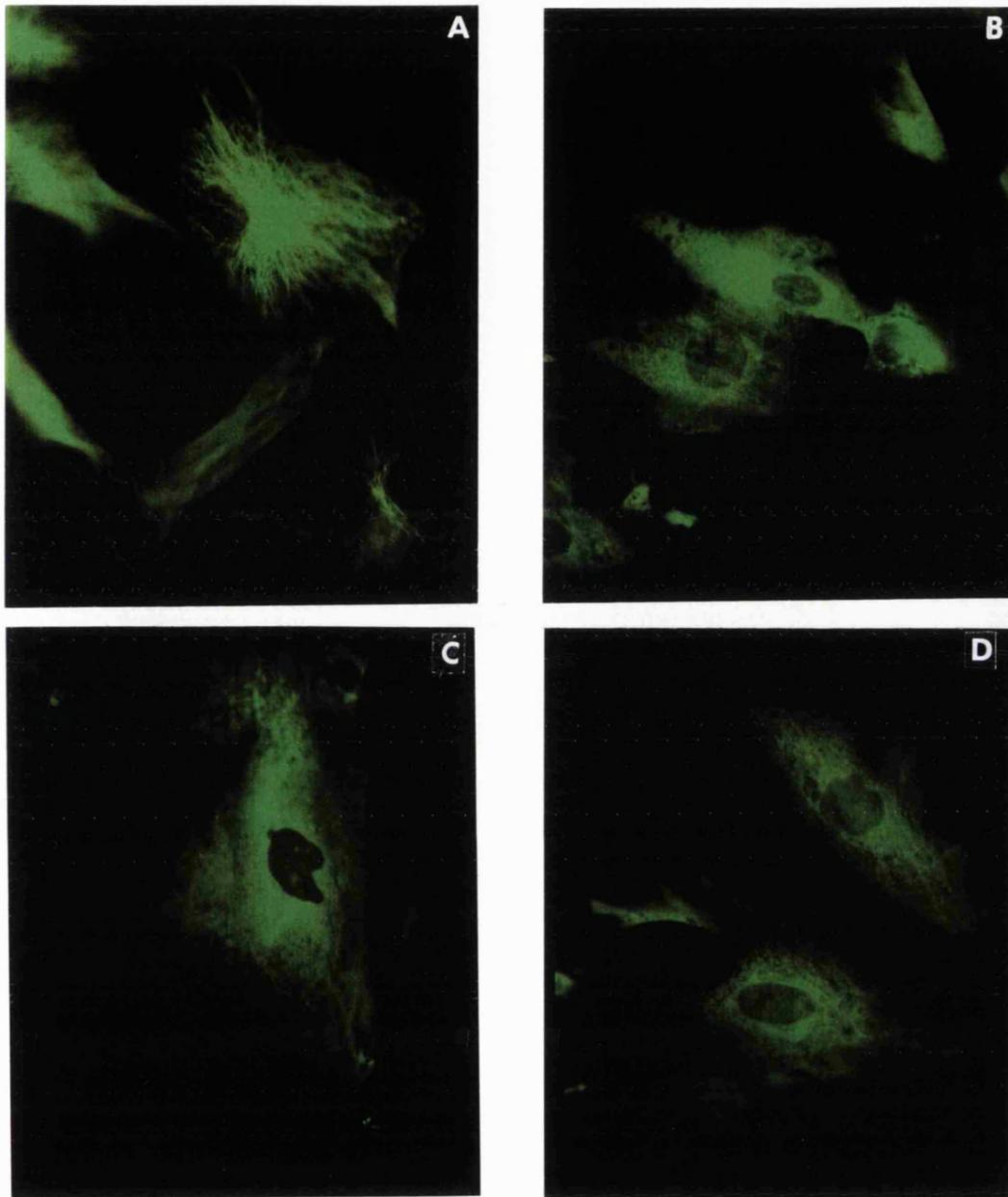


Figure 3.21. The effect of PHT pre-treatment treatment ($20 \mu\text{g/ml}$ or $7.3 \times 10^{-5} \text{ M}$ PHT made up in 'vehicle', for 5 days), on the structure of microtubules of normal fibroblasts (nf-4) and their response to COL (equimolar amount, 6 hours).

The micrograph shows indirect immunofluorescent (FITC) staining for β -tubulin

of A = Control cells (untreated); B = Cells treated with COL only;

C = PHT pre-treated cells plus COL (without PHT in culture media);

D = PHT pre-treated cells plus COL (with PHT in culture media). The structure of microtubules was intact in control cells (A) and disrupted after COL treatment (B). COL remained to be effective on PHT pre-treated cells both in the presence and absence of PHT in the medium (C&D).

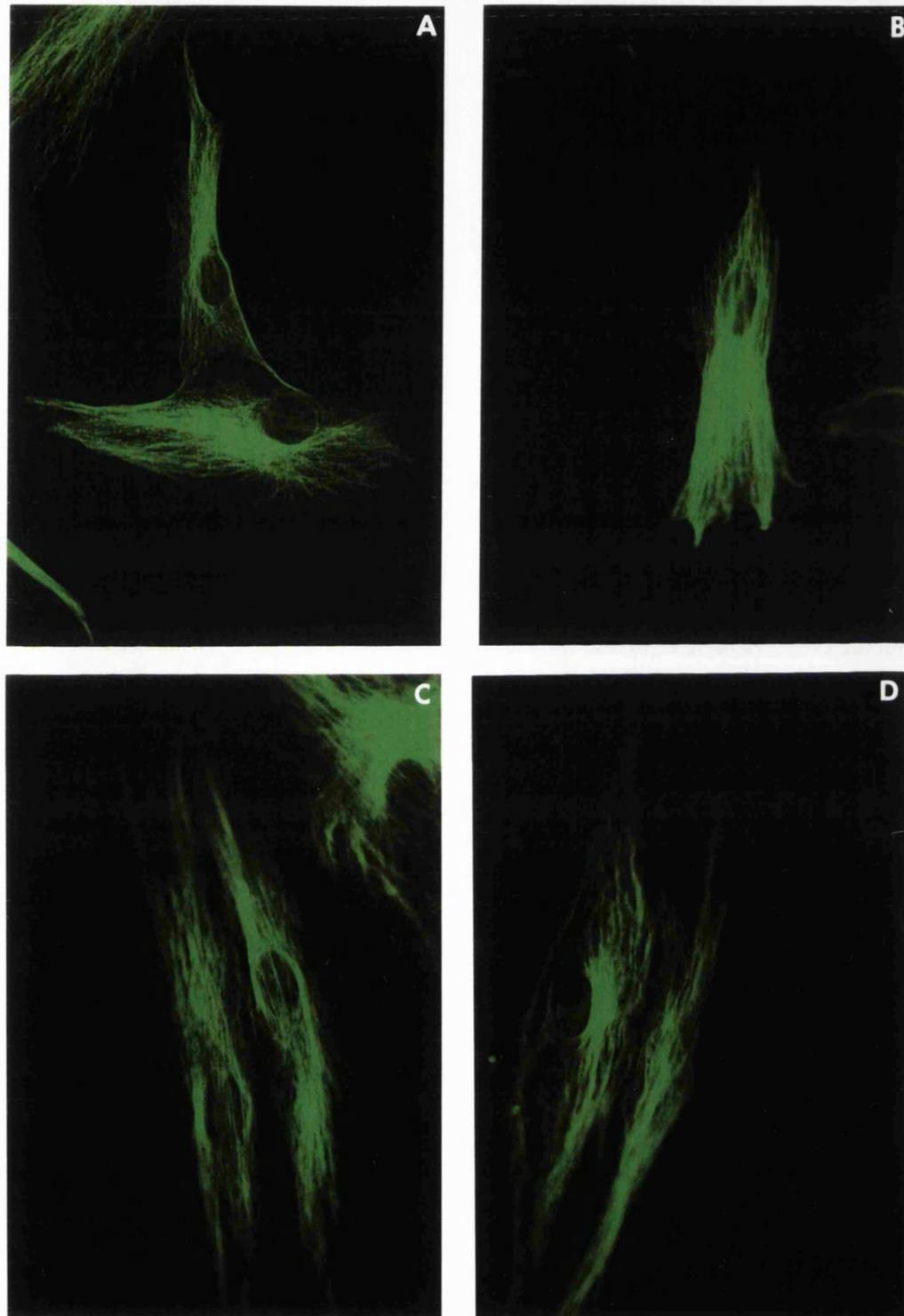


Figure 3.22. The effect of increasing concentrations of PHT on the structure of microtubules of normal fibroblasts (nf-4). The micrograph shows indirect immunofluorescent (FITC) staining for β -tubulin of A = Control cells (untreated), B = Cells incubated with PHT ($20\mu\text{g/ml}$, 7.3×10^{-5} M); C = Cell incubated with PHT ($50\mu\text{g/ml}$, 18.25×10^{-5} M); D = Cells incubated with PHT ($100\mu\text{g/ml}$, 36.5×10^{-5} M). PHT did not disrupt tubulin at any concentrations.

3.6. Investigations on the activities of compounds structurally related to Hydantoin

3.6.1. Comparison of Hydantoin-related compounds as chemoattractants

Compounds structurally related to HYD were compared for their ability to induce cell migration in the Micro Chemotaxis Chamber (Fig. 3.23), as described before. Although it was previously established that the optimum concentration was 1.82×10^{-5} M (equivalent to $5 \mu\text{g/ml}$ PHT) for normal fibroblasts in general (based on the pooled data from nf-1-5), the cell line used in this test displayed maximum migration in response to 3.64×10^{-5} M PHT and therefore such concentrations were used.

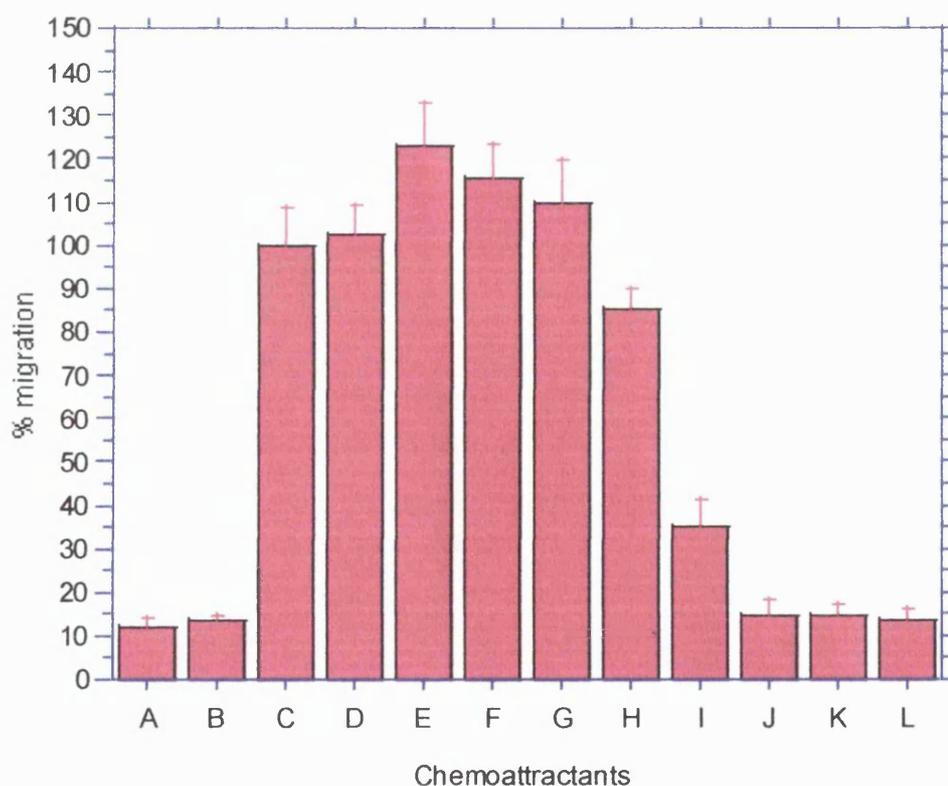


Figure 3.23. The effect of HYD derivatives on normal fibroblast (nf-4) migration. Values are expressed as percentages of the positive control, FN (100%), and were the combined results of two Micro Chemotaxis Chamber assays. A = Serum-free media; B = DMSO ($1 \mu\text{l/ml}$); C = FN ($2.5 \mu\text{g/ml}$); D = PHT; E = m-HPPH; F = p-HPPH; G = (MePh)-PH; H = Me-PH; I = ALLAN; J = HYD; K = HYDAC; L = HYDACAC.

DMSO, HYD, HYDAC, HYDACAC were found to have no chemotactic effects, while the remaining compounds all increased cell migration significantly (t-test, all $P < 0.0001$). Both m-HPPH and p-HPPH were better chemoattractants than PHT (t-test, $P = 0.0017$ and 0.0064 respectively), while (MePh)-PH was equal to it. Me-PH and ALLAN were both less potent than PHT.

Therefore, the activity of compounds structurally related to HYD could be ranked as follows:

m-HPPH > p-HPPH > (MePh)-PH \geq PHT > Me-PH \gg ALLAN \gg [HYD = HYDAC = HYDACAC = SFM]

3.6.2. The effects of Hydantoin-related compounds on cell contraction

The effects of compounds structurally related to HYD (single dose) were investigated using the CFM (Fig. 3.24-3.31). In addition, cytoskeletal poisons were tested for comparison (Fig. 3.32-3.33). The effects of PHT and COL were shown previously on Figures 3.17 and 3.18 respectively. Contraction profiles were analysed as described earlier in section 3.5.2.2. 'The effect of PHT (single dose) on cell contraction'.

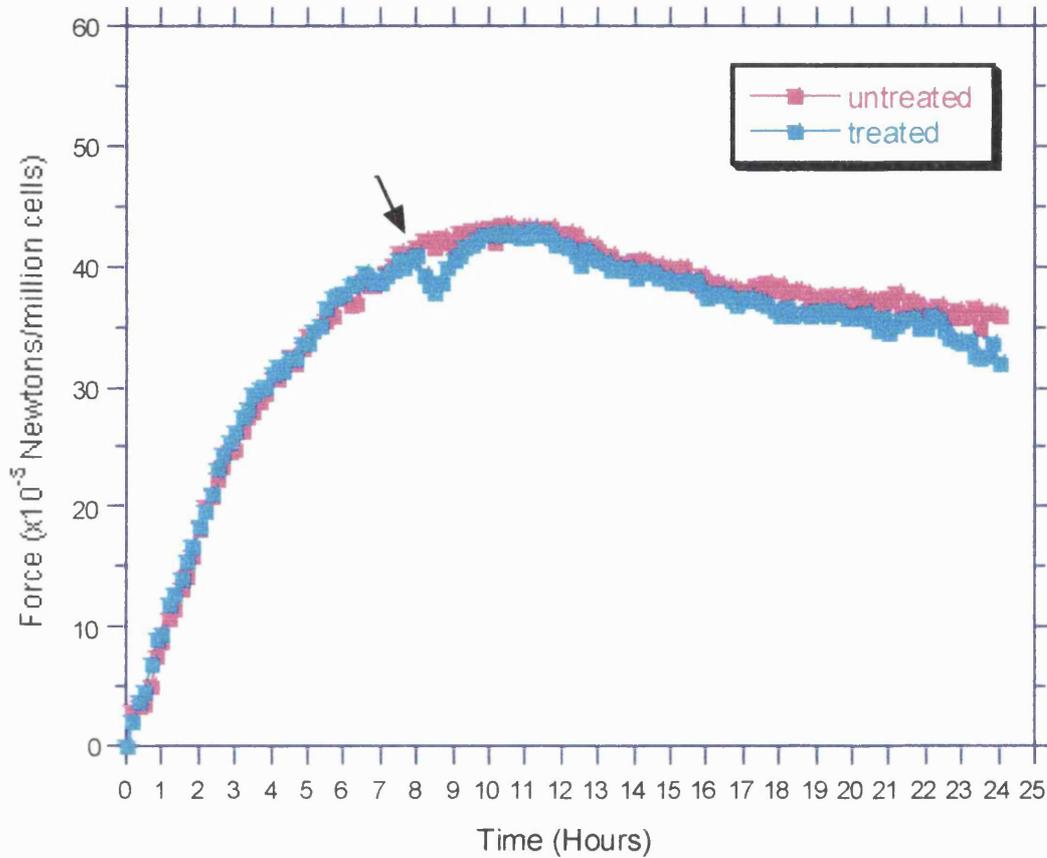


Figure 3.24. The effect of a single administration of HYD (7.3×10^{-5} M, represented by the arrow) at 8 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = -0.72 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -3.05 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 0.5 \text{ hours};$$

$$\Delta PF_2 = +4.99 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 1.83 \text{ hours};$$

$$\Delta PF_3 = -0.69 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = -3.89 \times 10^{-5} \text{ Newtons/million cells.}$$

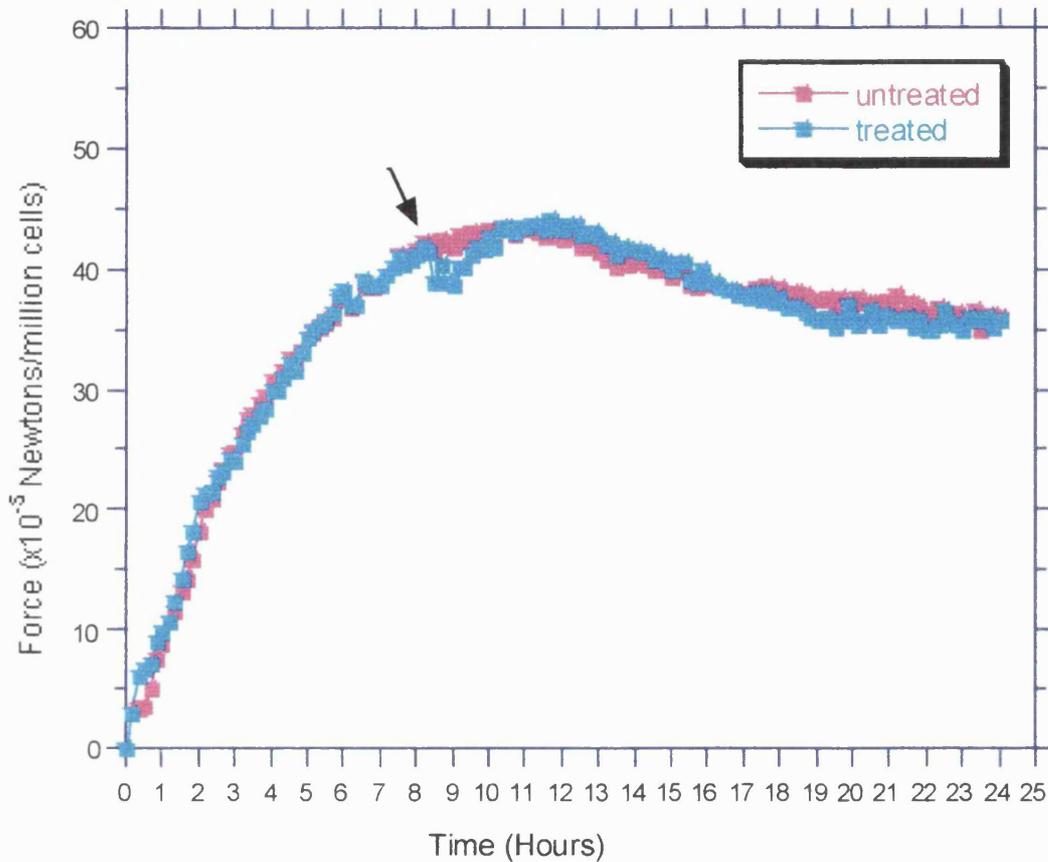


Figure 3.25. The effect of a single administration of HYDAC (7.3×10^{-5} M, represented by the arrow) at 8.3 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = -0.74 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -2.69 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_1 = 0.67 \text{ hours};$$

$$\Delta PF_2 = +4.78 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_2 = 1.5 \text{ hours};$$

$$\Delta PF_3 = +0.05 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = -0.24 \times 10^{-5} \text{ Newtons/million cells}.$$

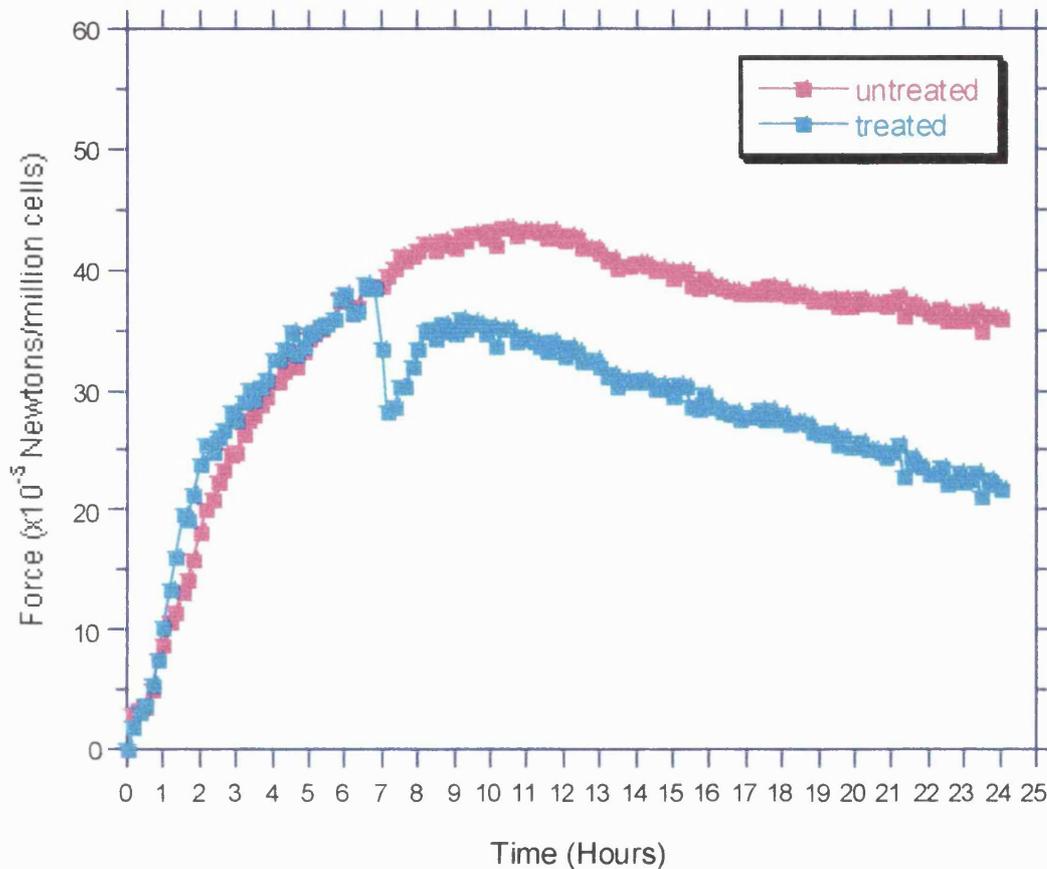


Figure 3.26. The effect of a single administration of ALLAN (7.3×10^{-5} M, represented by the arrow) at 6.67 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = -0.05 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -10.36 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_1 = 0.5 \text{ hours};$$

$$\Delta PF_2 = +7.36 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_2 = 1.5 \text{ hours};$$

$$\Delta PF_3 = +6.91 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = -14.11 \times 10^{-5} \text{ Newtons/million cells}.$$

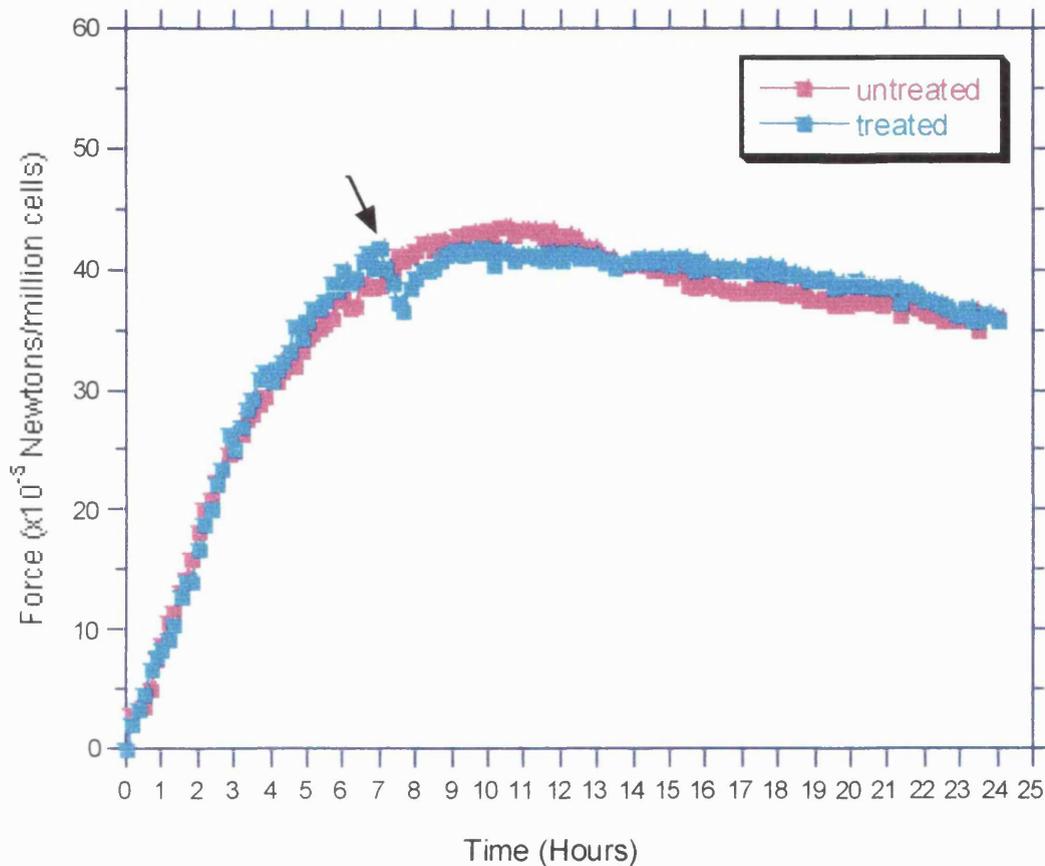


Figure 3.27. The effect of a single administration of HYDACAC (7.3×10^{-5} M, represented by the arrow) at 7 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +3.0 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -5.05 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_1 = 0.67 \text{ hours};$$

$$\Delta PF_2 = +5.0 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_2 = 1.4 \text{ hours};$$

$$\Delta PF_3 = -1.22 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = -0.1 \times 10^{-5} \text{ Newtons/million cells}.$$

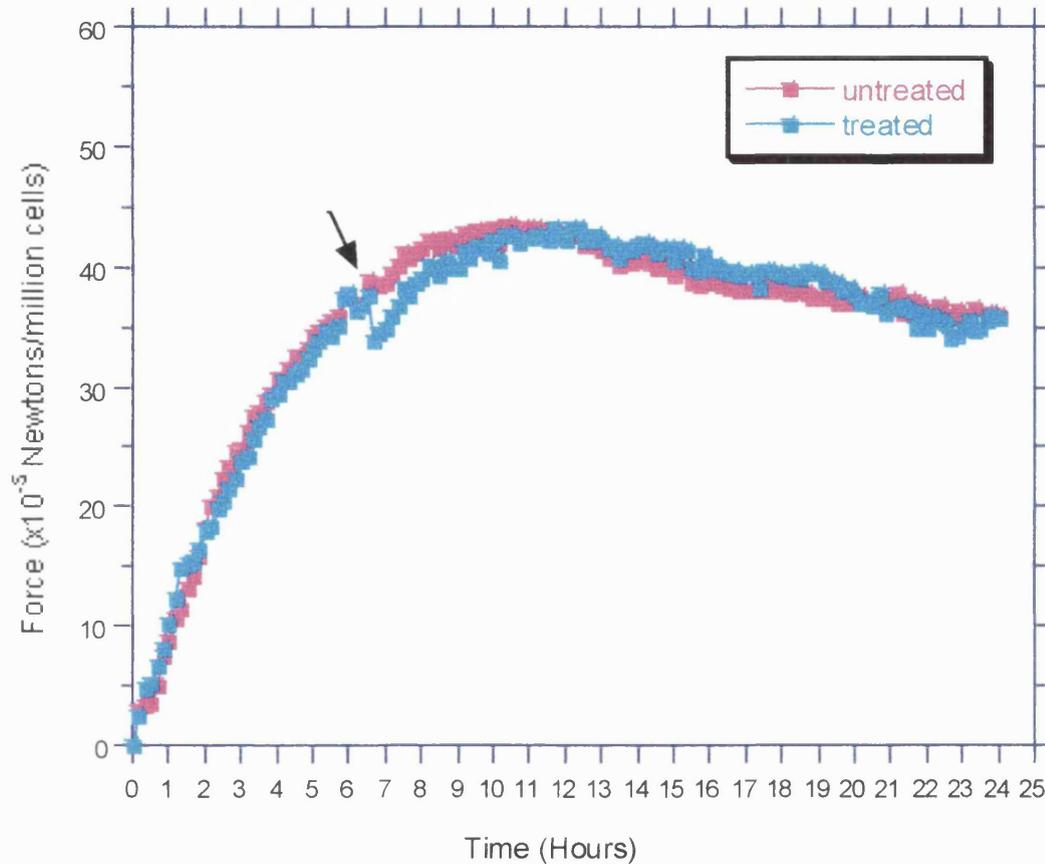


Figure 3.28. The effect of a single administration of Me-PH (7.3×10^{-5} M, represented by the arrow) at 6.5 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = -1.13 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_1 = -3.57 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_1 = 0.17 \text{ hours};$$

$$\Delta PF_2 = +5.65 \times 10^{-5} \text{ Newtons/million cells}, \Delta T_2 = +5.17 \text{ hours};$$

$$\Delta PF_3 = +0.004 \times 10^{-5} \text{ Newtons/million cells};$$

$$\Delta PF_4 = -0.1 \times 10^{-5} \text{ Newtons/million cells}.$$

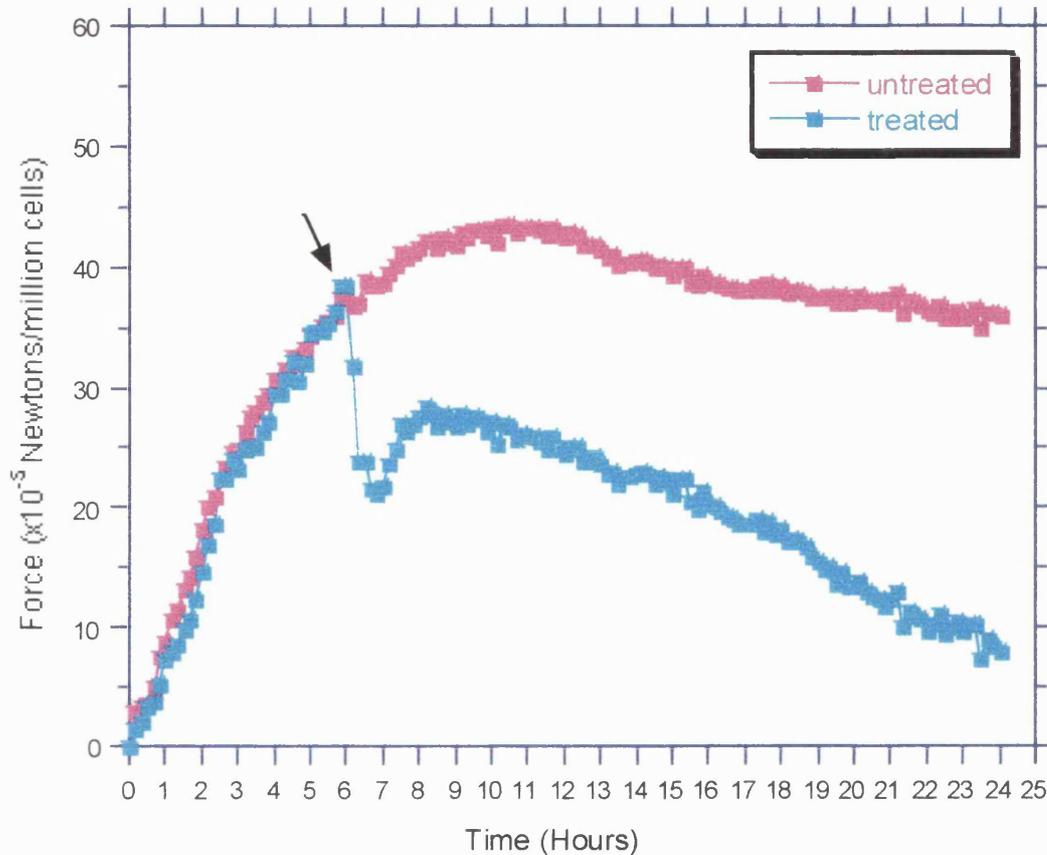


Figure 3.29. The effect of a single administration of (MePh)-PH (7.3×10^{-5} M, represented by the arrow) at 6 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +0.83 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -17.48 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 0.83 \text{ hours;}$$

$$\Delta PF_2 = +7.29 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 1.34 \text{ hours;}$$

$$\Delta PF_3 = -13.85 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -27.89 \times 10^{-5} \text{ Newtons/million cells.}$$

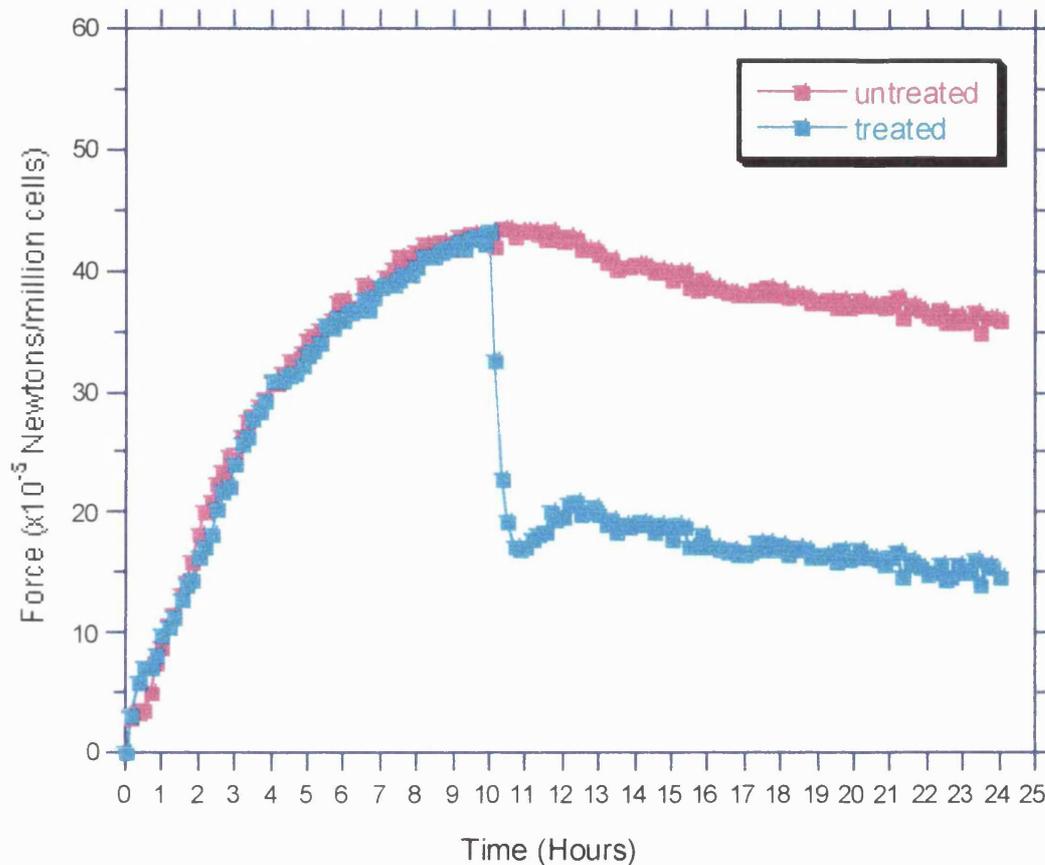


Figure 3.30. The effect of a single administration of p-HPPH (7.3×10^{-5} M, represented by the arrow) at 10 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +0.006 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -26.28 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 0.83 \text{ hours;}$$

$$\Delta PF_2 = +3.8 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 1.5 \text{ hours;}$$

$$\Delta PF_3 = -21.85 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -21.21 \times 10^{-5} \text{ Newtons/million cells.}$$

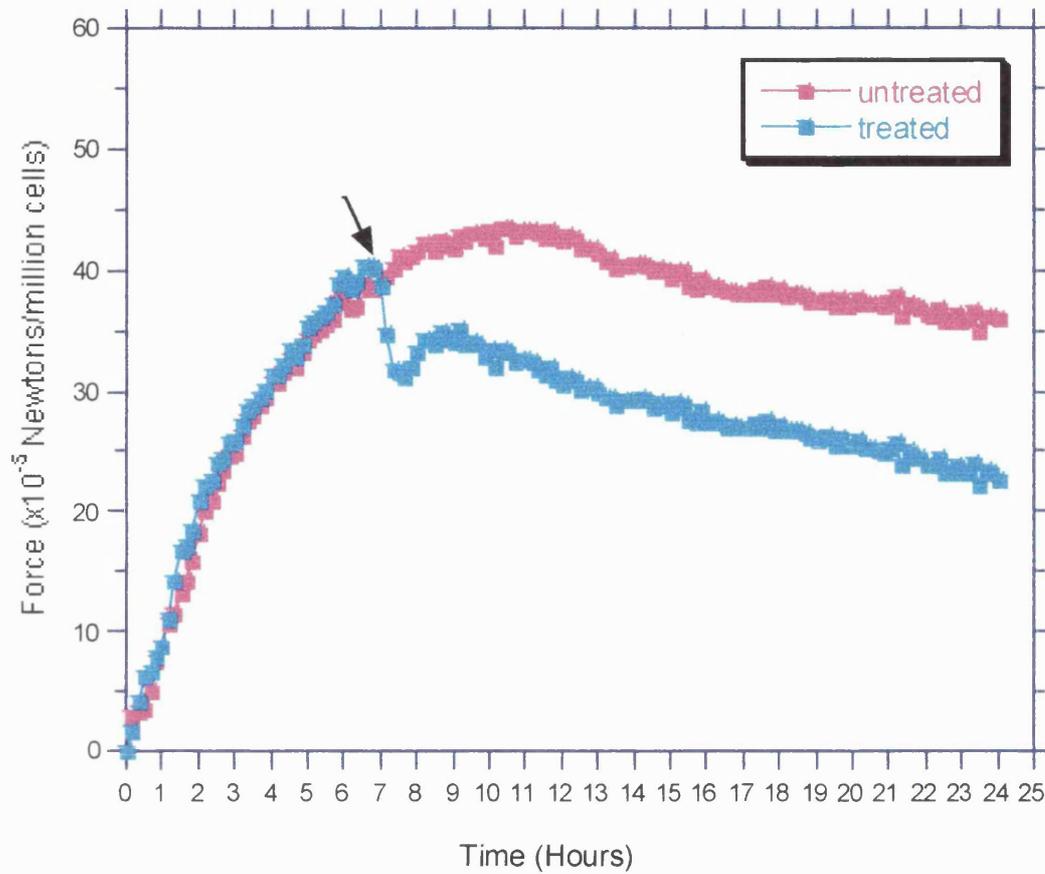


Figure 3.31. The effect of a single administration of m-HPPH (7.3×10^{-5} M, represented by the arrow) at 6.83 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +1.59 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -9.03 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 0.84 \text{ hours;}$$

$$\Delta PF_2 = +3.93 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 1.5 \text{ hours;}$$

$$\Delta PF_3 = -7.86 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -13.36 \times 10^{-5} \text{ Newtons/million cells.}$$

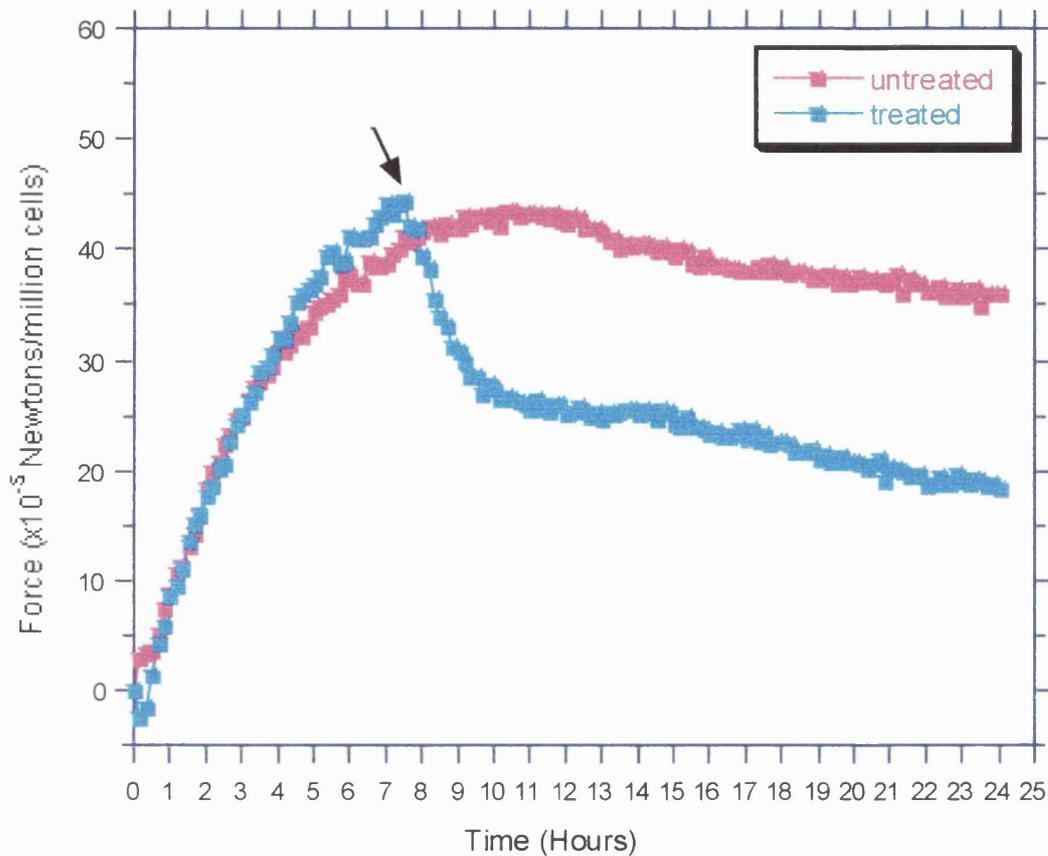


Figure 3.32. The effect of a single administration of TAX (7.3×10^{-5} M, represented by the arrow) at 7.5 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = +3.26 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -19.75 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 5.5 \text{ hours;}$$

$$\Delta PF_2 = +1.02 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 0.67 \text{ hours;}$$

$$\Delta PF_3 = -14.78 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -17.56 \times 10^{-5} \text{ Newtons/million cells.}$$

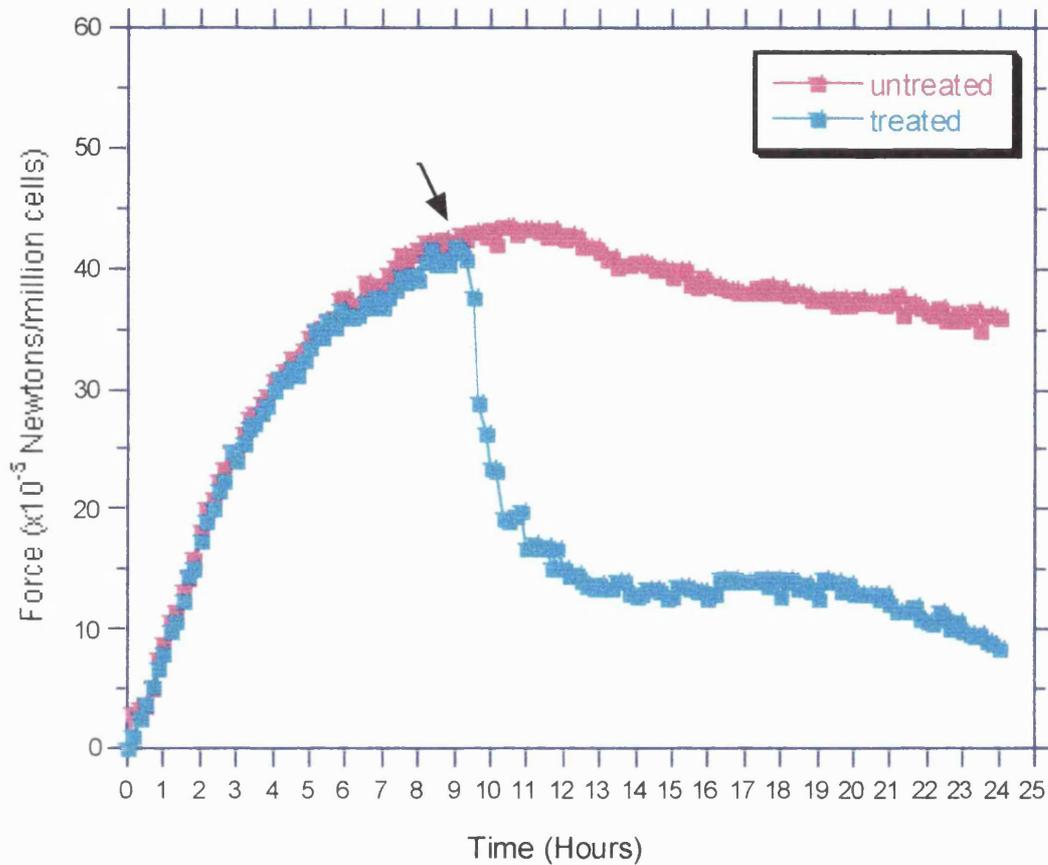


Figure 3.33. The effect of a single administration of CYTO (7.3×10^{-5} M, represented by the arrow) at 9.33 hours on the contraction of normal fibroblasts (nf-4).

Contraction analysis:

$$\Delta PF_0 = -1.63 \times 10^{-5} \text{ Newtons/million cells,}$$

$$\Delta PF_1 = -28.08 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_1 = 4.67 \text{ hours;}$$

$$\Delta PF_2 = +1.47 \times 10^{-5} \text{ Newtons/million cells, } \Delta T_2 = 2.33 \text{ hours;}$$

$$\Delta PF_3 = -24.27 \times 10^{-5} \text{ Newtons/million cells;}$$

$$\Delta PF_4 = -27.48 \times 10^{-5} \text{ Newtons/million cells.}$$

Based on these evaluations it was found that HYD, HYDAC, HYDACAC and Me-PH had negligible effects on contraction. Compounds found to be 'active' in these tests fell into two categories (Fig. 3.34) based on whether they induced or reduced contraction, and therefore could be compared with known cytoskeletal disrupter agents.

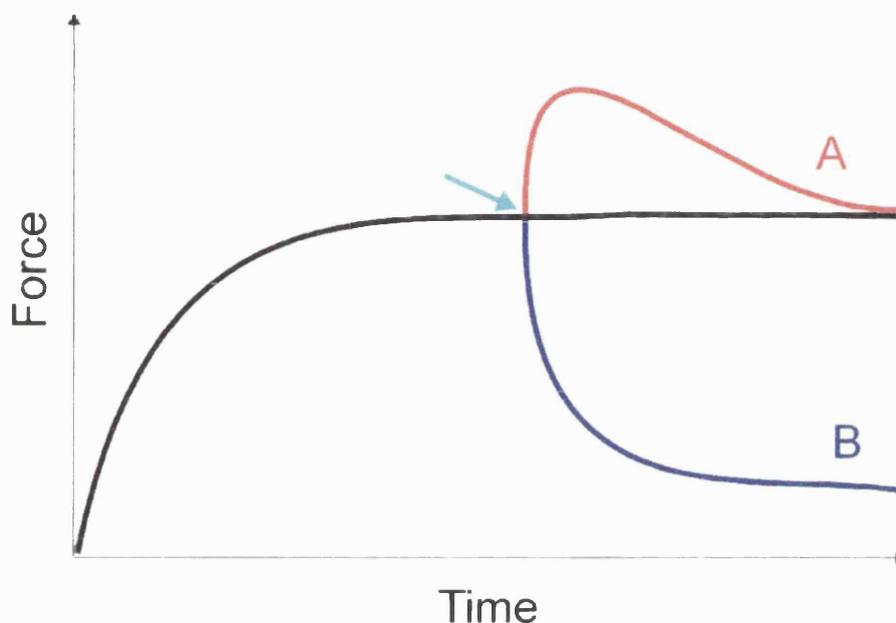


Figure 3.34. Schematic representation of the effects of various drugs on FPCL contraction in the CFM. The green arrow represents the point of drug addition. Compounds either stimulated contraction (type A, e.g. Colchicine-like activity) or reduced it (type B, e.g. Taxol- or Cytochalasin B-like activity).

Type A HYD derivative: PHT.

Type B HYD derivatives: ALLAN, (MePh)-PH, p-HPPH and m-HPPH.

Interestingly, out of all the HYD-related compounds only PHT stimulated contraction (type A). However, the magnitude of the peak was half of that induced by COL. ALLAN, (MePh)-PH, p-HPPH, and m-HPPH all induced a reduction in contractile forces (type B). Based on the similarities between the various components of their contractile profiles, the activities of these compounds were compared with those of TAX and CYTO (Table 3.5). It must be noted, however, that after the initial relaxation caused by the addition of these 'active' HYD derivatives, small secondary contraction peaks were seen which were uncharacteristic of TAX or CYTO.

In order to determine whether ALLAN, (MePh)-PH, and m-HPPH did indeed have a Taxol-like activity by binding and stabilising the microtubules, indirect immunofluorescence staining was carried out on treated cells. Similarly, further evidence was sought for the Cytochalasine B-like activity of p-HPPH in the disruption of microfilaments of treated cells. For these results see section 3.6.3. 'The effects of HYD-related compounds on cell morphology and cytoskeleton'.

'Active' compound	Cytoskeletal poison	Similarity
p-HPPH	CYTO	ΔPF_1
(MePh)-PH	TAX	ΔPF_1
PHT	COL	ΔPF_2
p-HPPH	CYTO	ΔPF_3
(MePh)-PH	TAX	ΔPF_3
ALLAN	TAX	ΔPF_4
m-HPPH	TAX	ΔPF_4
p-HPPH	TAX or CYTO	ΔPF_4
(MePh)-PH	CYTO	ΔPF_4

Table 3.5. Comparison of HYD derivatives found 'active' in the CFM with known cytoskeletal poisons.

The activity of these HYD derivatives was also ranked according to their ability to reduce the contractile forces:

- as measured by ΔPF_1 values associated with the initial relaxation in force:
p-HPPH > (MePh)-PH > ALLAN > m-HPPH.
- as measured by ΔPF_4 values associated with the maintenance of this relaxation after 24 hours:
(MePh)-PH > p-HPPH > ALLAN \geq m-HPPH.

To summarise, these results indicate that by inducing a contraction, PHT has a different effect from the other HYD derivatives investigated which relaxed FPCLs. Most activity was associated with HYD derivatives, which contained two Phenyl rings, and one of the rings was substituted at the 4 position with either a hydroxyl or a methyl group. Substitution at the 3 position reduced activity. The basic structures, e.g. HYD (closed ring), HYDAC (open ring) or HYDACAC (a substituted HYD) had no effects on contraction. Surprisingly, ALLAN was found to have significant activity (comparable or greater than m-HPPH) in this assay, even though it did not contain any substituted phenyl rings.

3.6.3. The effects of Hydantoin-related compounds on cell morphology and the cytoskeleton

Investigations carried out using the CFM indicated that some HYD-related compounds altered FPCL contraction in a comparable way to cytoskeletal poisons (COL, TAX, and CYTO). In order to test the idea that these compounds interfered with cytoskeletal functions, indirect immunofluorescence staining against F-actin, β -tubulin, vinculin and vimentin was performed on untreated cells and cells separately treated with COL, TAX, CYTO and HYD derivatives.

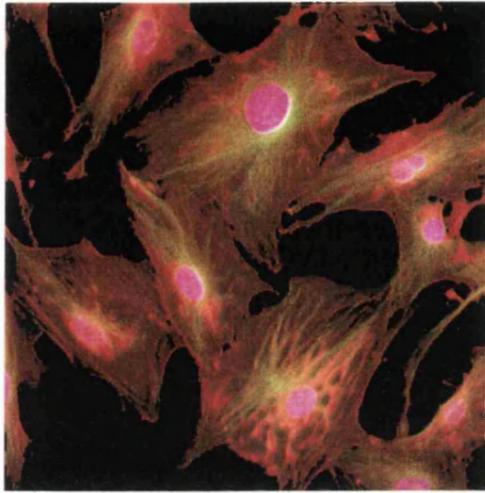
The morphology of control cells (Fig. 3.35) appeared to be a mixture of stellate and bipolar. Cells contained numerous microtubules and intermediate filaments radiating from the perinuclear zones into the peripheral lamellas and tail processes. Actin bundles were straight and orientated predominantly along the main cell axis. There were also numerous, discrete elongated vinculin-containing focal contacts in the lamellae. DMSO, the drug solvent, had no effects on the cytoskeleton at such concentrations (not shown).

COL decreased the elongation of the cells and they appeared to be somewhat ragged (Figure 3.36). Microtubules were completely depolymerised and absent, while intermediate filaments were collapsed into a perinuclear ring in most cases. Numerous straight microfilaments displayed criss-cross patterns with numerous large and elongated vinculin containing focal contacts at the cell periphery.

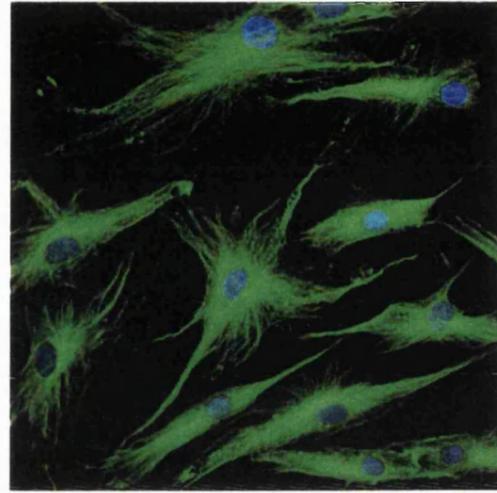
TAX decreased the polarisation and elongation of cells. Some cells became elliptical in their morphology (Figure 3.37). TAX-treated cells acquired a more smooth, ellipsoid or circular outline than the corresponding COL-treated cells. The densely packed microtubules were disorganised and the free ends were scattered in the cytoplasm. In discoid cells straight microfilaments were absent and became circumferential. Like microtubules, the loose network of intermediate filaments was confined to the central cytoplasm. Some vinculin containing focal contacts became dot-like or triangular and formed a nearly continuous circumferential line in discoid cells.

CYTO-treated cells had virtually no intact microfilaments, and the number of vinculin containing focal contacts was greatly reduced (Fig. 3.38) resulting in the collapse of the cells into stellate form. Remaining adhesion plaques were distributed in a star-like fashion on the cell periphery and microtubules were circular around the perinuclear areas.

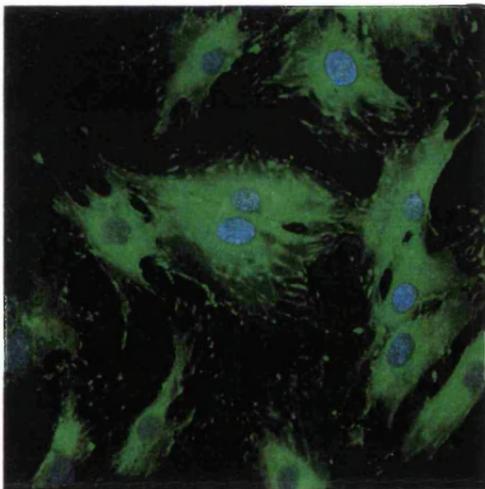
None of the HYD-related compounds were found to have comparable to these effects on the integrity of the F-actin, β -tubulin or vimentin-containing structures (Figures 3.39-47). Nevertheless, minor changes in cell shape, spreading and differences in vinculin-containing focal adhesion distribution were noted. However, these would require further quantitative investigation.



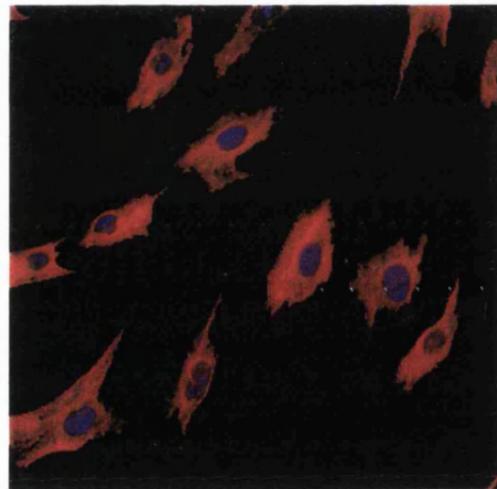
a



b



c

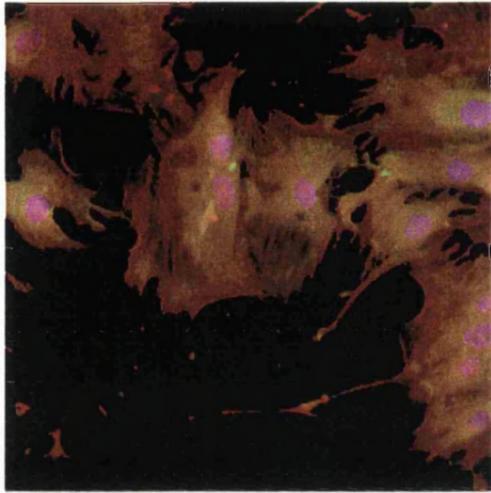


d

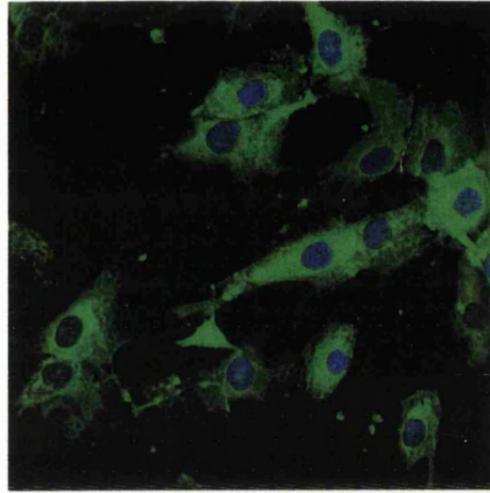
Figure 3.35. Immunofluorescent staining of control cells (nf-4).

(Magnification: x200.)

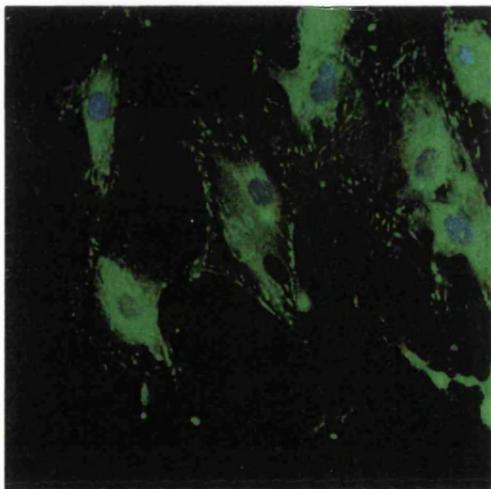
- A = microfilaments (red), microtubules (green) and nuclei (blue),
- B = microtubules (green) and nuclei (blue),
- C = vinculin-containing adhesion plaques (green) and nuclei (blue),
- D = intermediate filaments (red) and nuclei (blue).



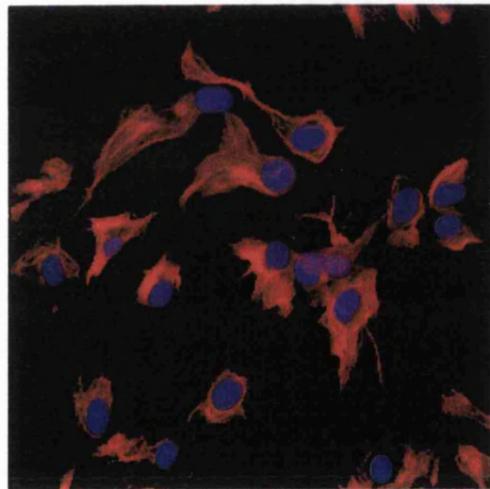
a



b



c



d

Figure 3.36. Immunofluorescent staining of cells (nf-4) treated with COL.

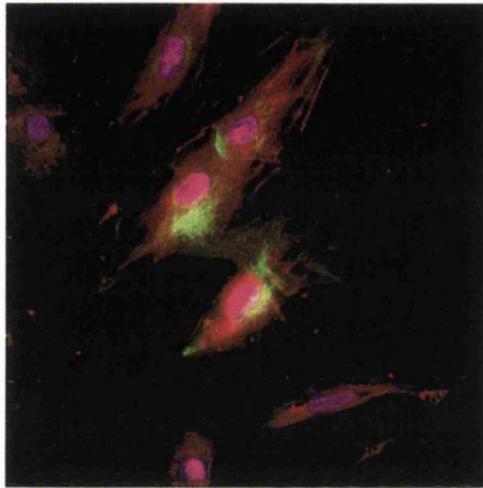
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

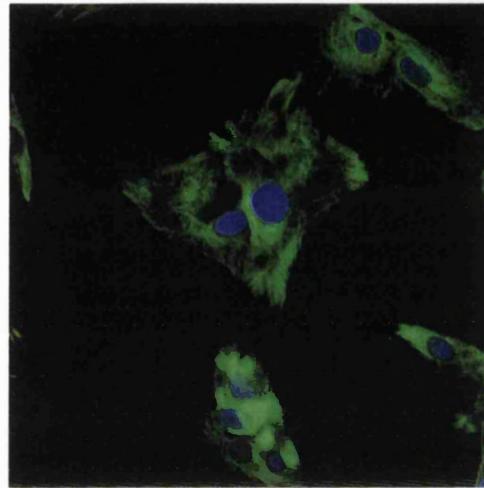
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

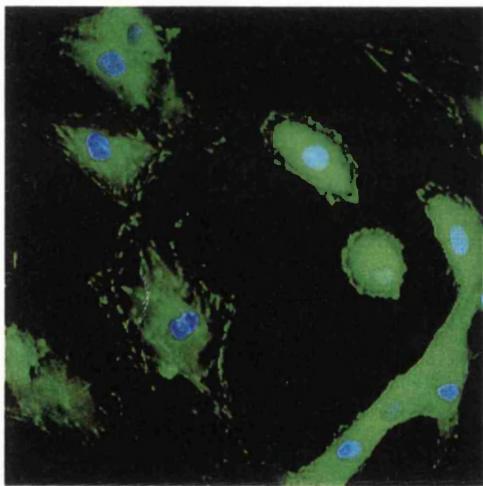
D = intermediate filaments (red) and nuclei (blue).



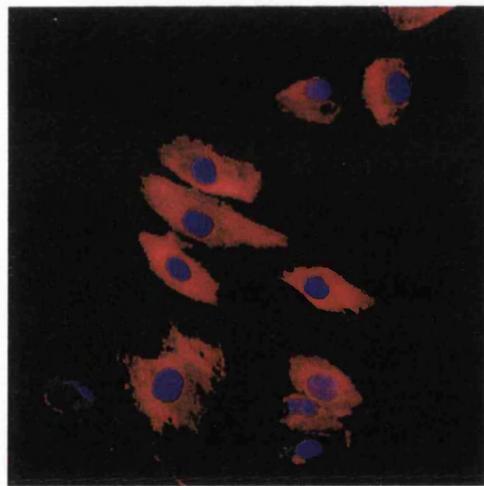
a



b



c



d

Figure 3.37. Immunofluorescent staining of cells (nf-4) treated with TAX.

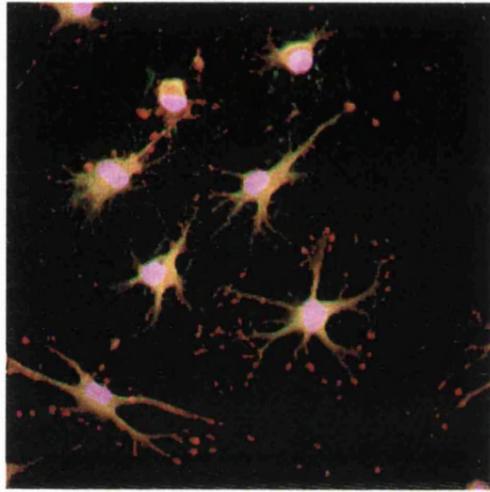
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

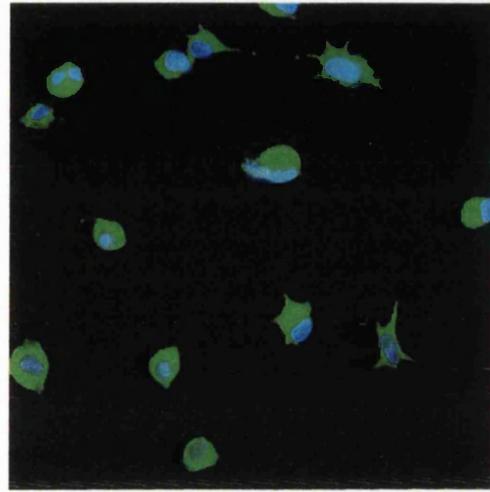
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

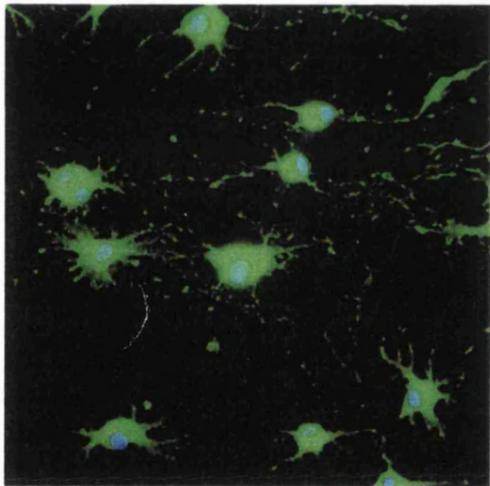
D = intermediate filaments (red) and nuclei (blue).



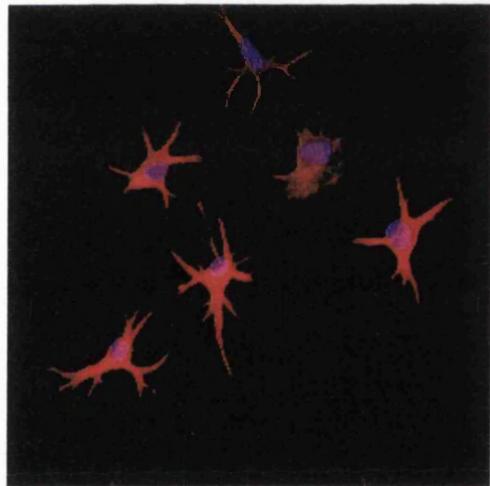
a



b



c



d

Figure 3.38. Immunofluorescent staining of cells (nf-4) treated with CYTO.

(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

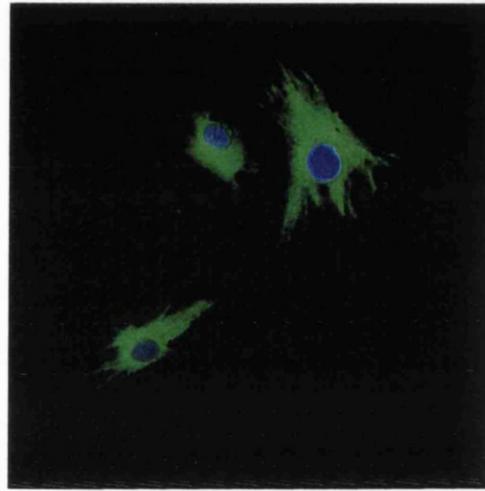
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

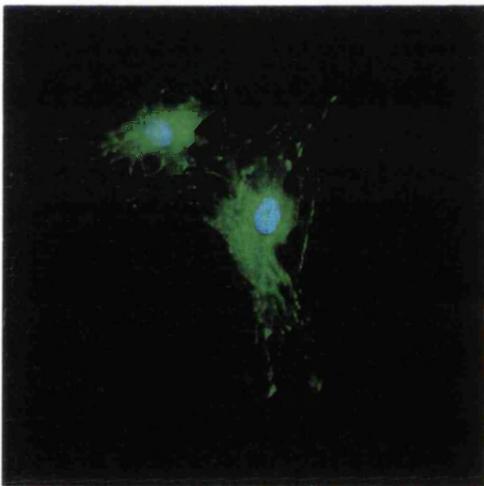
D = intermediate filaments (red) and nuclei (blue).



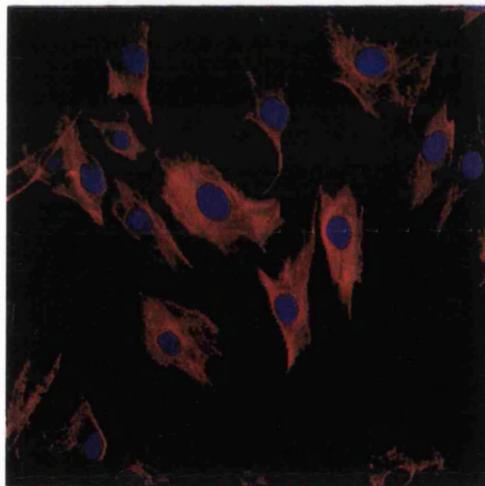
a



b



c



d

Figure 3.39. Immunofluorescent staining of cells (nf-4) treated with HYD.

(Magnification: x200.)

- A = microfilaments (red), microtubules (green) and nuclei (blue),
- B = microtubules (green) and nuclei (blue),
- C = vinculin-containing adhesion plaques (green) and nuclei (blue),
- D = intermediate filaments (red) and nuclei (blue).

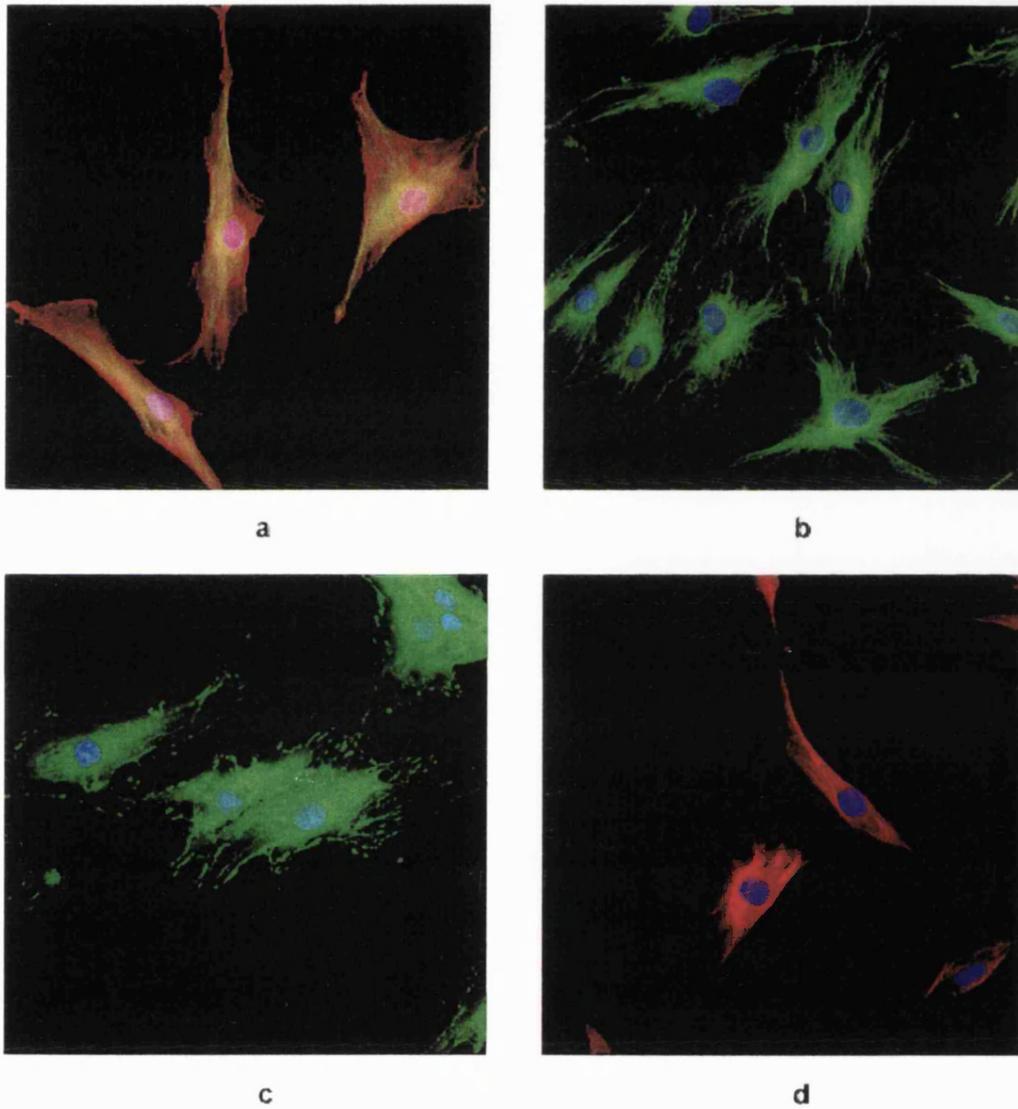


Figure 3.40. Immunofluorescent staining of cells (nf-4) treated with HYDAC.

(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

D = intermediate filaments (red) and nuclei (blue).

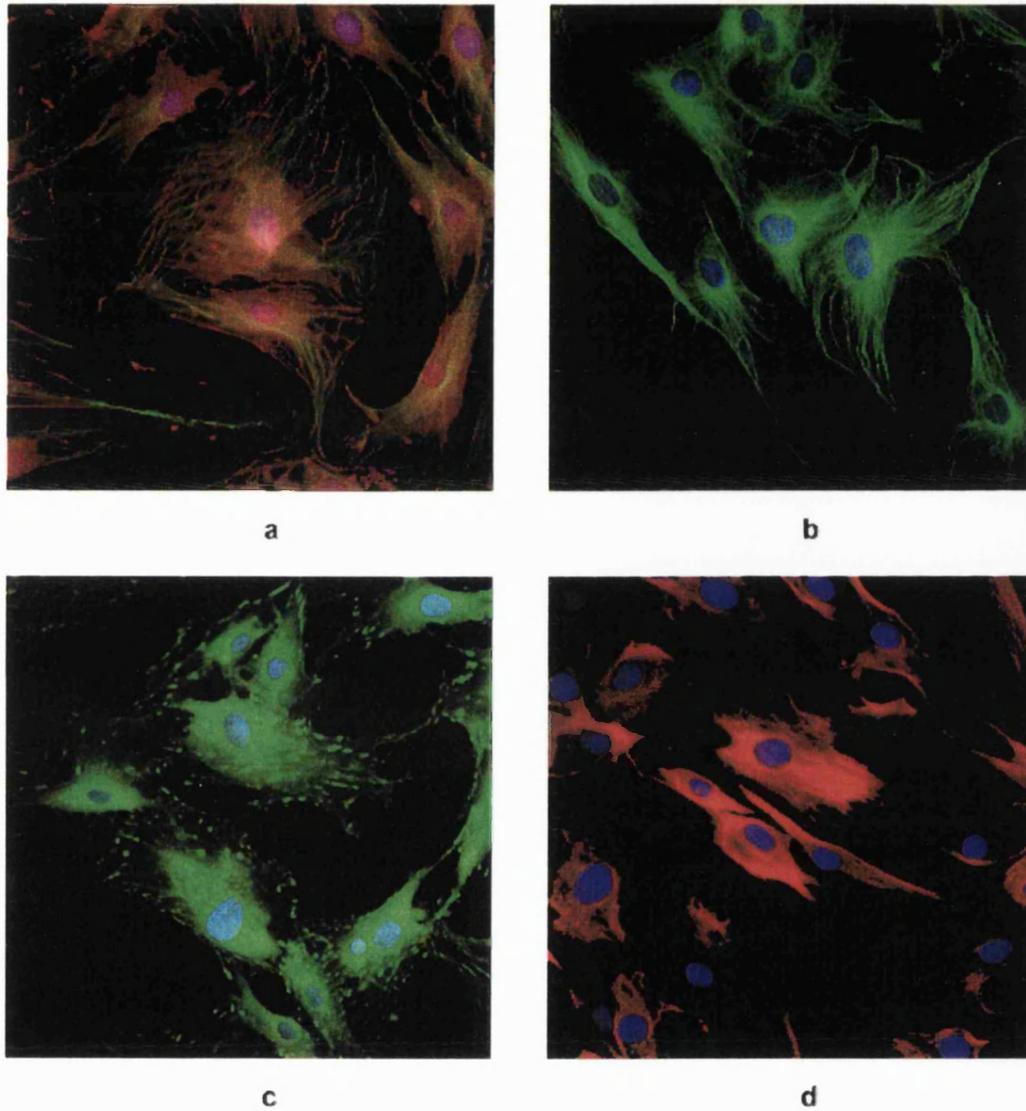


Figure 3.41. Immunofluorescent staining of cells (nf-4) treated with ALLAN.

(Magnification: x200.)

- A = microfilaments (red), microtubules (green) and nuclei (blue),
- B = microtubules (green) and nuclei (blue),
- C = vinculin-containing adhesion plaques (green) and nuclei (blue),
- D = intermediate filaments (red) and nuclei (blue).

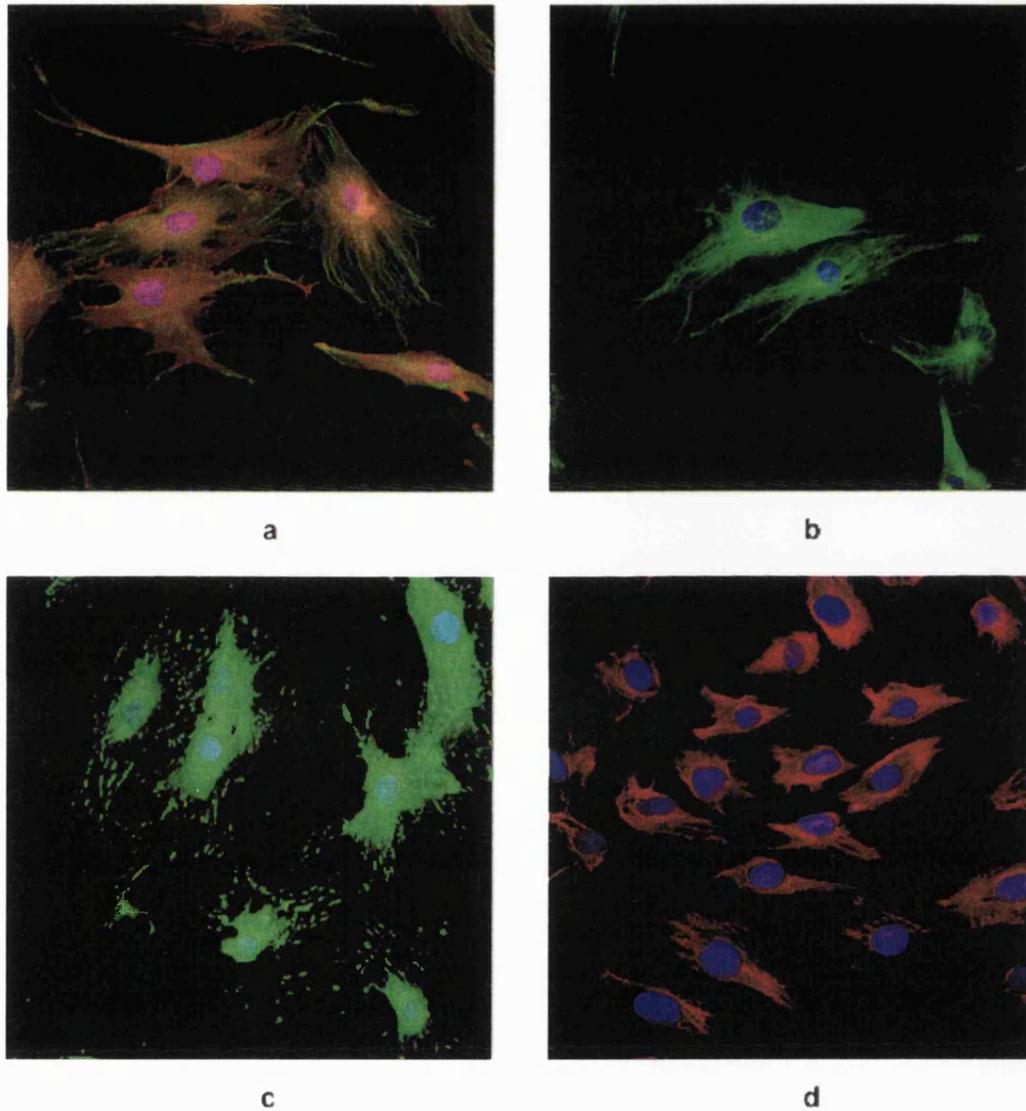


Figure 3.42. Immunofluorescent staining of cells (nf-4) treated with HYDACAC.

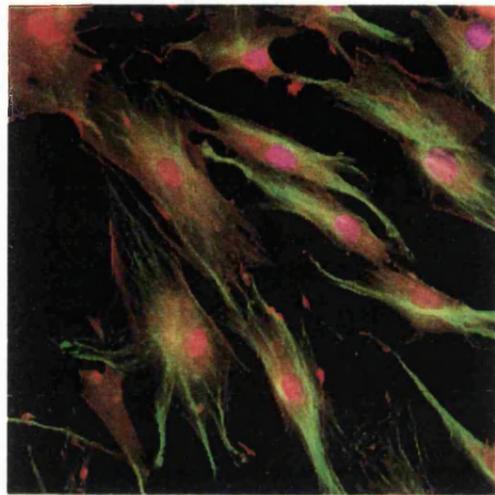
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

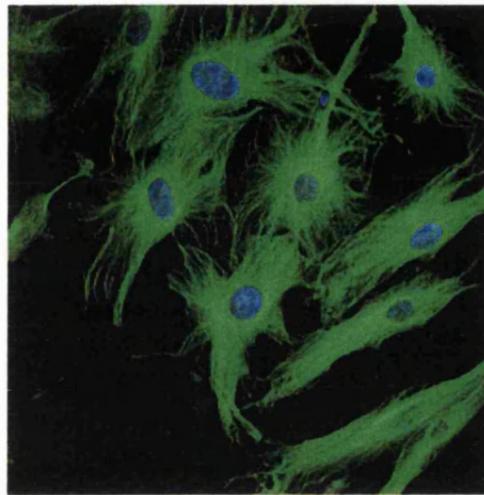
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

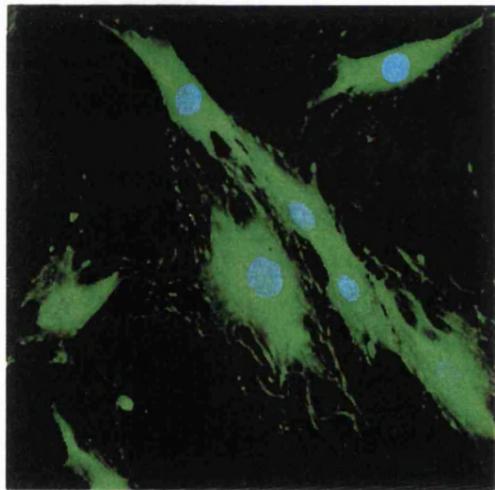
D = intermediate filaments (red) and nuclei (blue).



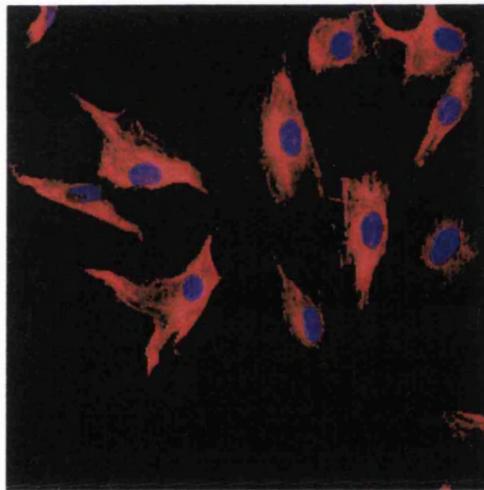
a



b



c



d

Figure 3.43. Immunofluorescent staining of cells (nf-4) treated with Me-PH.

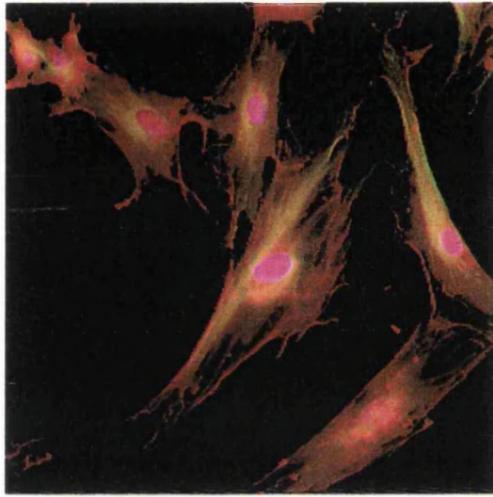
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

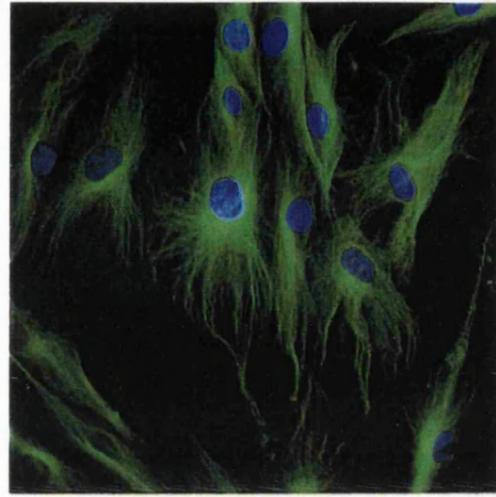
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

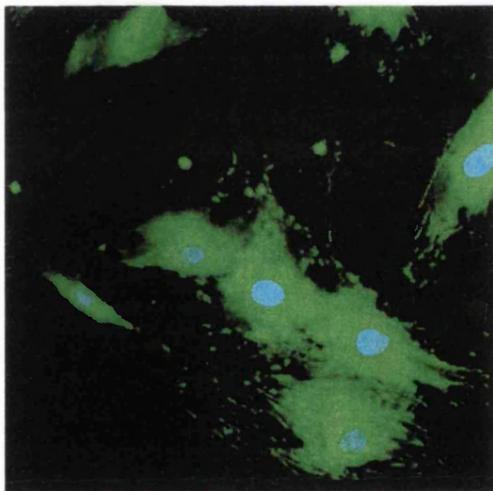
D = intermediate filaments (red) and nuclei (blue).



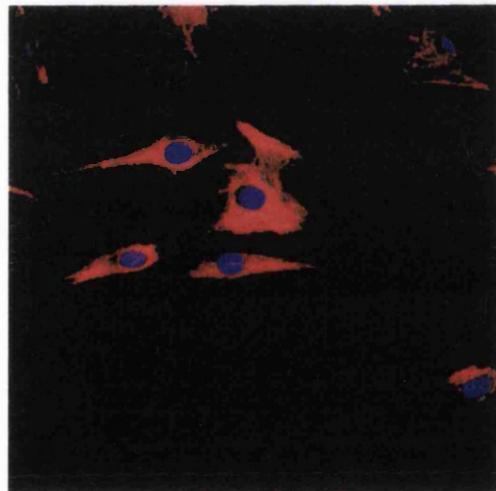
a



b



c



d

Figure 3.44. Immunofluorescent staining of cells (nf-4) treated with (MePh)-PH.

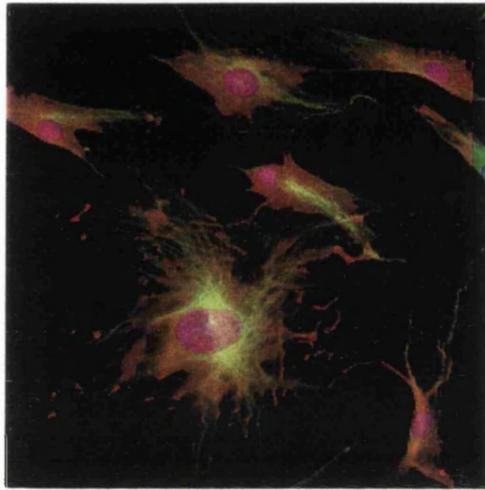
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

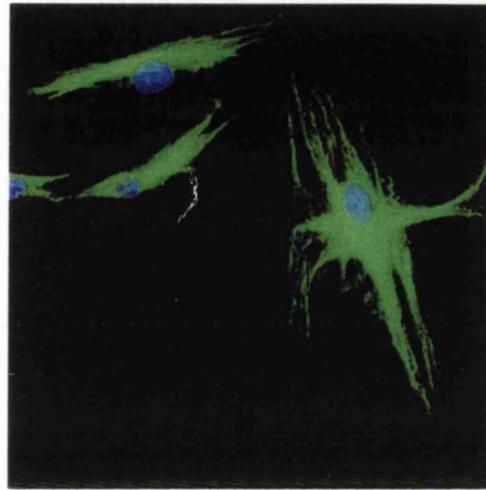
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

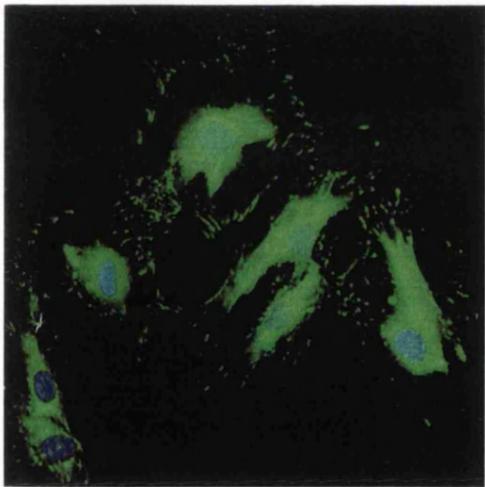
D = intermediate filaments (red) and nuclei (blue).



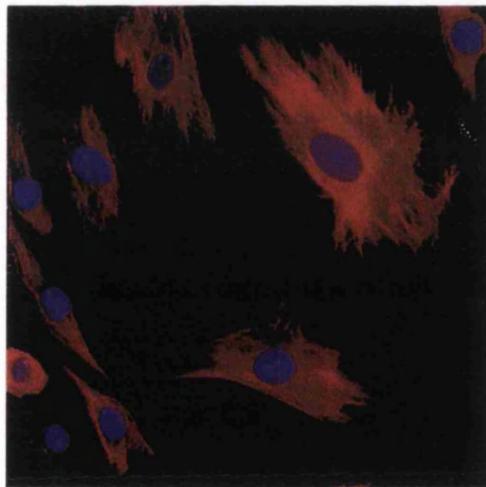
a



b



c



d

Figure 3.45. Immunofluorescent staining of cells (nf-4) treated with PHT.

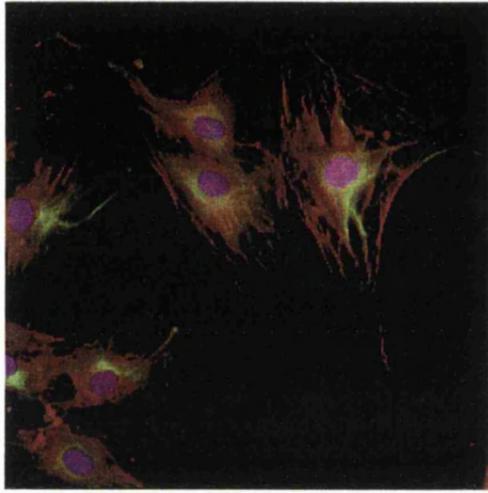
(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

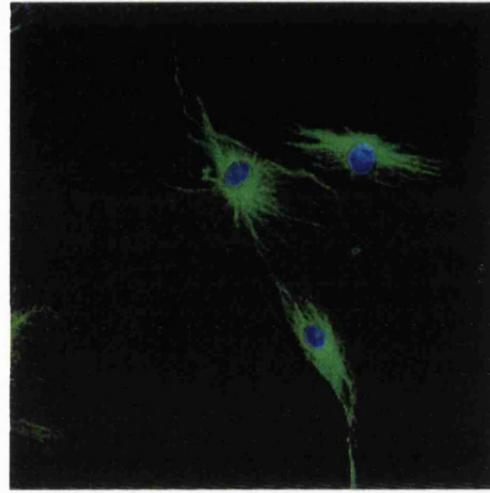
B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

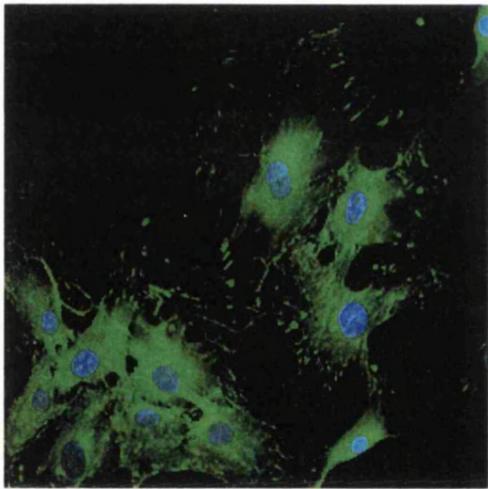
D = intermediate filaments (red) and nuclei (blue).



a



b



c



d

Figure 3.46. Immunofluorescent staining of cells (nf-4) treated with p-HPPH.

(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

D = intermediate filaments (red) and nuclei (blue).

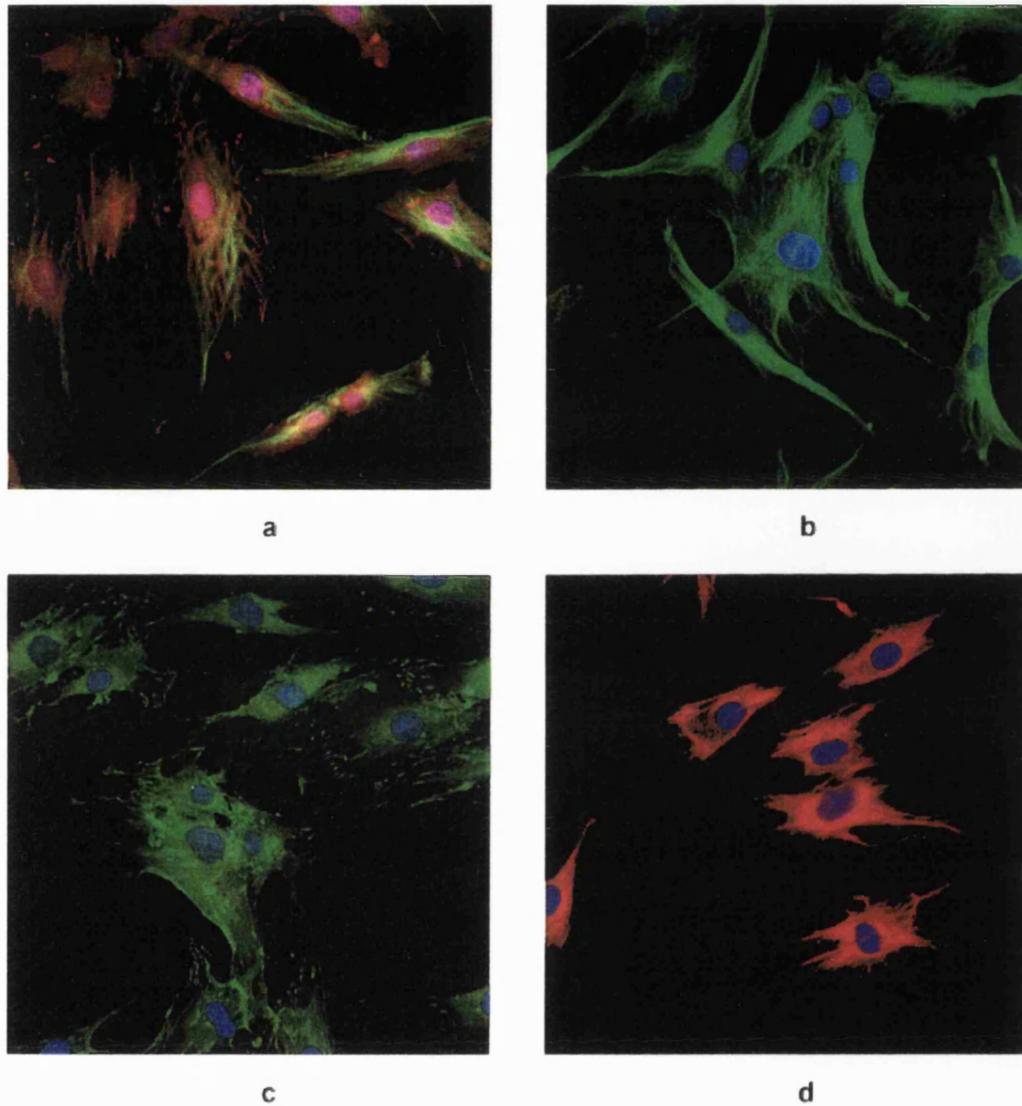


Figure 3.47. Immunofluorescent staining of cells (nf-4) treated with m-HPPH.

(Magnification: x200.)

A = microfilaments (red), microtubules (green) and nuclei (blue),

B = microtubules (green) and nuclei (blue),

C = vinculin-containing adhesion plaques (green) and nuclei (blue),

D = intermediate filaments (red) and nuclei (blue).

3.7. The effect of Phenytoin on porcine wound healing

3.7.1. The effect of Phenytoin powder (3-week wounds) , pigs 1&2

In this experiment PHT powder was evenly applied topically on to the woundbeds of two pigs and sealed with Opsite dressing. On visual inspection all wounds appeared to be completely epithelialised after 21 days post wounding. PHT induced significant inhibition of wound contraction (Fig. 3.48) in all treated wounds as analysed by Mann-Whitney test (95% confidence interval, all $P < 0.00001$). This effect was dose-dependent and the following ranking order could be established (Mann-Whitney test, 95% confidence interval): 0mg PHT < 6mg PHT ($P < 0.0001$) = 24mg PHT = 42 mg PHT < 84 mg PHT ($P = 0.0051$) < 120mg PHT ($P = 0.0024$). The maximum difference in wound area amounted to approximately 50% with 120mg PHT (149.99±16.08%, mean±1-SD).

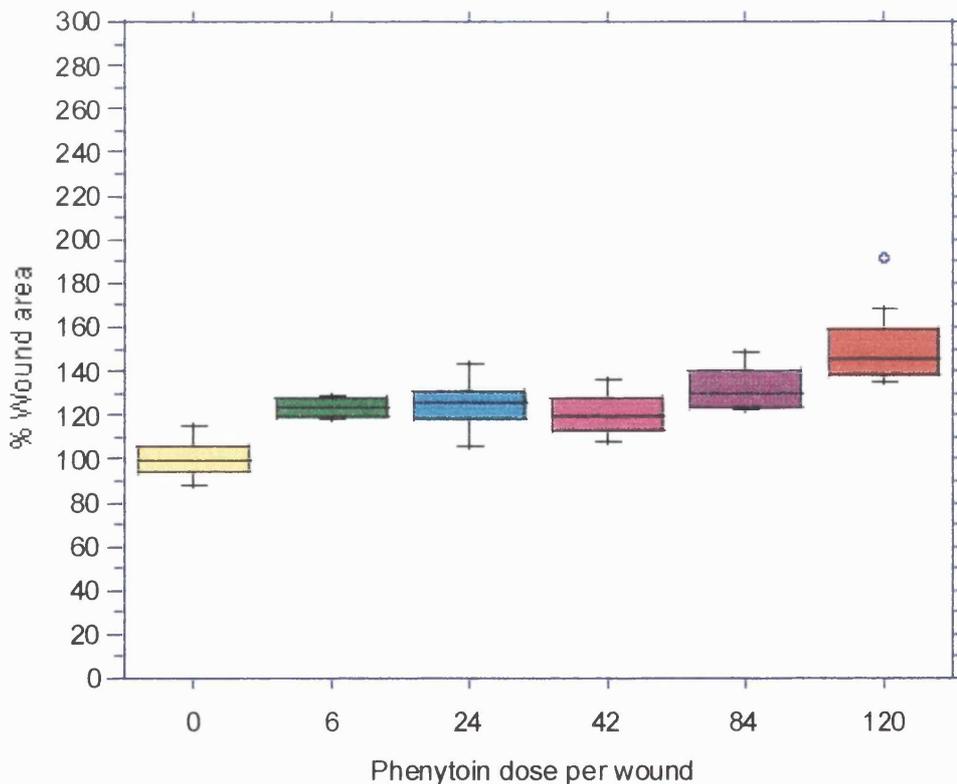
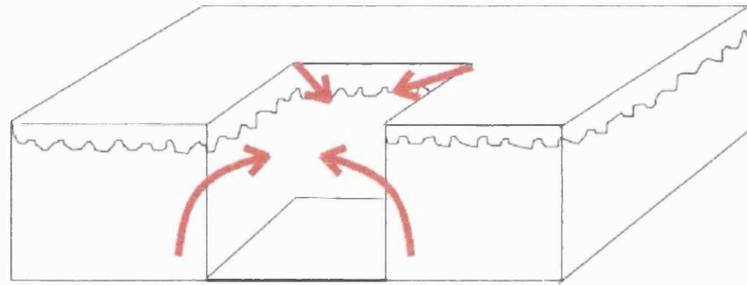


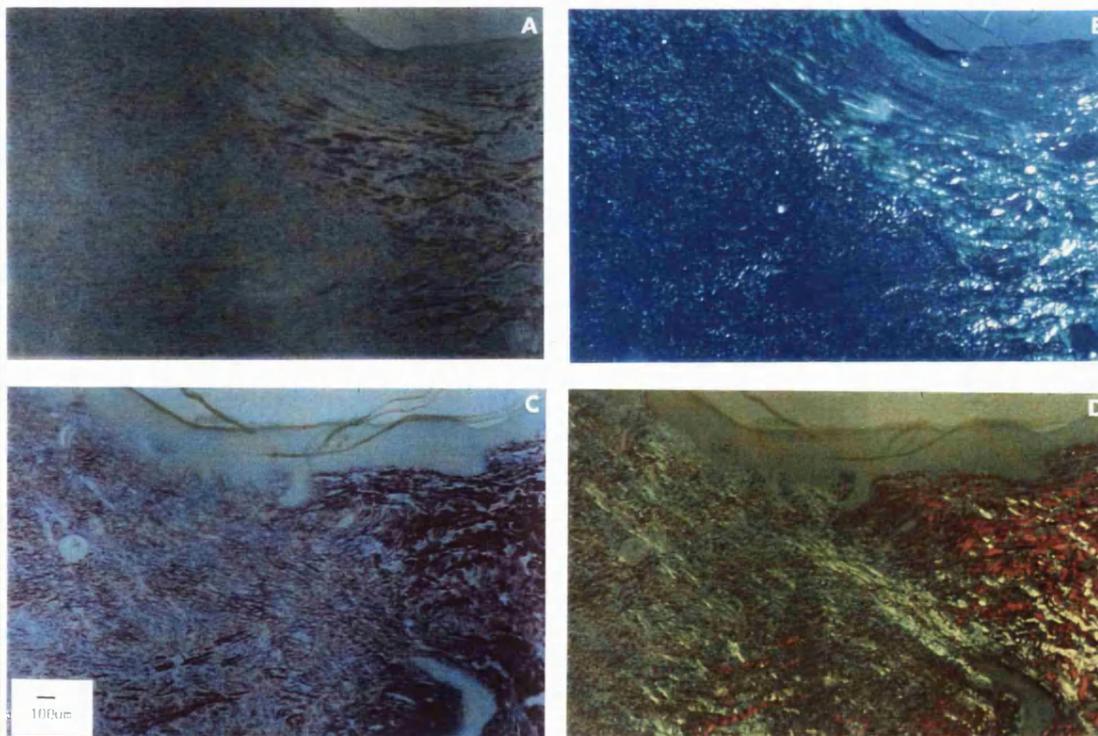
Figure 3.48. Boxplot representation of the effect of PHT powder on porcine wound contraction after 21 days. Values were expressed as percentage of the control wound area, which was taken as 100%. The horizontal lines within the boxes (25th to 75th percentiles) represent the median. Error bars denote 5th to 95th percentiles and unfilled circles indicate results outside of these ranges.

(See page 83 for n values)

Control and PHT-treated wounds were examined by histology using light microscopy and assessed for re-epithelialisation, granulation tissue formation, inflammatory cell infiltration, fibroblast density, and vascularity. The difference in contraction observed by image analysis of wound surface areas between control and PHT-treated wounds was also demonstrated by histological analysis (Fig. 3.49). However, this would require further quantitative investigation by image analysis of cross-sectional areas of histological sections (Naylor and Teo, 1994).



a.



b.

Figure 3.49. Schematic illustration of wound contraction (a) and corresponding representative Picro-Sirius red-stained tissue sections (b) of control (A&B) and 120mg PHT-treated wounds (C&D). Sections were also examined under

polarised light for birefringence of collagenous structures (B&D). Note the original, thicker collagen bundles are being pulled into the centre of the wound. The extent of this contraction was reduced when wounds were treated with PHT. Wounds treated with 120mg PHT contained areas where collagen deposition was greater than in untreated wounds.

Both control and PHT-treated wounds contained cell-dense granulation tissue with immature, aligned collagen fibres. The characteristic basket-weave pattern of collagen seen in intact skin (Fig. 3.50) was missing. Although there was no visible difference in the number of fibroblasts and vascularisation between control and PHT-treated wounds, the extent of collagen deposition was greater with higher concentrations (84 and 120mg PHT/wound) of the drug. A few lymphocytes and eosinophils were also seen in both treated and untreated wounds. These inflammatory cells were completely absent in unwounded skin (Fig. 3.50).

A distinct feature seen in all PHT-treated wounds was epithelial acanthosis, as greater than normal amounts of epithelium grew deep into the dermis with little differentiation. The severity and frequency of this feature intensified with increasing doses of the drug (Fig. 3.51). In contrast, the epithelial morphology of control wounds (Fig. 3.51A) was characterised by more flattened rete ridges compared with normal skin (Fig. 3.50A).

Additionally, sections of PHT-treated granulation tissue also contained above average number of 'holes', originally representing entrapped adipose tissue lost in the processing for histology (Fig. 3.52). (Miss. R.A. Porter later confirmed these 'holes' to be islands of fat cells by oily red staining of frozen sections.) Again, this feature intensified with increasing doses of PHT.

Measurements of PHT levels from blood taken up to 24 hours after topical drug administration failed to register measurable serum levels indicating that systemic absorption from the woundbed was minimal.

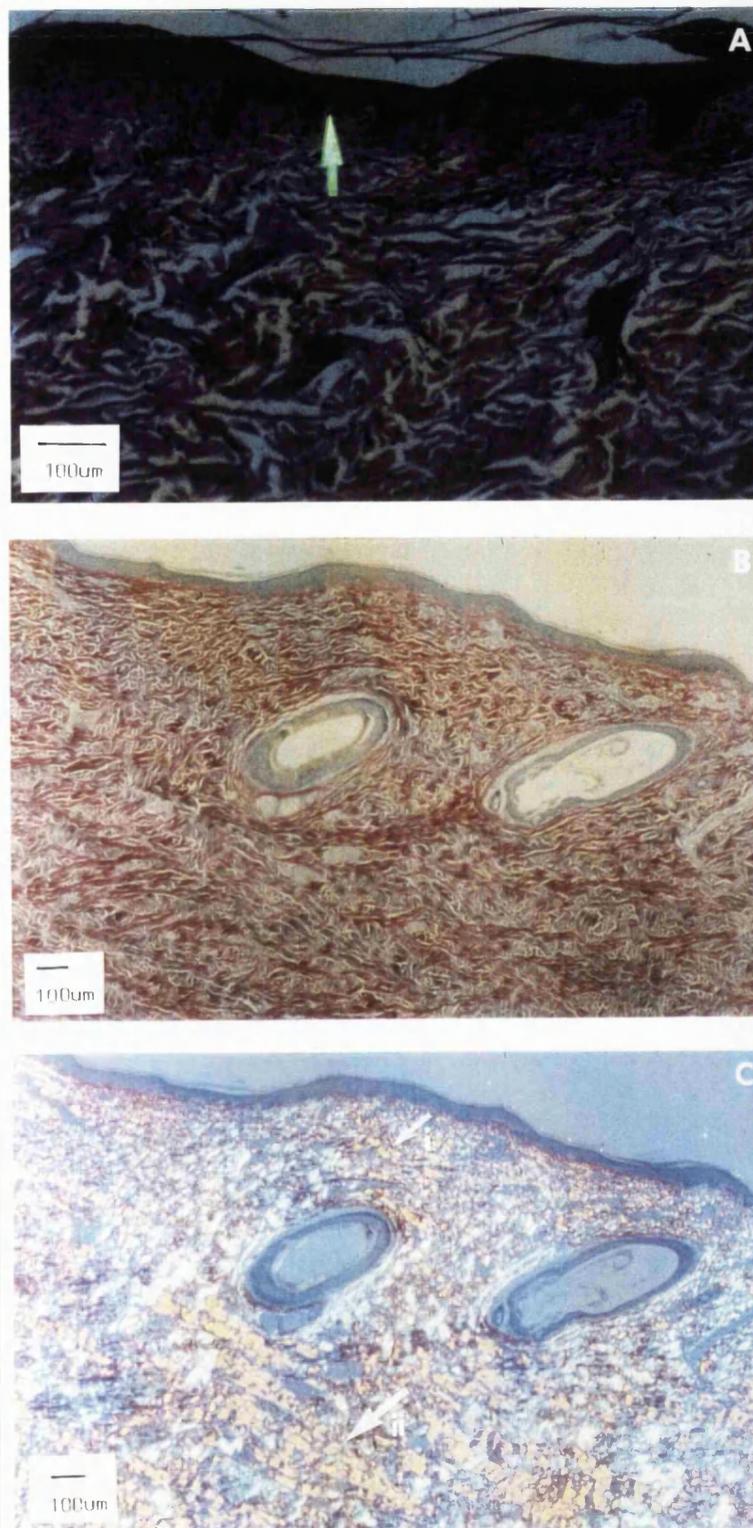


Figure 3.50. Representative Picro-Sirius red stained sections of unwounded pig skin. Note the abundant presence of rete pegs connecting the epidermis to the dermis (A, indicated by arrow), the change in collagen diameter from the papillary layer down to the reticular layer within the dermis (B&C; C = polarised view of B) and the characteristic basket-weave pattern of collagen fibres.

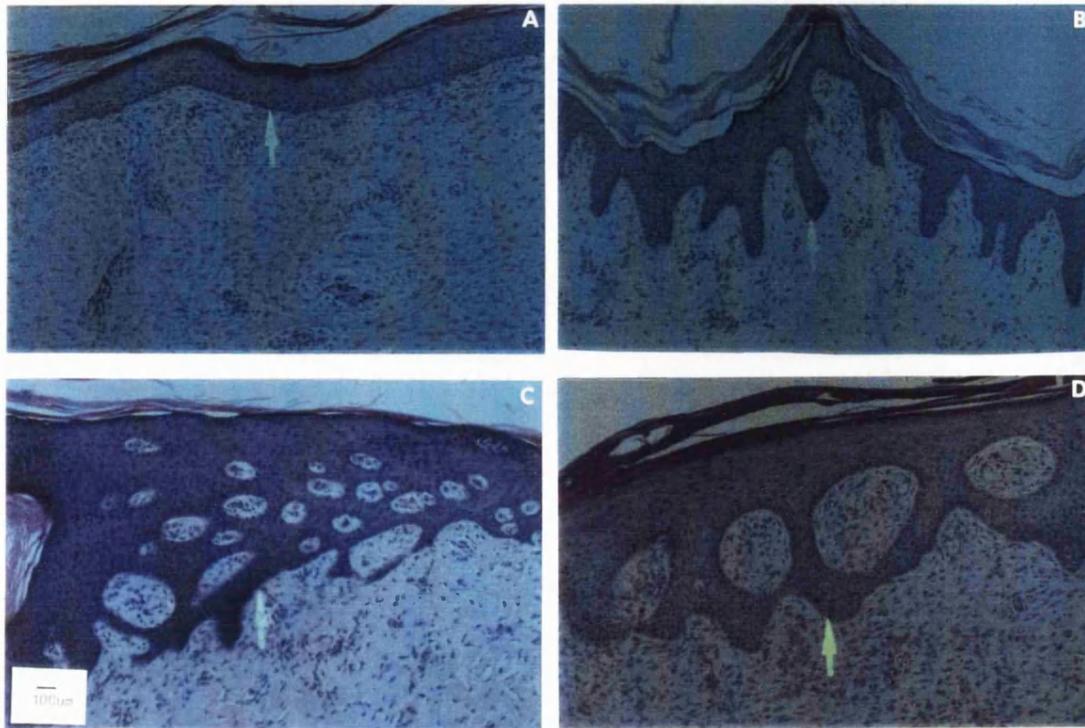


Figure 3.51. Representative H&E stained sections showing epithelial acanthosis in PHT-treated wounds.

A = control wound; B = 6mg PHT, C = 84mg PHT; D = 120mg PHT-treated wounds.

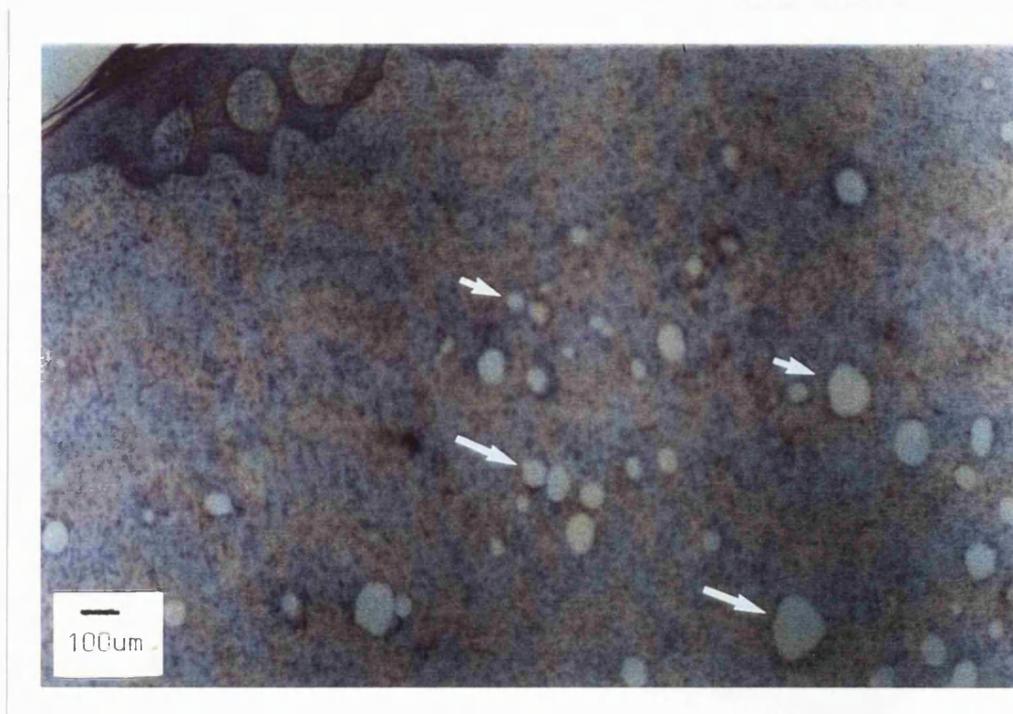


Figure 3.52. Representative H&E-stained wax tissue section (arrows) of a PHT-treated wound (120mg) displaying increased number of 'holes' in the dermis.

3.7.2. Phenytoin powder in combination with Fibrin Sealant (2-week wounds) , pigs 3&4

In this experiment topical PHT powder was tested alone or in combination with FS in randomised wounds of two pigs. All wounds were completely epithelialised within 14 days of wounding. As before, the wound areas were compared for each treatment regime (Fig. 3.53) and the results were analysed by Mann-Whitney test (95% confidence interval). FS and 6mg PHT powder both reduced wound contraction significantly ($P = 0.0235$ and 0.0046 , respectively). Surprisingly, when combined, they failed to have the same effect ($P = 0.058$). On the other hand, 24 mg PHT in FS and 42 mg PHT in FS both dose-dependently reduced contraction ($P = 0.0001$ and $P < 0.00001$, respectively). Interestingly, 42mg PHT in FS was 34% more effective than the equivalent PHT powder on its own ($P = 0.0073$).

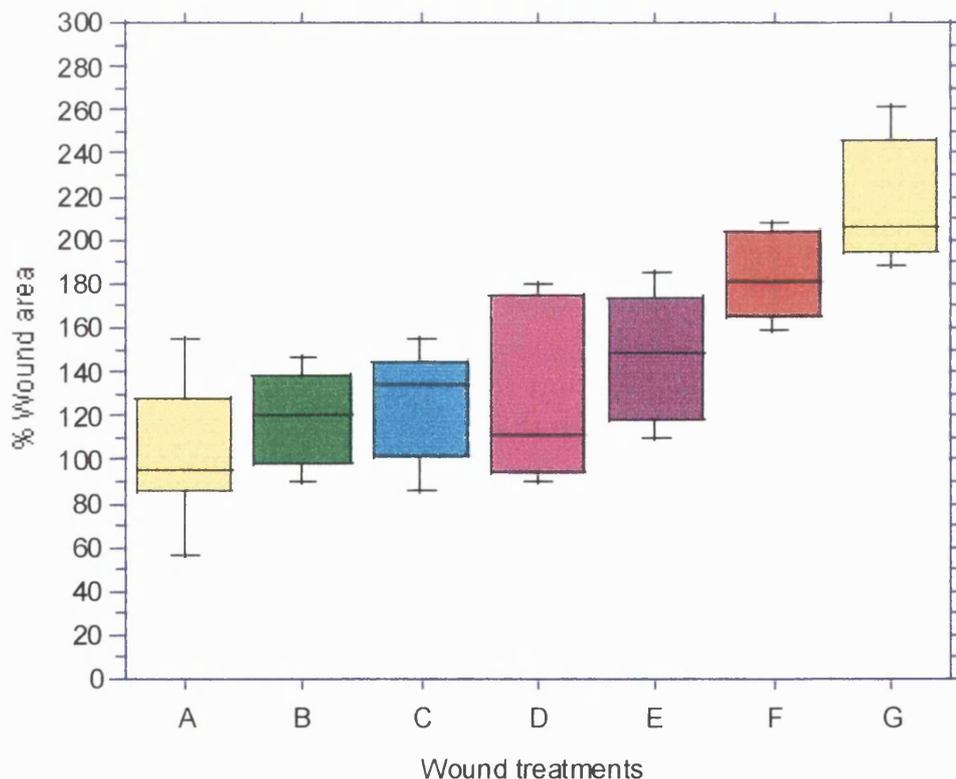


Figure 3.53. Boxplot representation of the effect of PHT and FS (alone or in combinations) on wound contraction after 14 days.

A = Control (no treatment); B = FS; C = 6mg PHT; D = 6mg PHT in FS;

E = 24mg PHT in FS; F = 42mg PHT; G = 42mg PHT in FS. Values were expressed as a percentage of the control wound area, which was taken as

(See page 84 for n values)

100%. The horizontal lines within the boxes (25th to 75th percentiles) represent the median. Error bars denote 5th to 95th percentiles and unfilled circles indicate results outside of these ranges.

As stated before, at three weeks post-operative, PHT powder alone (in all doses) reduced wound contraction significantly (all $P < 0.0046$). However, when incorporated into FS, only doses equivalent to 24mgPHT and higher reduced contraction significantly. Interestingly, Fibrin sealant alone also reduced contraction to the same extent as 6mg PHT powder. The additive effect of Fibrin sealant and PHT nevertheless became only significant with higher concentrations of PHT (42mg).

Based on Mann-Whitney test (using 95% confidence interval), the following ranking order could be established for wound size:

0mg PHT < FS ($P = 0.0235$) < 6mg PHT ($P = 0.0046$) = 6mg PHT (in FS) = 24mg PHT (in FS) < 42mg PHT ($P < 0.00001$) < 42 mg PHT (in FS; $P < 0.00001$).

N.B.: 0mg PHT = 6mg PHT (in FS).

In addition to its effect on contraction, FS induced a strong inflammatory reaction (Fig. 3.54B). It must be noted however, that control wounds also contained sites of inflammation (Fig. 3.54A). However, this effect was markedly diminished when FS was combined with PHT (Fig. 3.54D-F). There was no difference in vascularisation or fibroblast number between control and treated wounds. As seen previously, PHT induced acanthosis in all doses either in powder form or in combination with FS (Fig. 3.54C-G). Moreover, higher doses were again associated with entrapment of adipose tissue extending up to the papillary dermis (Fig. 3.54E-G).



Figure 3.54. Representative H&E stained tissue sections of A = untreated; B = FS; C = 6mg PHT powder; D = 6mg PHT (in FS); E = 24mg PHT (in FS); F = 42mg PHT; G = 42 mg PHT (in FS) treated full thickness porcine wounds. (i=epithelial acanthosis, ii=holes in the dermis)

CHAPTER 4. DISCUSSION

4.1. The *in vitro* effects of Phenytoin on cell proliferation and migration

The enigma of PHT-induced gingival overgrowth and its stimulatory effect on cutaneous connective tissue has occupied researchers for decades. Histological sections of gingival overgrowths (Brown *et al.*, 1991) and PHT-treated wound biopsies (Anstead *et al.*, 1996; Masgrau-Peya, 1995; Bansal NK and Mukul, 1993; Lodha, 1991; Muthukumarasamy *et al.*, 1991, Rodriguez-Noriega *et al.*, 1983) both showed increased cell number and connective tissue production.

It has been suggested (Benveniste and Bitar 1980; Hassell *et al.*, 1976) that during gingival overgrowth clonal selection of fibroblasts takes place, which can be of monoclonal or oligoclonal origin (Martin *et al.*, 1974). These so-called “responder cells” retain their phenotype in the absence of PHT through several passages *in vitro*. It is also possible that, during wound healing, PHT selects a fibroblast population in skin with either a higher proliferative or migratory capacity (Sempowski *et al.*, 1995).

Historically, the study of the *in vitro* effects of PHT has proven to be difficult. Contradictory results have been reported according to which PHT may either increase/decrease proliferative and synthetic activity or have no effect at all. Perhaps differences in experimental conditions such as cell type (normal gingival fibroblasts, fibroblasts grown from PIGO, dermal fibroblasts, HeLa cells, cells from rat fibrosarcoma and lymphocytes), cell density, phase of the cell cycle and PHT concentrations (Hou, 1993; Viyashingham *et al.*, 1991; Hassell and Gilbert, 1983; Al-Ubadi *et al.*, 1981; Benveniste and Bitar; 1980; Keith *et al.*, 1977; MacKinney *et al.*, 1975; Kasai and Yoshizumi, 1971; Noess, 1969; Shafer, 1961ab) may account for these inconsistencies in literature.

So far much research focussed on the influence of PHT on gingival fibroblast proliferation and little data is available on its effects on dermal cells. A recent break-through study by Ieiri and co-workers (Ieiri *et al.*, 1995) identified that (R)-p-HPPH, a metabolite of PHT, selectively stimulated dermal fibroblast growth of one cell line as measured by ³H-thymidine incorporation and cell counting using a haemocytometer. This enantiomer is also thought to be

responsible for the induction of PIGO and its production is genetically pre-determined (Ieiri *et al.*, 1995).

In order to gain further insight as to whether PHT increases the rate of healing by affecting cell division, the collective and individual responses of normal dermal fibroblast cell lines were evaluated at two time points in this thesis. These were then compared with the results obtained using EB fibroblasts and normal keratinocytes.

In fibroblast studies, the cells were made quiescent by serum starvation over 36 hours (Khan *et al.*, 1998), rendering the vast majority of the cells into the G₀ phase of their cell cycle. After establishing these synchronous monolayers, the cells were exposed to various concentrations of PHT in media containing low concentrations of FCS.

5µg/ml PHT was found to be stimulatory and optimal for normal, EB fibroblast and normal keratinocyte cell lines of this thesis, similarly to previous investigations using gingival fibroblasts (Benveniste and Bitar, 1980; Keith *et al.*, 1977; Houck *et al.*, 1972; Shafer, 1965), although as little as 2µg/ml PHT was reported to reduce the population-doubling time for human skin fibroblasts (Houck *et al.*, 1960). The magnitude of the increase seen here was in the range of 20-28% in two normal fibroblast cell lines, one of them being a late responder after 120 hours. Similarly in EB fibroblasts, the maximum stimulation seen was 26% in one cell line after 72 hours. However, it was transient and diminished after a further 48 hours. On the other hand, all keratinocyte cell lines displayed maximum response at either 5 or 10µg/ml PHT concentrations, which peaked after 120 hours and the increase in cell number ranged between 8-38%.

As in previous reports, there was considerable heterogeneity in the responses of the cell lines studied. It is yet to be confirmed whether differences were due to variations between patients, the presence of "responder cell" subpopulations in some individuals or differences in the metabolism of PHT. Moreover, differences between the age and sex of patients and those body sites from which the skin samples were taken may also be contributory factors to these variations seen both within and between groups. Further studies with large population of cell lines are necessary to establish the relationship between

(R)-p-HPPH production *in vitro* and direct cell responses to enantiomers of the major metabolites of PHT.

The effects of high concentrations of PHT were also compared in this thesis. As the solubility of PHT in wound fluid is thought to be similar to that in plasma (maximum 75µg/ml, pH=7.4, 37°C; Woodbury, 1989), concentrations of up to 100µg/ml PHT were investigated. Both normal and EB fibroblasts tolerated exposure to high concentrations of PHT, well even up to 120 hours, and there was little heterogeneity within these groups. This is in contrast with the findings of Noess (Noess, 1969) reporting that 40-60µg/ml PHT inhibited cell multiplication and 80-100µg/ml PHT resulted in cell death of human embryonic and adult gingival cells. Perhaps differences in their metabolic capacities may explain increased cytotoxicity of gingival but not of dermal fibroblasts. In contrast to dermal fibroblasts, keratinocytes were very sensitive to high concentrations of PHT. Pooled data analysis revealed that, after 72 hours, on average 46% of cells remained alive, but this figure dropped to only 38% after 120 hours. One cell line (nf-3) was however exceptional, as it tolerated PHT significantly better at 100µg/ml, than the other cell lines and 60% of cells were viable after 120 hours.

In conclusion, future studies using granulation tissue cells from PHT-treated and untreated wounds are needed to clarify the importance of clonal selection, as *in vitro* studies using previously untreated cells can only indicate that clonal selection is possible or perhaps likely to occur. Cell proliferation may also be confirmed and located *in vivo* using a proliferative marker (e.g. 5-bromo-2-deoxyuridine), or staining for proliferative cell nuclear antigens (e.g. Ki-67).

PHT, however, may not only act on or transform resident fibroblasts within the woundbed, but more importantly, could potentially recruit a different, more responsive subpopulation from the wound margins. A study by Nease (Nease, 1965) claimed that PHT (5µg/ml) increased fibroblast and keratinocyte outgrowths and migration from tissue explants of patients with either PIGO or periodontitis. However, the extent of the stimulation varied from individual to individual.

This observation was not reproducible, when normal, previously untreated cutaneous explants were incubated with PHT (Vijayashingham *et al.*, 1991). Vijayashingham and co-workers suggested that the failure of PHT to induce cell migration and proliferation *in vitro* were indicative of indirect modulation of these processes *in vivo*.

The results of this thesis nevertheless demonstrated that PHT was capable of exerting a direct effect on all three investigated cell types, and PHT was found to be a potent chemoattractant in Micro Chemotaxis Chamber assays. 5µg/ml PHT was found to elicit maximal responses for normal fibroblast and keratinocyte chemotaxis, whereas EB fibroblasts required a slightly higher concentrations of PHT (10µg/ml). It is possible that within EB fibroblast populations there are fewer cells, which could be induced to migrate or, alternatively, the cellular features of cell surface properties of EB fibroblasts may be different, requiring more chemoattractant stimulus. Normal and EB fibroblasts were similar in their response to the chemoattractants (except for the levels of PHT required to achieve maximum migration). Future work may concentrate on the assessment of changes to the surface markers of these activated fibroblasts in terms of their integrin and vinculin expression.

Keratinocytes, on the other hand, behaved differently from normal fibroblasts, as desensitisation with concentrations greater than the optimum diminished cell migration much more rapidly. It is possible that concentrations greater than 10µg/ml dose-dependently reduced cell viability and therefore their motility.

Checkerboard analysis of fibroblast migration revealed that PHT predominantly stimulated chemotaxis, with some chemokinesis. This suggests that PHT may play a role both in the initiation of cell recruitment and in the directional maintenance of fibroblast migration during granulation tissue formation. Therefore, a topical application of PHT directly into the wound is desirable, where it could potentially attract cells into the wound cavity from the margins and also mobilise cells already in the woundbed.

The transition of a stationary fibroblast into a motile fibroblast is associated with cytoskeletal changes together with the secretion of various enzymes, which degrade the extracellular matrix, facilitating cell movement.

PHT (10 μ g/ml) was found to induce the *in vitro* expression of one such protease, u-PA, in both normal and EB fibroblasts. The u-PA system consists of two serine proteinases (plasmin and u-PA), its inhibitors (α_2 -anti-plasmin, PAI-1, PAI-2) and the u-PA receptors (u-PAR), as shown on Figure 4.1.

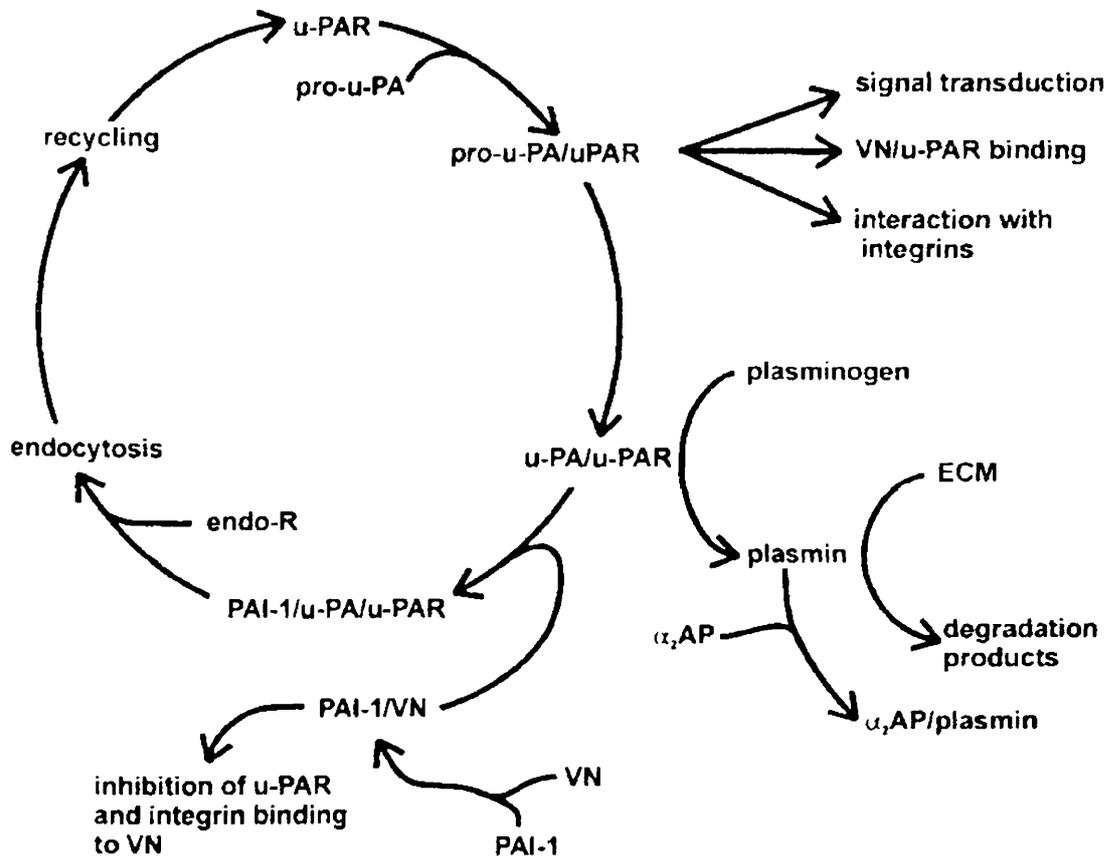


Figure 4.1. The dynamic state of the u-PA system on the cell surface (reproduced from Andreasen *et al.*, 1997).

The activation of this system can have manifold functions in regulating cell behavior (Andreasen *et al.*, 1997). Most importantly, it may upregulate cell migration simultaneously via both proteolytic as well as a non-proteolytic mechanisms. The proteolytic pathway involves the generation of plasmin at focal adhesion sites, catalysed by u-PAR-bound u-PA, resulting in matrix degradation and detachment of the trailing edge (Pollanen *et al.*, 1988; 1987). The non-proteolytic mechanism implies the enhancement of cell adhesion at the leading edge. This may occur via stimulation of the interactions between u-PA receptor and integrins (β_1 -, β_2 -) or vitronectin (Wei *et al.*, 1994) and additionally

signal transduction cascades may also be initiated. U-PA also activates PDGF and TGF- β .

U-PA expression is also crucial part of keratinocyte motility (Grondahl-Hansen *et al.*, 1988; Morioka *et al.*, 1987). Further work is needed to establish the effect of PHT on epithelial cells in order to investigate whether the acanthosis seen in PHT-treated pig wounds was a result of increased proliferation or u-PA-stimulated migration.

In addition to its direct action on skin cells, it can also be postulated that PHT may have an indirect effect on wound healing, via growth factor expression or responsiveness to growth factors:

- PHT was shown to increase PDGF release from macrophages and monocytes (Dill *et al.*, 1993). PDGF is important in the regulation of the proliferative and synthetic activity of fibroblasts, and is also a potent chemoattractant (Stoker *et al.*, 1991). During impaired wound healing both PDGF and its receptor expression are reduced (Beer *et al.*, 1997) and FN is extensively degraded (Wysocki and Grinnell, 1990), the latter as a result of perturbed proteinase inhibition mechanism (Grinnell and Zhu, 1996; Rao *et al.*, 1995; Grinnell *et al.*, 1992). The lack of FN may retard cell migration and the orderly deposition of collagen as it acts as a template for collagen fibrillogenesis (McDonald, 1982; Kurkinen *et al.*, 1980). Application of topical PHT may not only compensate for this imbalance by activation of macrophages and monocytes, but also by affecting the attachment, migration and proliferation of cells either directly or indirectly. The rate of collagen deposition and maturation may also be affected. Analysis of the *in vivo* changes in wound fluid profiles of PHT-treated wounds in the future may shed light on its effects on growth factors and cytokines and their roles in chronic wound healing.
- PHT was suggested to increase proliferative responsiveness to EGF (Reddy *et al.*, 1996) by upregulating its receptor expression in both normal (untreated) gingival fibroblasts and fibroblasts from PIGO (Huang *et al.*, 1997; Modeer and Andersson, 1990). Enhanced responsiveness to EGF

may increase migration speed and directional persistence of fibroblasts (Ware *et al.*, 1998). Analysis of the receptor profiles of granulation tissue fibroblasts (before and after PHT treatment) are needed to validate this hypothesis.

In conclusion, these *in vitro* studies (proliferation and migration) have shown that PHT may directly affect granulation tissue formation by stimulating cell migration, although a slight increase in cell division was also noted with low concentrations of PHT in all three cell types from some individuals. Differences in metabolic capacities or host responses (presence of genetically predetermined “responder” fibroblasts) may explain variations between individual responses to PHT. The recruitment of fibroblasts *in vivo* may enhance fibroplasia, and the upregulation of keratinocyte migration would speed up the re-epithelialisation process. Additionally, it is hypothesised, that indirect effects of PHT may also enhance this process via growth factor expression or alterations in responsiveness to growth factors.

4.2. The effect of Phenytoin on contraction (*in vitro*)

Two *in vitro* models of wound contraction have been used to assess drug effects on fibroblasts cultured in collagen matrices. Although neither of these models truly represents the *in vivo* situation completely, they provide useful insights into mechanisms that may cause contraction. Grinnell (1994) has excellently reviewed the differences between traditional tethered and untethered FPCL's. Further investigations were recently carried out as to the differences between the traditional untethered FPCL and the novel CFM (tethered gel) models (Porter *et al.*, 1998). In these systems, a floating collagen matrix resembles dermis, while gels under tension (traditional anchored gel or CFM) are akin to granulation tissue. Therefore, the latter model is more consistent in representing the *in vivo* mechanical restraint of the wound margins during healing, as cells are highly responsive to stimuli from the matrix (Eastwood *et al.*, 1998a). Free-floating FPCLs on the other hand provide an easy means of measuring changes over longer time periods (up to 7 days), while the CFM is

limited to the accurate measurement of force changes exerted by fibroblasts during short periods of time (up to 30 hours).

4.2.1. Comparison of contraction of normal and Epidermolysis Bullosa fibroblasts

Before the effects of PHT were investigated on normal fibroblasts and fibroblasts grown from pathological tissues any differences in contraction was measured between these two cell types. The EB fibroblasts investigated in this study were all hypercontractile, both as measured by the conventional untethered FPCL and the CFM methods. However, they were a highly select population since all derived from Recessive Dystrophic EB patients receiving surgery for contracture and mitten deformities. A more extensive investigation by Eisen and co-workers (1987), using also untethered FPCLs, established a broad range of variability in contraction (normal, poor and hypercontractile) between RDEB cells. This might be due to variations between the behaviour of cell derived from patients from different subclasses of RDEB.

Erlich and co-workers (1983) in a previous study examining 2 RDEB fibroblast lines in untethered FPCLs found that their cells showed retarded behaviour in contraction compared to normal dermal fibroblasts. They suggested this was due to their inability to elongate and spread out when incorporated into a collagen matrix and their reduced survival compared to normal fibroblasts. Moreover, an abnormal pattern of F-Actin immunostaining in microfilaments was also reported implying an altered cytoskeletal organisation in RDEB fibroblasts (Erlich *et al.*, 1984). Comparison of the results of this thesis with this study is difficult since they used pepsin-extracted collagen from human leiomyoma, compared with type I rat tail collagen used here. Pepsin digestion of collagen and inclusion of other collagen types alters fibrillogenesis (Leibovich *et al.*, 1962) and may change the nature of collagen lattice and cell responses (Erlich, 1988). In accordance with Erlich's (1983) findings referred to above, Adams and co-workers (1986) in a similar study suggested that impaired wound healing in RDEB patients is due to lack of contraction by fibroblasts, but no abnormal morphology was observed in their cell lines.

Normal and EB fibroblasts were also assessed in the CFM, where peak forces of contraction were compared at the end of their primary contraction peak, at approximately 8 hours. The contractile forces generated by EB cell lines were 2.5 times greater than that of normal cell lines. Morphological examination of EB cells did not reveal any aberrations as EB and normal cells were indistinguishable in their appearance in the tethered gels during a 24 hour contraction. This is in line with previous findings by Eastwood *et al.*, (1996) establishing that the pattern and magnitude of contraction can be correlated with cell attachment and the extension of cell processes. Although the morphological findings of the EB cells of this thesis were the opposite of those reported by Erlich (1983), both results underline the correlation between contractile capacity and cell morphology, both regulated by the cytoskeleton and focal adhesions.

The ability of the two experimental systems (conventional untethered FPCL and CFM) to determine differences in contraction was also compared. One EB cell line (ebf-2) produced 6.4 times more force than a normal cell line (nf-6) in the CFM but only 1.3 times in free-floating gels after 24 hours. This is probably due to the absence of mechanical restraint in untethered gels, which is known to alter cell behaviour (see section 4.5.1. 'The relationship between normal fibroblast migration and contraction'), together with the fact that the CFM is a much more precise instrument.

4.2.2. The effect of Phenytoin (single dose) on cell contraction

Experiments using free-floating gels established that PHT was capable of reducing the contraction of normal cells at 20 μ g/ml concentrations, but not at 10 μ g/ml. When tested, 20 μ g/ml PHT was also effective on the hypercontractile EB fibroblasts reducing their contraction to near normal levels.

In order to dissect its precise mechanism of action, a single application of PHT at 20 μ g/ml was tested in the CFM around the end of the primary contraction peak once peak levels were established, cells were attached and their processes extended. Interestingly, PHT induced an additional peak after an initial dip in contraction. The latter could be equated with a drop in force due to the mechanical disturbance of drug addition. However, at the end of the experiment (at 24 hours), the contraction force of both treated and untreated

gels was the same, as indicated by the ΔPF_4 value. This suggests that either the action of PHT diminishes with time or PHT is being converted into metabolites, which have relaxing effects. Both metabolites of PHT (P-HPPH and m-HPPH) were found to dramatically reduce contraction when administered at the end of the contraction peak (Fig. 3.30 and 3.31). Moreover, as drug metabolism occurs, PHT may also bind intracellular organelles (Figure 3.9.) further affecting cell functions.

EB fibroblasts responded similarly to normal fibroblasts, albeit peak forces (ΔPF_2) induced by PHT varied greatly, and took longer to achieve ($\Delta T_2 = 1.4 - 3.7$ times greater than normal). However, the difference between the forces of contraction of treated and untreated EB cells was similar (ranging between $\Delta PF_3 = 8.7-10.7 \times 10^{-5}$ Newtons/million cells), and was twice that seen in normal fibroblasts (Fig. 3.17). This perhaps reflects the maximum effect PHT may exert on EB cells at this concentration. Nevertheless, as before, ΔPF_4 values were small, indicating either the transient nature of PHT effects or its potential metabolism. Further work is required to establish whether differences in peak forces (ΔPF_2) in response to PHT in EB fibroblasts reflect on their pathological nature or simply vary between patients.

The CFM has proven to be a powerful tool for the analysis of the mechanism of contraction and drug effects. Previous work with the CFM has shown that contractile forces were generated as soon as the gel becomes solid and corresponds with fibroblast attachment to the collagen matrix and extension of cell processes (Eastwood *et al.*, 1996). This was confirmed in this present study using EB cells and correlation of cell contraction and cell morphology (Fig. 3.15). Work from our laboratory by Miss. K. Sethi has also shown that blockage of cell-matrix interactions through FN and VN reduced and abolished contraction, supporting the importance of cell-matrix interactions in force generation. As changes in cell morphology and contraction require a functional cytoskeleton, the CFM has been used to observe the effects of cytoskeletal disrupter drugs on contraction. Based on these findings Eastwood and co-workers (Eastwood *et al.*, 1998a) have suggested three cell-mediated components to the generation of force (Fig. 4.2).

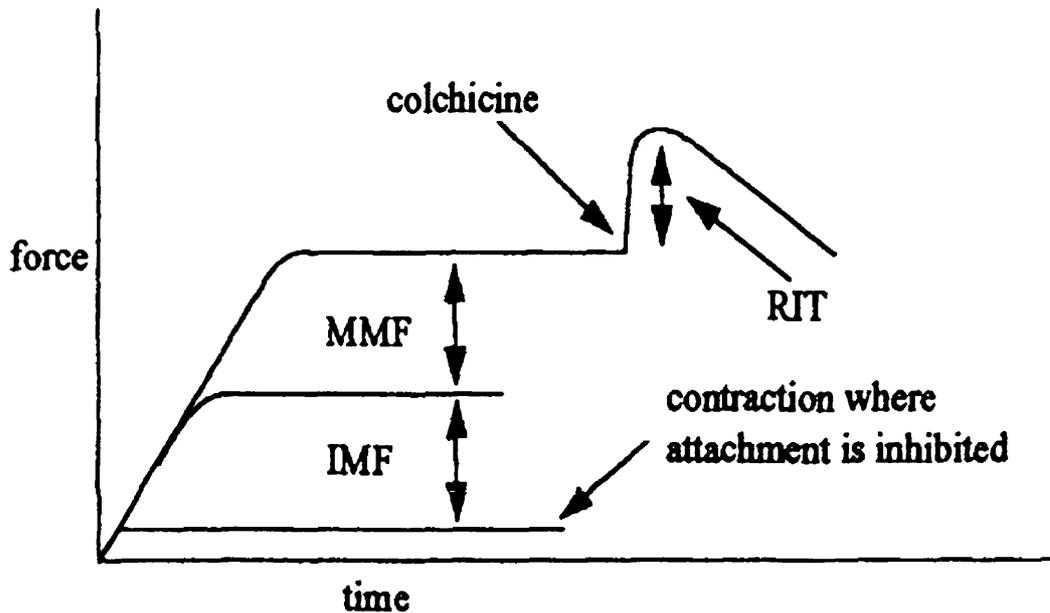


Figure 4.2. Composite curve showing isolated components of the fibroblast-mediated contraction (reproduced from Eastwood *et al.*, 1998a).

IMF = integrin-mediated force, MMF = microtubule-mediated force and RIT = residual internal tension.

According to their concept, the initial force generated is due to cell-matrix binding termed integrin-mediated force (IMF). It is thought, that $\alpha 2\beta 1$ integrin is primarily responsible for this initial contraction (Carver *et al.*, 1995; Klein *et al.*, 1991). This is followed by microtubule-mediated force (MMF) associated with the elongation of the cell processes. The final component is produced when microtubules are disrupted (i.e. COL treatment) and the residual internal tension (RIT) within each cell is released.

In this study, the contraction peak induced by COL was compared with that of PHT to investigate any similarities of action between these two drugs. (For detailed mechanistic analysis of the action of COL see section 4.5.2. 'The action of cytoskeletal poisons on normal fibroblast contraction and migration'.) This contraction (ΔPF_2) produced by PHT was half that of COL, a known microtubule poison at equimolar concentrations in the same normal fibroblast cell line. Furthermore, the difference in contraction between treated and

untreated cells (ΔPF_3) was 5.5 times greater with COL. In both cases, however, at 24 hours drug effects were diminished. Immunostaining for β -tubulin in normal fibroblasts after incubation with various concentrations of PHT (20-100 μ g/ml) revealed that microtubules were intact and in a dynamic state, therefore any similarity in action between these two drugs was ruled out. Although numerous reports claimed that PHT inhibits tubulin polymerisation (thereby inducing metaphase arrest), and that PHT has Colchicine-like activity in various cell types (Estus and Blumer 1989; MacKinney *et al.*, 1984, 1980, 1977), this was not found to be the case in human dermal fibroblasts.

The mechanistic detail of contraction induction by PHT (single dose) is yet to be evaluated. It is hypothesised that PHT may alter contraction by influencing EGF-induced focal adhesion disassembly and cell motility (Xie *et al.*, 1998) as it was shown to upregulate EGF receptor expression in normal (untreated) gingival fibroblasts and fibroblasts from PIGO (Huang *et al.*, 1997; Modeer and Andersson, 1990).

It is also possible, that PHT, although not a classical calcium channel antagonist (Messing *et al.*, 1985), may influence cell migration by blockage of calcium movement through the cell membranes (Twombly *et al.*, 1988) thus altering intracellular calcium levels. Although calcium is thought to be an important secondary messenger in cells generally, it also plays a key role in the activation of calpain, a calcium-dependent protease. Calpain facilitates the release of cytoskeletal linkages (both $\beta 1$ and $\beta 3$ integrin-mediated) during rear traction and therefore cell migration.

Additionally, it is feasible that PHT induces changes in the function of motor elements in the fibroblasts resulting in a small contraction.

4.2.3. The effect of Phenytoin pre-treatment on cell contraction

Pre-treatment of normal and EB fibroblast resulted in the reduction of primary peak force of contraction, which was repeatable with different cell lines in both cell types. The degree of this reduction was however more variable in EB fibroblasts (26-63% reduction) than in normal fibroblasts (48-54% reduction). However, more cell lines need to be investigated in both cases to draw significant conclusions from this study.

This reduction in contraction with prolonged exposure to PHT may be due to accumulation of its metabolites. Both p-HPPH and m-HPPH (racemates) were demonstrated to reduce contraction dramatically in the CFM (see section 4.5.3. 'Evaluation of the action Hydantoin derivatives on normal fibroblast contraction and migration').

Interestingly, the magnitude of this reduction seen with PHT pre-treatment remained similar in the presence or absence of PHT in the CFM culture media, confirming previous suggestions that PHT-induced phenotypic changes are retained in the absence of PHT through several passages (Benveniste and Bitar, 1980; Hassell *et al.*, 1976). It is important to note that in both normal cell lines (nf-8 and nf-4) investigated, peak times remained the same when PHT was present in the CFM culture media (Fig. 3.19 versus 20), whereas in the absence of PHT peak contraction was achieved much later (approximately $\Delta 10$ hours, Fig. 3.20). The significance of this phenomenon requires further investigation.

PHT pre-treatment of cells also resulted in the alteration of their RIT. In untreated cells RIT was equal to 50% of their PF. PHT pre-treated cells, on the other hand, displayed only 30-32% more force (RIT), with or without PHT present in the CFM culture media, upon COL treatment. Again, drug induced phenotypic changes may be responsible for this.

4.3. The effect of Phenytoin on porcine wound healing and contraction

The effects of PHT on porcine wound healing were observed at two time points in two different formulations.

In the first experiment various concentrations of PHT powder were used to treat non-randomised wounds and evaluated after three weeks. Full-thickness wounds, as in this case, heal by re-epithelialisation and contraction (Pollack, 1984). The repair sites were visibly re-epithelialised and the control wounds had contracted approximately 63% of their original size, which was in line with previous reports (Rudolph, 1977). PHT powder was found to dose-dependently reduce wound contraction *in vivo*, a feature indicated previously by *in vitro* experiments of this thesis. Prolonged exposure of human dermal

fibroblasts to PHT reduced their contraction significantly as measured both by untethered FPCLs and the CFM.

Although the surface areas of PHT-treated wounds were larger than the controls, there was no impairment of healing suggesting that the rate of filling was increased. Therefore, reduced contraction was compensated for by upregulated matrix production and vice-versa. Although collagen deposition did appear to be greater in PHT-treated wounds by light microscopy, it is not known if reduction in wound contraction was indeed the result of the emergence of phenotypically altered fibroblasts with more synthetic rather than contractile ability or perhaps the action of metabolites shown to reduce contraction *in vitro*. A possible way to investigate this phenomenon would be to culture granulation tissue fibroblasts from treated and controls wounds and measure their contractile and synthetic capabilities. Moreover, in the absence of a suitable control powder, it is also not known what effects can be attributed to the sole physical presence of a bulky material inside the wound. Additionally, apart from the presence of PHT powder in the wounds, increased amounts of entrapped adipose tissue could also be responsible for reduction in wound contraction (De Vries *et al.*, 1995). These islands of fat cells seen in PHT-treated wounds might have once served as a reservoir for PHT, which is a lipophilic compound. However, it is unclear by what mechanism do these adipocytes escape from the woundbed.

At this time point there were no differences noted in fibroblast number, vascularisation or inflammatory status between the PHT-treated and control wounds. High concentrations of PHT (84 and 120mg/wound) appeared to enhance collagen deposition. This feature is, however, yet to be confirmed by Transmission Electron Microscopy and quantified by radiolabelling (Hou, 1993).

All PHT-treated wounds, regardless of the dose administered, displayed more extensive re-epithelialisation and areas of acanthosis. This epidermal morphology associated with PHT treatment was unusual, since the healing of full-thickness wounds is associated with a more flattened rete ridge pattern compared with normal skin. In no cases were there immune infiltration into the acanthotic areas, suggesting that it could be distinguished from abnormal conditions such as psoriasis and eczema. Further research is required to establish if the effect seen is transient or permanent and localise the

proliferative pattern by staining with either 5-bromo-2-deoxyuridine or Ki-67 antibody.

This *in vivo* phenomenon also confirms previous *in vitro* results of this thesis suggesting that PHT may enhance keratinocyte proliferation and migration in susceptible individuals. The increased presence of keratinocytes may in turn lead to the secretion of elevated levels of PDGF (Ansel *et al.*, 1992; Krane *et al.*, 1991) thus potentially influencing the growth, migration and synthetic capacity of fibroblasts and vascular elements (Stoker *et al.*, 1991).

Acanthosis is also a common feature seen in PIGO. Therefore, the mechanism of action of PHT in promoting wound healing may lie in its ability to stimulate epithelial proliferation and decrease cell transit time. These early *in vitro* and *in vivo* indications suggest that PHT may mediate a better attachment of the epidermis to the dermis. This would provide an increased resistance to mechanical forces likely to produce re-traumatisation of the recently healed wound. Topical PHT may be a particularly useful therapeutic agent in the treatment of mechanobullous diseases such as EB, as it improves epidermal-dermal cohesion.

In the second experiment, randomised wounds were treated with PHT powder or in combination with FS and evaluated two weeks post-operatively. Fibrin Sealants are tissue adhesives that have been extensively developed and tested for clinical use in Europe and are awaiting regulatory approval in the USA, as they are manufactured from pooled blood products (Sierra, 1993). They afford biocompatibility, biodegradability, and haemostasis over artificial sealants such as cyanoacrilates and marine adhesives. Fibrin Sealants may also be used to deliver drugs. If developed, PHT incorporated into FS may have potential uses in increasing the tensile strength of surgical wounds and reducing unwanted contractures.

In contrast to 3-week wounds, 2-week control wounds contained more inflammatory cells, fibroblasts, and blood vessels. The presence of predominantly macrophages, lymphocytes and eosinophils was also indicative of the late inflammatory phase. Wounds treated with FS especially contained increased immune infiltrates. This may have been a reaction to human fibrin. However, when FS was combined with PHT, there was a remarkable reduction in inflammation. This is in line with previous clinical data reporting the ability of

PHT to enhance wound healing by speeding up the inflammatory process (Flanagan and Flanagan, 1992; Lodha *et al.*, 1991; Malhotra and Amin, 1991; El Zayat, 1989; Modaghegh *et al.*, 1989; Smith *et al.*, 1988).

Both FS and PHT reduced wound contraction when compared with controls, although they failed to have an additive effect at low PHT doses. Also, there was no significant difference between the contraction of controls and wounds treated with 6mg PHT in FS/wound. Perhaps these discrepancies were due to the wounds being randomly located on the back of the pig, where certain areas of the skin were submitted to more motion. As with 3-week wounds, PHT reduced wound contraction dose-dependently and all treated wounds manifested epithelial acanthosis and entrapped islands of fat. These findings indicated that FS was a good drug delivery system, and PHT in combination with autologous FS may be a useful candidate to be developed clinically.

4.4. Wound Pharmacology of Phenytoin and its clinical implications on therapy

Currently, much attention and faith is placed on the development of expensive, topical molecular factors. The efficacy of such agents remains yet to be evaluated in clinical trials and they may not prove to be useful. This could be due to the fact that these agents may not remain active when placed in a hostile wound environment. Moreover, no single factor could be the answer to remedy a chronic wound (Harding and Boyce, 1998). PHT on the other hand is cheap and is readily available in most countries.

Clinical studies using topical PHT therapy have suggested that it may be useful for the treatment of both acute and chronic wounds of various aetiologies. Although these results are encouraging, the efficacy of topical PHT therapy has yet to be confirmed by double blind, placebo-controlled studies. Surprisingly, most of the clinical trials with topical PHT were based on the daily application of a uniform layer of PHT powder of unknown quantity. Only Lodha *et al.* (1991) specified the dose to be 20mg/cm², which was sufficient to promote wound healing, but it could not be taken to be optimal. Clearly, dose-response studies are needed with topical PHT in the various wound aetiologies.

It has been concluded (although few investigators have measured serum levels), that systemic absorption is not significant (Anstead *et al.*, 1996; Masgrau-Peya *et al.*, 1995; Pierce *et al.*, 1995; Lewis and Rhodes, 1994; Smith *et al.*, 1988) in accordance with our *in vivo* results. A very recent report is perhaps the most striking of all case studies. Anstead *et al.* (1996) treated an obese man with a massive sacral-lumbar pressure ulcer which required 12.5g/day of bulk-grade PHT as a slurry in NaCl (0.9%) to cover it. Despite the application of these great amounts of PHT, which represented several times the lethal daily oral dose, the serum concentrations were only 4.3mg/l a month after therapy was commenced. This is the only account, which measured appreciable serum concentrations. However, this was also the largest wound ever to be treated with topical PHT.

The solubility of powdered PHT in wound fluid (pH=7.07-7.2) is expected to be similar to or less than that of plasma (pH=7.4, 75µg/ml; Woodbury, 1989) and reasonable amounts will either be washed out with any exudate or lost on the dressing. The presence of bacteria will, moreover, lower the pH of wound fluid, decreasing the solubility of PHT. It is not known, however, if bacteria are capable of metabolising PHT. Both our *in vitro* and clinical studies suggested that PHT is well tolerated by resident fibroblasts. Migration studies of this thesis showed that PHT might play a role both in the initiation of cell recruitment and in the directional maintenance of fibroblast migration during granulation tissue formation, which clearly demonstrated the importance of topical, rather than systemic administration of PHT for wound healing purposes.

Moreover, these results also bear implications on the dose and the formulation of the topical PHT treatment. Therefore, future studies should be aimed at the production of various formulations of PHT suitable for applications during the changing course of wound healing and their optimisation for topical delivery in animal models. Although Modaghegh (Modaghegh, 1989) claimed that PHT powder gave the most favourable results in a rat experimental model, testing four formulations (gel, cream, PHT sodium powder, and PHT powder), no further details were given to substantiate his results.

In attempting to develop the ideal topical formulation of PHT for wound healing purposes, various factors have to be considered:

- The wound site: Gingival or dermal location?

Formulations for the enhancement of gingival wound healing used low concentrations (1%) of PHT in gels in line with the fact that systemic uptake from the oral mucosa is a likelihood. Contrary to this, copious amounts of PHT powder were applied to chronic wounds where the entire epithelium was absent, without significant systemic absorption. (One reason for this could be, apart from the low water solubility of PHT, that vascularity and perfusion are also compromised in non-healing wounds.)

- State of dermal wound: Full-thickness or partial thickness?

In the early stages of wound healing high concentrations of PHT may be applied directly to the woundbed to stimulate dermal and vascular functions. Later, re-epithelialisation will increasingly impede drug delivery to the dermis, even though a fully differentiated SC is not yet present (Walker *et al.*, 1997), requiring a suitable method of delivery (e.g. in combination with penetration enhancers) to ensure that adequate amounts of PHT will reach the dermis. Alternatively, depending on the progress of the wound, PHT may be used to stimulate epidermal activity in low concentrations (incorporated into an appropriate vehicle), as keratinocytes are more sensitive to it.

- Powder or formulated application?

Although many of the trials indicated the efficacy of PHT powder, its use poses practical limitations, namely inaccuracy of dose, potential hazard to patient/carers by airborne particle exposure. A recent study by Masgrau-Peya *et al.* (1995) reported the use of PHT cream (2 or 5% in a hydrophilic base) in EB Simplex patients. Cream formulations are not only easily administered, but also act as emollients. Currently Laboratorios Rubio, Spain, is developing an ointment for wound healing purposes.

Another way of administering this drug would be to incorporate it into FN mats (Brown *et al.*, 1997; Ejim *et al.*, 1993) designed to deliver therapeutic agents (Whitworth *et al.*, 1996). It has been suggested that the addition of exogenous plasma FN in solution form aids wound healing (Wysocki *et al.*, 1988; Kono *et al.*, 1985; Nishida *et al.*, 1983). This combination of solid state FN and PHT would provide a scaffold that would promote the adhesion, orientation and movement of a variety of cells *in vitro* (Prajapati *et al.*, 1996; Wojciak-Stothard *et al.*, 1996) and *in vivo* (Whitworth *et al.*, 1996), and in turn the released PHT would potentially enhance the activity of a variety of cells. Provision of exogenous FN would also ensure that the fibroblasts retain their migratory phenotype, as endogenous FN production leads to a switch to stationary phenotype (Mensing *et al.*, 1984).

Thirdly, as has been demonstrated in this thesis, PHT can be readily mixed with FS, whilst retaining its activity and would afford a useful tool for the sealing of surgical wounds. Moreover, PHT could also be incorporated into wound dressings based on materials such as alginate, collagen or other dermal devices.

The 'universal ability' of PHT to heal wounds was demonstrated in patients from diverse ethnic backgrounds. Its effectiveness therefore indicates that most individuals possess in their skin either enzymes capable of converting PHT to an active species, or alternatively there are subpopulations of skin cells capable of responding directly to PHT itself. This is in contrast to the incidence of PIGO (approximately 50% of treated patients), which may be linked to differential individual metabolic capacities by liver enzymes and the presence of responder cell populations in gingival tissues.

Little is known about the distribution and metabolism of PHT within the wound. Given the nature of PHT and poor circulation in chronic wounds, it is feasible that PHT is retained there for longer periods and could be metabolised by cytochrome P450 isozymes present in the epidermis, sebaceous glands, the outer root sheath of the hair follicles and the dermis (Streinstrasser and Merkle, 1995). Immunohistochemical evaluation of PHT uptake by normal fibroblasts of this thesis demonstrated that cells were able to internalise and metabolise PHT. The drug was predominantly bound around the nucleus, in accordance with previous reports (Vernillo and Schwartz, 1987). Therefore, future, meaningful *in*

in vitro studies into the absorption of PHT in wounds should consider both diffusion and its cutaneous biotransformation.

It is clear that no single target/cell could be isolated, which is responsible for accelerated healing of PHT-treated wounds (Fig. 4.3). Also, it is likely that PHT has direct and indirect effects on a variety of cells.

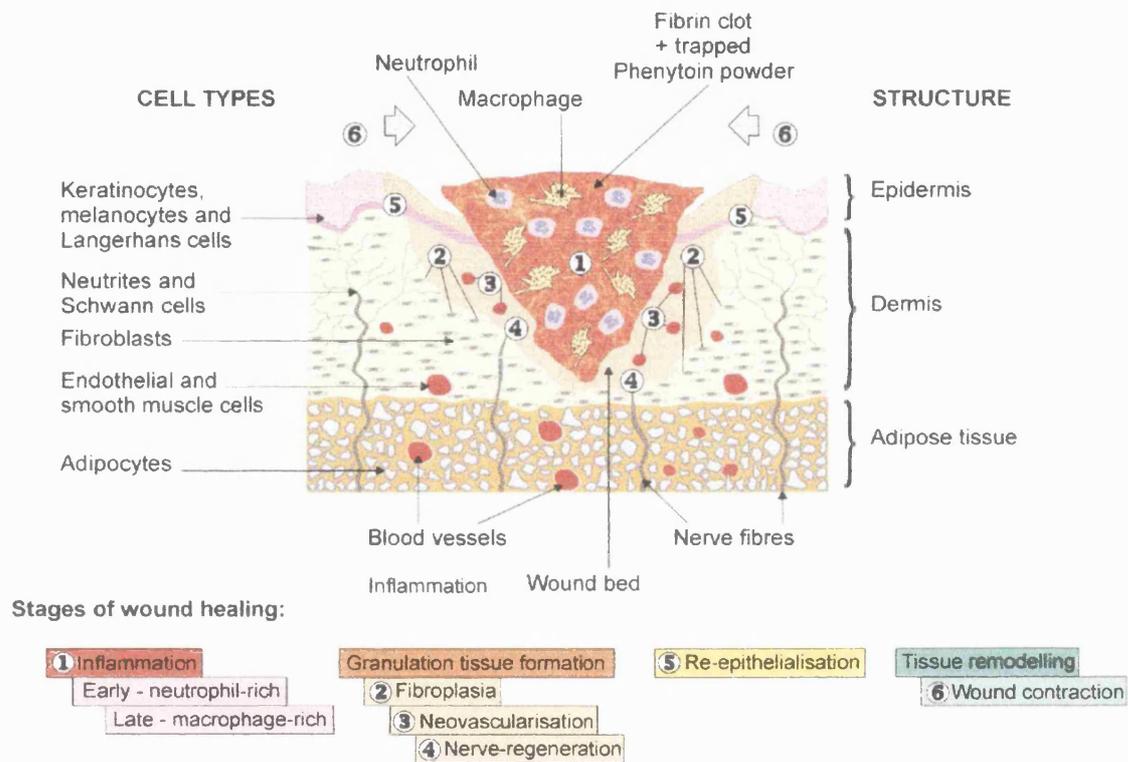


Figure 4.3. Schematic overview of wound healing. No single target/cell could be identified which is responsible for accelerated healing of PHT-treated wounds. *In vivo* and clinical studies suggest that topical PHT accelerates the inflammatory process, granulation tissue formation and re-epithelialisation. Interestingly, it also reduces wound contraction, whilst upregulating matrix accumulation and thereby healing is not impaired.

Although there is evidence from studies using gingival fibroblasts that PHT alters ECM deposition (collagen and FN) in gingival tissues, further work is needed to confirm this in dermal fibroblasts for wound healing. In particular collagen and FN production may be assayed for by radiolabelling (Hou, 1993) or by RT-PCR (Tarnuzzer *et al*, 1996). Furthermore, the effect of PHT should also be tested *in vitro* on endothelial (microvascular and large vessel cells) and

nerve cells (Schwann cells, neurites) as both vascularisation and innervation were reported to be increased upon treatment.

More importantly, the active enantiomer of the pharmacophore [(R)-p-HPPH] needs to be confirmed for wound healing. In this context it seems to be important that racemic mixtures m-HPPH and p-HPPH were found to be stronger chemoattractants than PHT for human dermal fibroblasts *in vitro*, further emphasising the need for continued interest in the Wound Pharmacology of PHT. This also raises the question, is it possible to design a new generation of safer, HYD-related drugs, by replacing the phenyl rings without losing their activity? In order to address this question, the activity and effects of HYD-related compounds were evaluated in search for a new wound healing agent.

4.5. In search of a new vulnerary agent

Having identified two *in vitro* models in which the effects of PHT on dermal fibroblasts could be measured, other related compounds were tested to establish a structure activity relationship. The aim was to identify the active component of the PHT molecule that is responsible for inducing cell migration and alteration of contraction.

The main questions regarding structure and activity were as follows:

- Does the basic structure, HYD, have any activity on its own or are further substituents required?
- Are both phenyl rings (associated with potential carcinogenicity) necessary for activity?
- What is the effect of ring substitution?

In order to analyse the effects of HYD-related compounds, firstly the behaviour and the cytoskeleton of untreated fibroblasts (controls) were investigated. Then, the effects of known cytoskeletal poisons were tested for comparison and for mechanistic clues. Finally the effect of HYD-related compounds were observed.

4.5.1. The relationship between normal fibroblast migration and contraction

PHT was found to influence cell migration and contraction *in vitro*. In order to appreciate the significance of these effects when searching for active PHT analogues, the relationship between these two important events must be discussed.

Cell migration (Fig. 4.4) is an essential part of wound healing. It involves morphological polarisation, membrane extension, formation of cell-substratum attachments, exertion of contractile force and traction, and finally the release of attachments by the cells moving into the wound cavity. Locomotion involves at least two separate types of force generation. Firstly, a protrusive force to extend the membrane processes, and secondly a contractile force to move the body forwards. Lauffenburger and Horwitz (1996) have recently reviewed these independent though co-ordinated phenomena.

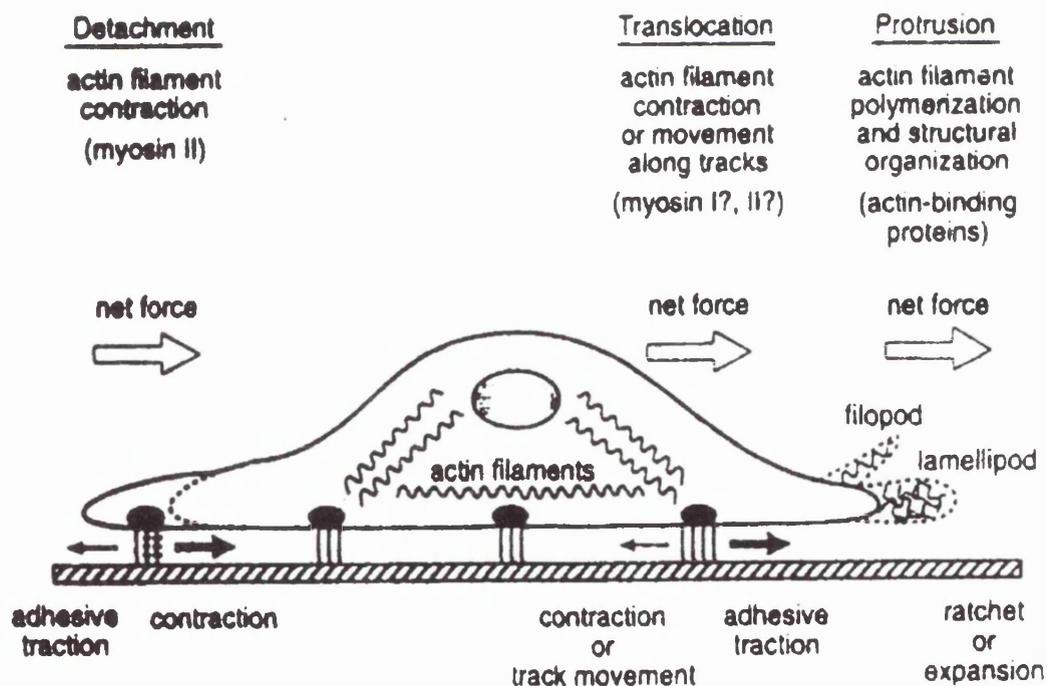


Figure 4.4. "Illustration of the different forces involved in cell migration. Protrusion of the membrane lamellipodia or filopodia requires force generated by actin polymerisation, by the Brownian ratchet mechanism, the cortical expansion mechanism, or a combination of these. Translocation of the cell body forward, once the membrane protrusion has become adherent to the

substratum, may occur by myosin interactions with actin filaments; possible mechanisms for this include contraction of filaments connecting cell-substratum adhesion complexes with intracellular structures, or relative movement of adhesion complexes across cortical actin filament "tracks". In either case, the magnitude of the traction is greater than the rearward pull of the adhesion complexes. Detachment of the cell rear involves disruption of cell-substratum attachments, perhaps accelerated by myosin-mediated actin filament contraction pulling on adhesion complexes. Here, the magnitude of traction is less than the contraction force." (Quote and diagram from Lauffenburger and Horwitz, 1996).

The tractional force exerted by a moving cell is related to the intracellularly generated contractile force, albeit they are not equal, as cell deformation and disruption of the cell-substratum attachments may occur. Likewise, a non-compliant substratum will exert an equal, opposite traction force on this cell through these attachment plaques (Choquet *et al.*, 1997). Therefore, the magnitude of the cell-generated internal contraction is not the only determining factor of cell migration speed. It is predicted that the ratio of the contractile force to cell substratum adhesiveness determines locomotion in a biphasic manner (DiMilla *et al.*, 1991). Maximal speed is associated with intermediate ratio of contractile force to substratum adhesiveness (Keely *et al.*, 1995; DiMilla *et al.*, 1993). This dependence of locomotion speed suggests that adhesion complexes in the tail region may be one means by which cell migration rate is regulated (Palecek *et al.*, 1998; 1996).

Another important aspect of wound healing involving cell migration is wound contraction. It has been suggested that tractional forces of normal migrating fibroblasts (Grinnell, 1994; Erlich and Rajaratnan, 1990) bring about wound contraction. Impairment of cell migration, on the other hand, was demonstrated to lead to reduced contraction of untethered FPCLs (Andujar *et al.*, 1992).

This complex *in vivo* phenomena was excellently represented in a mathematical model by Tranquillo and Murray (1993) outlining the importance of the interaction between both cells and the surrounding extracellular matrix during wound contraction. To summarise, their model accounted for:

- the presence of fibroblasts and their activities (random migration, passive convection with ECM, logistic growth),
- the extracellular matrix and its passive convection,
- the cell/ECM composite (simple viscoelastic material containing traction-exerting cells and elastic subdermal attachments).

Although the manifold aspects of wound contraction can not be reproduced accurately *in vitro*, the use of FPCL's has provided important mechanistic information. Firstly, contraction is cell-mediated and its force is dependent on cell type (Porter *et al.*, 1998; Eastwood *et al.*, 1996; Kolodney and Wysolmerski, 1992) and the integrity of the cytoskeleton.

Secondly, stress conveyed by the extracellular matrix is a key factor in fibroblast morphology and potentially phenotype development. In anchored collagen matrices cells are bipolar and orientated along the lines of maximum strain (Eastwood, 1998b), whereas fibroblasts in floating collagen matrices are stellate, having no preferred or substantial strain (Grinnell, 1994; Nakagawa *et al.*, 1989). Moreover, in stressed lattices, cells continue to synthesise DNA and proliferate whereas, in unstressed lattices, cells arrest in G₀. Stressed lattices are also characterised by increased collagen biosynthesis and decreased collagenase activity (Grinnell, 1994; Lambert *et al.*, 1992; Nakagawa *et al.*, 1989).

Thirdly, soluble factors present within collagen lattices were also important in controlling cell migration and contraction. FN has been identified as one of the serum factors controlling contraction and cell migration (Asaga *et al.*, 1991; Gillery *et al.*, 1986, Knox *et al.*, 1986), whilst FN and VN are important early mediators of tractional forces. Several growth factors (TGF- β , PDGF) have also been identified which promote cell contraction even in the absence of serum (MacNeil *et al.*, 1996; Anderson *et al.*, 1990; Clark *et al.*, 1989; Montesano and Orci, 1988). It may be that such stimulation in contraction and

migration can be correlated with up-regulation of integrin synthesis (Keely *et al.*, 1995; Kirschberg *et al.*, 1995; Carver *et al.*, 1995; Klein *et al.*, 1991).

In view of the above mentioned factors affecting cell migration and contraction, the comparison of results obtained from the Micro Chemotaxis Chamber assays in the absence of serum with those of the CFM are difficult. Nevertheless, these tests in their own right provide useful information regarding the effects of HYD derivatives on different aspects of cell migration and contraction.

4.5.2. The action of cytoskeletal poisons on normal fibroblast contraction and migration

The actions of three cytoskeletal poisons, Colchicine, Taxol, and Cytochalasine B were investigated in this thesis. Previous studies have shown that the addition of either of these drugs inhibited fibroblast migration to FN as measured in Micro Chemotaxis Chambers (Joseph *et al.*, 1989). This further supports the idea that a functional cytoskeleton is required for alterations in cell shape and migration (Tomasek and Hay, 1984). Treatment of cells with COL or CYTO was also found to interfere with their protein synthesis and secretion (Evangelisti *et al.*, 1995).

COL, the first agent tested, prevents microtubule polymerisation by tubulin binding. In accordance with this, cytoskeletal staining of normal fibroblasts grown on glass slides confirmed their disruption at concentrations used in this thesis. COL was reported to reduce the contraction of untethered FPCLs as measured after 24 hours (Kasugai and Ogura, 1995) whilst Brown and co-workers (Brown *et al.*, 1996) reported gel contraction of FPCLs in the CFM within two hours of its addition. However, this effect was transient, as peak contractions returned to normal after several hours depending on whether COL was added at the primary or secondary phase of contraction. Similar induction in contraction was observed at COL concentrations used in this thesis. It is postulated that the disruption of microtubules (which are under compression, i.e. loading) results in a displacement of the load unto the matrix leading attached to the cells. This leads to an increase in contractile force exerted on the matrix (Brown *et al.*, 1996; see also Fig. 4.2). Retraction of cell processes

produced a peak in contraction, which is indicative of the mechanically load (Fig.4.5) stored in the 'space frame', i.e. the RIT. Kolodney and Elson (1995), on the other hand, suggested that myosin activation by phosphorylation of LC₂₀ is responsible for contraction stimulated by microtubule poisons.

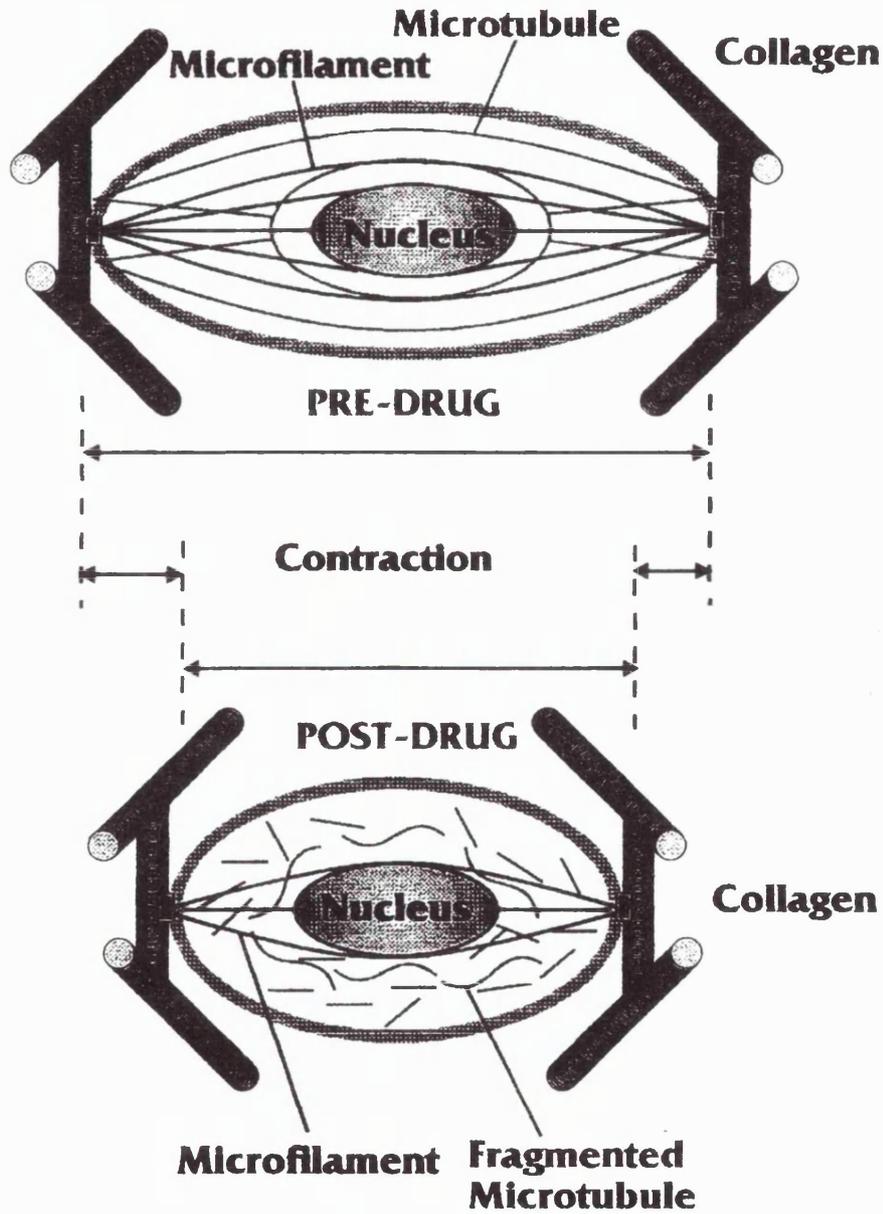


Figure 4.5. Diagram showing the "Balanced Space Frame" model of microtubular function in fibroblasts (reproduced from Brown *et al.*, 1996).

A = Untreated cell. B = Cell after COL treatment. Note the disruption of the load-bearing microtubules by COL treatment.

TAX, another cytoskeletal agent studied here, operates through an opposite mechanism to COL. It binds and stabilises microtubules causing all the free tubulin to assemble into microtubules. This was confirmed by cytoskeletal staining of normal fibroblasts grown on glass slides at concentrations used in this thesis. TAX has been previously reported to relax the contraction of FPCLs in a system similar to the CFM (Kolodney and Elson, 1995; Kolodney and Wysolmerski, 1992). This finding was well reproduced in the CFM at concentrations used in this thesis. Again, invoking the “Balanced Space Frame” model, an increase in microtubule density would result in an increase in RIT, as it would take some load off the surrounding collagen, registering as a fall in force.

CYTO, the third cytoskeletal agent studied, acts by depolymerising actin microfilaments. Cytoskeletal staining of fibroblasts grown on glass slides revealed total cell collapse into a star shape as a result of the disappearance of microfilaments, with little remaining attachment sites. This loss in force generation was well reproduced in the CFM and has also been previously reported in both tethered (Kolodney and Wysolmerski, 1992) and untethered systems (Kasugai and Ogura, 1993; Bell *et al.*, 1979).

In summary, to date many theories are in existence for the action of cytoskeletal poisons on FPCL contraction giving an explanation through a mechanical mechanism, based on Ingber’s tensegrity model (Ingber, 1993) or by biochemical regulation of motor cell elements (Kolodney and Elson, 1995; Kolodney and Wysolmerski, 1992). The space frame model proposed by Brown and co-workers (1996) complements previous hypothesis of tensegrity (the concept that the external load e.g. on the collagenous matrix is counterbalanced by internal forces in the cell) by Dennerll *et al.* (1988) and Ingber (1993, 1991).

4.5.3. Evaluation of the action Hydantoin derivatives on normal fibroblast contraction and migration

Having considered the complex interaction of cell migration and contraction, a rationalisation of the activity of HYD derivatives was attempted using the CFM and Micro Chemotaxis Chamber assays.

The CFM proved to be a valuable tool for the assessment of precise force changes induced by these test compounds. Based on their abilities to influence the contractile profile of tethered FPCLs, these drugs fell into 'active' and 'inactive' categories. 'Active' compounds induced either a relaxation or a contraction in the gels, while 'inactive' compounds had no effects at all. The behaviour of 'active' compounds was further classified according to the changes induced in peak forces resembling the action of cytoskeletal inhibitors. Cytoskeletal staining (against β -tubulin, F-actin, vimentin and vinculin) of fibroblasts treated with HYD-related compounds at equimolar concentrations has however ruled out any similarity in action with either COL, TAX or CYTO.

Moreover, all compounds found 'active' in the CFM also induced cell migration in normal fibroblasts, albeit at lower concentrations. (Interestingly, Me-PH, although chemotactic for fibroblast, failed to elicit any response in the CFM.) This further confirmed that cytoskeletal function was not impaired and cell migration was not abrogated as reported to be the case with cytoskeletal poisons (Joseph *et al.*, 1989).

PHT was found to be unique amongst the 'active' compounds in that it had an opposite effect in the CFM to all other 'active' compounds, even though, they all induced chemotaxis. Potential mechanisms of action of PHT in inducing cell migration and contraction has already been discussed (see section 4.2.2. 'The effect of Phenytoin (single dose) on cell contraction'). It is possible that these other 'active' HYD derivatives may also act by affecting cell-matrix adhesions, as differences in the distribution of vinculin-containing focal adhesions together with minor changes in cell shape were noted. It is hypothesised that these compounds may elicit a favourable ratio of contractile force to substratum adhesiveness (e.g. by regulation of $\alpha 2\beta 1$ integrin expression).

Nevertheless, good correlations were observed between the results obtained from migration and contraction studies. Therefore, the main

conclusions regarding structure-activity relationship combined from these two assay systems are as follows (Fig. 4.6-8):

A. The basic structure, HYD, had no activity indicating that further substituents are required at the 5-position.

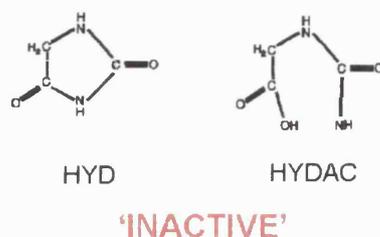


Figure 4.6. Structure-activity relationship of HYD derivatives to cell migration and contraction (step 1).

HYD, which represents the basic structure of PHT without substituents, had no effect on either contraction or migration. HYDAC, a compound resulting from the ring opening of HYD, was also inactive.

B. Both phenyl rings are preferred for maximum activity.

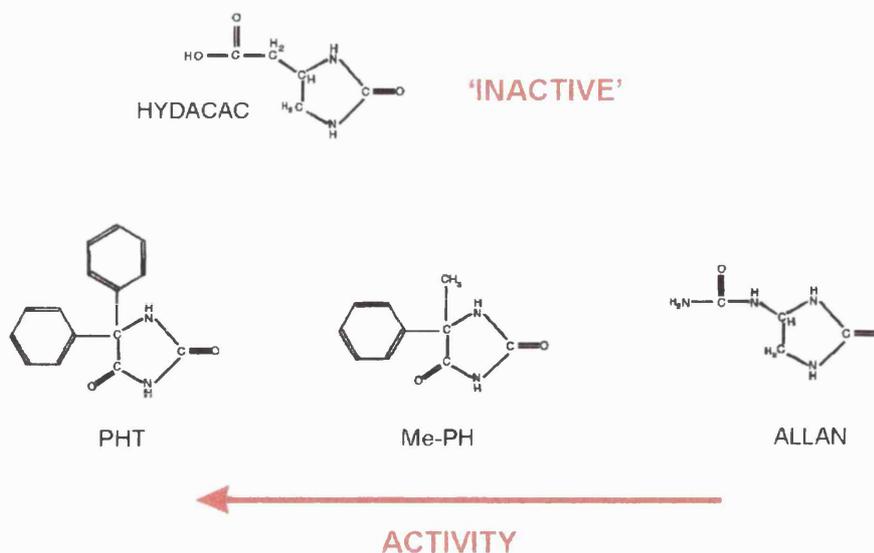
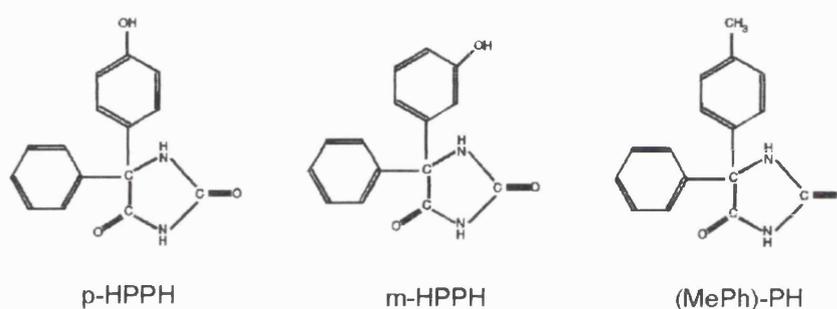


Figure 4.7. Structure-activity relationship of HYD derivatives to cell migration and contraction (step 2).

Substitution of a single side chain (3 atoms' length) at the 5-position of the HYD ring increased activity to some extent. Although both ALLAN and HYDACAC contain similar length side chains at the 5-position, differences in charges and spatial configurations resulted in variations in their activities. ALLAN was mildly chemotactic and relaxed gel contraction in the CFM. HYDACAC, on the other hand, had no effect in either test systems.

Substitution of one phenyl and one methyl group at the 5-position (Me-PHT) further increased activity, which was enhanced, when both phenyl groups were present (PHT).

C. Ring substitution is an important factor in activity.



MOST 'ACTIVE' COMPOUNDS

Figure 4.8. Structure-activity relationship of HYD derivatives to cell migration and contraction (step 3).

Racemic mixtures of metabolites of PHT (m-HPPH and p-HPPH) together with (MePh)-PH were found to be the most active compounds. Although the ranking of these compounds was different in the two test systems, it can be concluded that a negatively charged group was preferred at the meta or para positions.

In conclusion, the *in vitro* results obtained from migration and contraction studies indicated that maximum activity was seen with analogues of PHT containing substituents at the meta or para positions of the phenyl rings. The parent compound, HYD, was found to be inactive. These results also suggested that the two phenyl rings, which are also associated with potential carcinogenicity, play an important role in influencing fibroblast functions. However, the design of new compounds and application of metabolites of PHT with substitutions on both phenyl rings (Fig. 4.9-10) would block epoxide formation during metabolism and may lead to safer, yet effective PHT analogues.

Further studies may also concentrate on the evaluation of the following:

- Which enantiomer is responsible for the activity of PHT metabolites and analogues?

As a previous study implicated the least abundant metabolite, (R)-p-HPPH, in the pathogenesis of both PIGO and the growth stimulation dermal fibroblasts (Ieri *et al.*, 1995), the same isomer may also be responsible for inducing cell migration and alteration of fibroblast-mediated gel contraction.

- Is it possible to enhance activity by replacing the hydroxyl groups with other polar or apolar groups?

Almost twenty years ago, Savini and co-workers (Savini *et al.*, 1980) reported the testing of synthetic PHT analogues in gingival wound healing. They claimed that 1% para-chlorophenytoin was three times more effective than PHT itself. However, the details of their studies were not published and systematic *in vitro* evaluation of synthetic PHT analogues remains the goal.

Molecular modelling of new synthetic analogues (Fig. 4.9) and further metabolites (Fig. 4.10) of PHT may also lead to the 'mapping' of their target(s), whilst correlations of their *in vitro* activities in the test systems reported in this thesis may identify new vulnerable products with improved safety and efficacy profiles.

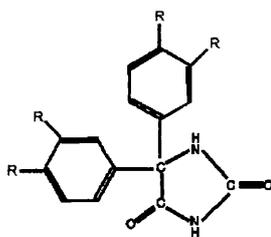


Figure 4.9. Proposed new generation of PHT analogues to be tested. R = alkyl, halogen, hydroxyl etc.

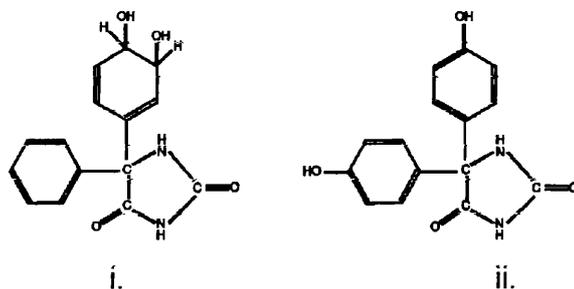


Figure 4.10. Proposed metabolites of PHT to be tested.

i = DHD; ii = 5,5-bis-(p-hydroxyphenyl)hydantoin

CHAPTER 5. CONCLUSION

Phenytoin (PHT), an agent widely used for the control of convulsive disorders, has also been reported to enhance wound healing. About half the patients receiving long-term PHT therapy develop a fibrous overgrowth of the gingivae (PIGO) as a side effect. This apparent stimulatory effect on connective tissue has inspired its use for wound healing.

There are many proposed mechanisms put forward for PIGO. It is thought that the local metabolism of PHT in gingiva and the presence of so-called "responder" fibroblasts may play a key role in its pathogenesis.

(R)-p-HPPH, the least abundant metabolite of PHT, is thought to trigger the emergence of fibroblast populations with potentially elevated proliferative and synthetic rates. Similarly, PHT and/or its metabolites may also induce the selection of such fibroblasts in PHT-treated wounds.

In this thesis it was attempted to rationalise the mechanism by which PHT may upregulate connective tissue activity, using *in vitro* and *in vivo* studies which targeted specific stages of wound healing.

The results of this thesis, together with previous investigations reviewed, suggest that PHT may have both direct and indirect actions on a variety of cells during wound healing which affects virtually all of its stages.

Proliferation studies revealed that low concentrations of PHT (5-10 μ g/ml) were stimulatory to both normal and EB fibroblasts and keratinocytes. However, their tolerance to higher concentrations of the drug was different. It must be noted that not all individuals responded similarly to low concentrations of PHT, which may reflect on their metabolic capacities and/or the absence of cells capable of responding to the drug. PHT (5-50 μ g/ml) was found to be chemotactic for both fibroblasts and keratinocytes, indicating that it may enhance both fibroplasia and re-epithelialisation by recruitment of cells into the wound. PHT may also exert indirect effects on cells via growth factor expression or alterations in responsiveness to growth factors.

Measurements of forces generated by fibroblasts within tethered collagen matrices recorded by the CFM determined that pre-treatment of cells with PHT (20 μ g/ml) for 5 days reduced contraction of both the normal and the

hypercontractile EB fibroblasts *in vitro*. This effect may be due to the accumulation of its metabolites (m-HPPH and p-HPPH), as they were demonstrated to reduce contraction dramatically. Alternatively, PHT may induce phenotypic changes in fibroblasts in the long term.

Good correlations were noted *in vivo* in reducing wound contraction in pigs without impairment of healing as observed by histology after two or three weeks post dosing (with either PHT powder or incorporated into FS).

Study of the Wound Pharmacology of PHT revealed that topical application is favoured and systemic absorption is not significant. Furthermore, different formulations are required for its application depending on the site and stage of wound healing. It was concluded that, like PIGO, elucidation of its local metabolism is vital in understanding its mechanism of action.

Having identified two potential, direct modes of action of PHT in wound healing, measured *in vitro*, a rationalisation of the structure-activity relationship of HYD-related compounds was attempted. It was established that activity was closely related to the unique structure of PHT and the parent compound, HYD, was inactive. Metabolites (m-HPPH and p-HPPH) and an analogue [(MePh)-PH] of PHT were shown to be most active in these assays. This indicated that the presence of both phenyl rings and substituents at the meta or para position were preferred. Future *in vitro* studies should isolate and rationalise the precise structure of the pharmacophore in its enantiomeric purity, which may also lead to the development of a new vulnerary agent.

In the meantime, the study of both drug-induced gingival overgrowth and the mechanistic role of PHT in wound healing will give further insight into various aspects of fibrosis, its prevention, and its enhancement in wound healing.

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Appendices

Appendix 1. List of publications

1. Talas G, Brown RA, McGrouther DA (1999), The role of Phenytoin in wound healing – a Wound Pharmacology perspective. *Biochem Pharm* (in press) – a commissioned Commentary.
2. Talas G, Brown RA (1999), The stimulatory effects of Phenytoin on normal, Epidermolysis Bullosa fibroblasts and keratinocytes. *Int J Biochem Cell Biol* (submitted).
3. Talas G, Adams TST, Eastwood M, Rubio G, Brown RA (1997), Phenytoin Reduces the contraction of Recessive Dystrophic Epidermolysis Bullosa fibroblast populated collagen gels. *Int J Biochem Cell Biol: Directed issue on mechanisms of tissue repair*, **29(1)**: 261-270.
4. Talas G, Adams TST, Porter RA, Eastwood M, Brown RA (1996), Phenytoin alters dermal fibroblast contraction *in vitro* and wound contraction *in vivo*. *Wound Rep Reg* **(4)**: A177.
5. Brown RA, Talas G, Porter RA, McGrouther DA, Eastwood M (1996), Balanced mechanical forces and microtubule contribution to fibroblast contraction. *J Cell Phys*, **169**: 439-447.
6. Talas G, Porter RA, Rubio G, Brown RA (1994). Analysis of Phenytoin stimulation of human dermal fibroblast activity. *Wound Rep Reg* **(2)3**: A226.

Appendix 2. List of oral presentations

1. Epidermolysis Bullosa – Intercalated Orthopaedic Science, Connective Tissue Biology course.
2. “The role of Phenytoin in Wound Healing” (1998) – Pan Thames Registrar training in Plastic Surgery, Middlesex Hospital.
3. “The role of Phenytoin in Wound Healing” (1996) – Pan Thames Registrar training in Plastic Surgery, inaugural teaching meeting at The Royal College of Surgeons of England.
4. “Quantitative measurement of contractile forces generated by fibroblasts from normal and pathologic skin” at the 5th Annual Meeting of the European Tissue Repair Society, Padova, Italy (30 August-2 September 1995).
5. I participated in a consensus meeting held under the auspices of the European Tissue Repair Society - industry liaison group at Sitges, Barcelona, Spain (19-21 May 1995) discussing the science of Wound Pharmacology (the fate of vulnarary agents in wounds, delivery systems and regulatory issues).

Appendix 3. List of conferences attended (poster presentations)

1. Delivery of active substances to wounds: problems, pitfalls, and potential. A joint meeting between UK Association of Pharmaceutical Scientists and the European Tissue Repair Society held at University College of Medicine, Cardiff, Wales (3 December 1997).
2. Talas G, Adams TST, Porter RA, Eastwood M, Brown RA (1996), Phenytoin alters dermal fibroblast contraction *in vitro* and wound contraction *in vivo*. Poster presentation at the 2nd Joint Meeting of the Wound Healing Society and European Tissue Repair Society, Boston, MA, USA (15-19th May 1996).
3. G. Talas, R.A. Schasfoort, R.A. Brown, R.A. Porter, The Migratory Effect of Phenytoin: Potential for Wound Healing? Poster presentation at the 2nd Joint Meeting of the Wound Healing Society and European Tissue Repair Society, Boston, MA, USA (15-19 May 1996).
4. Talas G, Porter RA, Rubio G, Brown RA (1994), Analysis of Phenytoin stimulation of human dermal fibroblast activity. Poster presentation at the 4th Annual Meeting of the European Tissue Repair Society, Oxford, UK (25-28 August 1994).
5. Talas G, Porter RA, Rubio G, Brown R (1994), Influence of Phenytoin on human dermal fibroblast activity in culture. Poster presentation at XIVth Meeting of the Federation of European Connective Tissue Societies, Lyon, France (30 August-3 September 1994).