Studies on Cytotoxic and Neutrophil Challenging Polypeptides and Cardiac Glycosides of Plant Origin

BY

SENIA JOHANSSON
ABSTRACT

This thesis examines the isolation and characterisation (biological and chemical) of polypeptides from plants. A fractionation protocol was developed and applied on 100 plant materials with the aim of isolating highly purified polypeptide fractions from small amounts of plant materials. The polypeptide fractions were analysed and evaluated for peptide content and biological activities. A multitarget functional bioassay was optimised as a method for detecting substances interacting with the inflammatory process of activated neutrophil granulocytes. In this assay, the neutrophil was challenged with an inflammatory mediator, \( \tilde{N} \)-formyl methionyl-leucyl-phenylalanine (fMLP), or with platelet activating factor (PAF), to induce exocytotic release of the enzyme elastase, which then was quantified by photometric determination of the product p-nitroanilide (pNA) formed from a chromogenic substrate for elastase. Of the tested extracts, 41% inhibited pNA formation more than 60%, and 3% stimulated formation.

Phoratoxin B and four new peptides, phoratoxins C-F, were isolated from *Phoradendron tomentosum*. In addition, the cardiac glycoside digitoxin was isolated from *Digitalis purpurea*. All these substances expressed cytotoxicity and a neutrophil challenging activity.

Phoratoxins C-F were similar to earlier described phoratoxins A and B, which belong to the group of thionins. All the peptides were evaluated for cytotoxicity in a human cell line panel. Phoratoxin C was the most potent towards the cell lines (mean \( IC_{50} \) 160 nM), and was therefore investigated further on tumour cells from patients. Correlation analysis of the log \( IC_{50} \) values indicated a mechanism of action different from clinically used archetypal cytotoxic drugs. Phoratoxin C also showed selective toxicity to the solid tumours when compared to the haematological cancer types. The phoratoxin C was 18 times more potent towards the solid tumour samples from breast cancer cells (87 nM) compared to the tested haematological malignancies.

The structure-activity relationship concerning cytotoxicity was evaluated for digitoxin and related cardiac glycosides. Digitoxin was shown to be potent, with the average \( IC_{50} \) 37 nM being within the therapeutic concentration used for cardiac congestion (13-45 nM). Digitoxin expressed selective toxicity towards solid tumours from patients compared to haematological malignancies.

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Printed in Sweden by Tryck & Medier, Uppsala 2001
Iblad jag finner en klöver med fyra magiska blad.
Jag griper den ivrigt och spar den så barnsligt förtjust och glad.

Ni alla förnumstigt kloka får gärna åt därskapen le.
Jag tror ändå att den myten kan något av sanning ge:
att lycklig blir den som finner där andra inget kan se.

Docent Maj Levander-Lindgren
List of Original Publications

This thesis is based on the following articles. They are referred to in the text by their Roman numerals (I-IV).


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# Studies on Cytotoxic and Neutrophil Challenging Polypeptides and Cardiac Glycosides of Plant Origin.

by Senia Johansson

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1. Introduction

Plants and animals have diversified differently through evolution, but because of the same origin, it is thought that they might have the same ancestral proteins. The defence mechanism is one example where it might be evident. Studies of innate immunity led to the discovery of common molecular mechanism used for host defence in plants, insects, and mammals (Bergey et al., 1996; Borregaard et al., 2000; Klessig et al., 2000; Marx, 1996). This includes the various receptors that recognise classes of microbial cell-surface molecules, the similar signal transduction pathways that activate transcription of genes related to host defence, and the ubiquitous cationic peptides and proteins that act as antimicrobial effectors. Among the diverse defensive chemicals that are synthesised in plants in response to herbivore or pathogen attacks are protease inhibitor proteins (Ryan, 2000; Ryan et al., 1993). These proteins can inhibit proteases of pests and pathogens, limiting protein digestion and retarding both growth and development of the attacker in response to wounding. Protease inhibitor genes have recently been proposed as modulators of programmed cell death (PCD) in plants (Solomon et al., 1999) and of defence by preventing unwanted cell death (Prasad et al., 1994).

Antimicrobial peptides have been identified as key elements in the innate host defence against infection. Recent studies indicate that the activity of antimicrobial peptides may be decreased in cystic fibrosis, suggesting a major role of these peptides in host defence against infection. They have also been shown to kill mammalian target cells and microorganisms by a common mechanism that involves an electrostatic interaction with membranes (Florack and Stiekema, 1994; Hughes et al., 2000; Lehrer et al., 1993). One type of these antimicrobial peptides is small (3-5 kDa), basic, and rich in cysteine. These are the mammalian, insect, and plant defensins. Another group of small cysteine-rich, highly basic peptides that are thought to play a role in the protection of plants against microbial infection are the thionins (Florack and Stiekema, 1994; García-Olmedo et al., 1998).

Human neutrophils are responsible for our first line of cellular defence against invading pathogens. In this work, highly purified plant fractions were obtained by a fractionation protocol for the isolation of polypeptides from plant biomass. The isolated purified plant fractions were further evaluated in the optimised neutrophil multitarget functional bioassay. The substances responsible for the observed effect in the neutrophil bioassay were isolated from *Phoradendron tomentosum* and *Digitalis purpurea*. The active substances were found to be polypeptides and cardiac glycosides, respectively. To gain further insight, and in particular, in case substances from plants with the neutrophil challenging activity also have a potential role in inducing death of cancer cells, the isolated substances were further investigated for cytotoxicity in a mechanism-based cell line panel, and in tumour cells from patients.
Background

2. Background

Peptides in Plants

Numerous peptides, such as hormones, neurotransmitters, and snake toxins, have been isolated from human and animal sources. In contrast, only a limited number of peptides have been found in plants. Nevertheless, the isolation of peptides from plant biomass has recently received attention for three main reasons. First, plants containing unique pharmacologically active peptides have been found within natural-products-based drug discovery programs (Gustafson et al., 1994; Witherup et al., 1994). Second, genetically transformed plants ("transgenic plants") are now considered an attractive and cost-efficient alternative to fermentation-based systems for production of, for example, high value recombinant peptides (Goddijn and Pen, 1995; Whitelam, 1995). Third, like animals, plants are known to make use of peptides as signal substances (Bergey et al., 1996; Marx, 1996). Examples of plant peptides are systemin, cyclic peptides, defensins, thionins, and several protease inhibitors.

Cyclic peptides

Cyclic peptides are an important group of compounds with potent, specific biological activities. A large number of small cyclic peptides have been isolated from plants. Small cyclic peptides occur to a great extent in the Caryophyllaceae and Rubiaceae families. Cyclic peptides are also found in Jatropha spp. within the family Euphorbiaceae (Horsten et al., 1996; van den Berg et al., 1995; van den Berg et al., 1996). Among the cyclic peptides that are found today, the most common biological activities appear to be antitumor and cell-growth-inhibitory effects (Hamanaka et al., 1987; Itokawa et al., 1984). This might be misleading since the most prevalent biological assays seem to be antitumour assays, and therefore, there might be many other possibilities that have not yet been examined. However, the octapeptides dichotomins from Stellaria dichotoma var. lanceolata exert both cytotoxic and cyclooxygenase inhibitory activities (Morita et al., 1996).

Macrocyclic peptides

Kalata B1 was isolated from the African plant Oldenlandia affinis based on ethnopharmacological reports of African women making use of that plant for accelerating contractions and childbirth (Gran, 1970). To obtain the drug kalata-kalata, natives boil the dried plant leaves to produce an extract, which is then sipped during labour. Kalata B1 was isolated in the beginning of the 1970s from extracts of the plant, and was considered an uterotonic agent; however, not until 1995 was the primary structure of the peptide confirmed (Saether et al., 1995). The peptide contains 29 amino acids and three disulphide bonds; and for a peptide this large, it has the unusual feature of a cyclized backbone. Although cyclic peptides are produced naturally in microbes and plants, they are usually restricted to peptides with fewer than 15 amino acids (Itokawa et al., 1997). Over the past few years, some other cyclic peptides with similar sizes and with cysteine residues have been reported. Together, they form a new family of macrocyclic peptides, recently named cyclotides (Craik et al., 1999).
Most of these macrocyclic peptides have been found in plants from the Rubiceae family, of which *Olenlandia affinis* is a member. Circulin A and B were isolated from *Chassalia parvifolia* (Gustafson et al., 1994); and cyclopsychotride A, from *Psychotria longipes* (Witherup et al., 1994). All these peptides have a cyclic backbone, and sequences somewhat homologous with that of kalata B1. Violapeptide 1 from *Viola arvensis* Murray (Schöpke et al., 1993) was the first reported cyclotide isolated from the Violaceae family.

In addition to their cyclic nature, these macrocyclic peptides also have an inhibitor type of cysteine-knot motif in their disulphide connectivity (Daly et al., 1999a; Derua et al., 1996; Saether et al., 1995; Tam and Lu, 1997; Tam and Lu, 1998). This motif contains a bonding pattern of Cys I-IV, II-V, and III-VI, characterised by the Cys III-VI disulphide bond threading through the other two to give a knotted motif. The knotted motif together with small triple-stranded $\beta$-sheets has been found in peptides and proteins with diverse functions. These include ion channel toxins, protease inhibitors, and growth factors (McDonald and Hendrickson, 1993; Pallaghy et al., 1995).

This cyclotide family is of particular interest because the individual members exhibit a diverse range of biological activities. The circulins exhibit anti-HIV activity (Gustafson et al., 1994); cyclopsychotride A inhibits neurotensin binding to cell membranes (Witherup et al., 1994), and kalata B1 exhibits uterotonic activity. Originally, violapeptide I was discovered for its haemolytic activity (Schöpke et al., 1993); but recently, Daly et al. (1999b) and Tam et al. (1999) have shown that in addition kalata B1, the circulins, and cyclopsychotride A have relatively weak haemolytic activity. Circulin B and cyclopsychotride are antimicrobial against both Gram-positive and Gram-negative bacteria, whereas kalata B1 and circulin A are specific for the Gram-positive *Staphylococcus aureus*. After a modification of the sole arginine residues in kalata B1, the antimicrobial activity was markedly decreased, whereas the modification of arginine residue of circulin A resulted in no significant loss of activity. The function of these cyclic peptides within plants, although yet to be established, most likely involves a defence mechanism (Tam et al., 1999).

**Thionins**

Thionins are low-molecular-weight proteins (5 kDa) occurring in seeds, stems, roots, and leaves of a number of plant species. Thionins can be divided into at least four types depending on the plant species and tissue in which they occur, and on the overall net charge and the number of amino acids and disulphide bonds present in the mature protein (Garcia-Olmedo et al., 1989). Type 1 thionins, which are abundantly present in the endosperm in the family Poaceae, are highly basic, and consist of 45 amino acids, 8 of which are in the central disulphide loop. Type 2 thionins have been extracted from leaves and nuts of the parasitic plant *Pyrularia oubera* (Vernon et al., 1985), and from the leaves of barley *Hordeum vulgare* (Bohmann and Apel, 1987; Bohmann et al., 1988). They consist of 47 and 46 amino acids, respectively, and are slightly less basic than the type 1 thionins. Both type 1 and type 2 have eight cysteines that are involved in four disulphide bonds. Type 3 thionins, which consist of 45 or 46 amino acids, 9 of which are in the central disulphide loop, have been extracted from leaves and stems of mistletoe species:
Background

*Viscum album, Phoradendron tomentosum, Phoradendron liga, and Dendrophthora clavata* (Mellstrand and Samuelsson, 1974a; Samuelsson and Pettersson, 1970; Samuelsson and Pettersson, 1977; Thunberg and Samuelsson, 1982). These thionins, which have three disulphide bridges that are conserved with respect to the previous types, are as basic as type 2 thionins. Type 4 thionins, which consist of 46 amino acids with three disulphide bonds, but neutral in charge, have been extracted from seeds of Abyssinian cabbage (*Crambe abyssinica*) (van Etten et al., 1965). All four types of thionins appear to be highly homologous at the amino acid level. The cysteine residues and the tyrosine residue at position 13 are highly conserved, except for the type 4 thionins.

Thionins are toxic to either Gram-positive or Gram-negative bacteria, fungi, yeast, and various mammalian cell types. Toxicity requires an electrostatic interaction of thionins with the negatively charged membrane phospholipids, followed by either pore formation or a specific interaction with a certain domain in the membrane (Florack and Stiekema, 1994). The purely electrostatic interaction of thionins with membranes, the first step in the exposure of toxicity, can be inhibited by divalent cations, such as calcium (Florack and Stiekema, 1994; Sauviat, 1990). Upon thionin treatment of mammalian cells, the specific interaction of thionins with certain phospholipids, mediating transduction of cellular signals, can explain the release of specific compounds along with the activation of calcium channels and specific enzymes (Florack and Stiekema, 1994). Cytotoxicity correlates well with ability to form ion channels. In fact, Hughes et al. (2000) propose that the primary mode of action for the toxicity of thionins is due to their ability to form ion channels in cell membranes; and they go on to propose that the passage of ions through the channel probably involves Tyr-13.

Antimicrobial activity has been demonstrated for several thionins (Bohlmann et al., 1988; Fernandez de Caley et al., 1972; Florack and Stiekema, 1994; Molina et al., 1993; Terras et al., 1993; Terras et al., 1996), supporting the suggestion that thionins are defence proteins (Fernandez de Caley et al., 1972). Other evidence for such a possible function comes from observations that the expression of several thionins is inducible by phytopathogenic fungi (Bohlmann et al., 1988; Boyd et al., 1994; Epple et al., 1995; Titarenko et al., 1993; Vale et al., 1994). Furthermore, high-level expression of viscotoxin A3 cDNA in transgenic *Arabidopsis thaliana* has been shown to enhance the resistance against the pathogen *Plasmidiophora brassicae* (Holtorf et al., 1998).

**Viscotoxins**

*Viscum album* L contains two groups of toxic components: lectins and viscotoxins (Samuelsson and Jayawardene, 1974). The peptides from the European mistletoe, *Viscum album* L, called viscotoxins, were first isolated and sequenced by Samuelsson and co-workers (Samuelsson, 1958; Samuelsson, 1961), and were found to belong to the group of thionins. Five viscotoxins, A1, A2, A3, B and 1PS, have been isolated; and their primary structures are determined (Olson and Samuelsson, 1972; Orrù et al., 1997; Samuelsson, 1973; Samuelsson and Jayawardene, 1974; Samuelsson and Pettersson, 1971; Samuelsson et al., 1968; Schrader and Apel, 1991). In addition, cDNAs encoding viscotoxins from *Viscum album* L. have been isolated and characterised for the full-length preprotein of viscotoxin A3 and for
the precursor of viscotoxin B. Besides the viscotoxin domain, the precursor contains a signal sequence and a acidic polypeptide domain. Since both the negative charge and the position of the cysteine have been conserved within the acidic domain, (Schrader-Fischer and Apel, 1993) propose that this may play an important role in keeping the thionin inactive within the plant cell. Another cDNA of thionin precursors in *Viscum album* L. has a highly divergent thionin domain with eight instead of six cysteine residues, while the acidic polypeptide domain and the signal sequence is conserved among all of the variants (Schrader-Fischer and Apel, 1993). Viscotoxins contain three S-S bridges, Cys3-Cys40, Cys4-Cys32, and Cys16-Cys26 (Orrù et al., 1997).

**Phoratoxins**

In 1967 Samuelsson and Ekblad isolated for the first time phoratoxin (later called phoratoxin A) from a Californian mistletoe, *Phoradendron tomentosum* (DC) Engelm. subsp. *macrophyllum* (Cockerell) Wiens. In 1974, Mellstrand and Samuelsson reported the amino acid sequence for this peptide, which consists of 46 amino acids tightly bound together by three disulphide bridges in the same way as for the viscotoxins (Mellstrand and Samuelsson, 1974a). Thunberg (1983) isolated phoratoxin B, which was shown to be very similar to phoratoxin A. Some known activities of viscotoxins and phoratoxins are summarised in Table 1.

**Table 1.** Listing of various activities displayed by viscotoxins and phoratoxins.

<table>
<thead>
<tr>
<th>Name</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscotoxins</td>
<td>Bradycardia, negative inotropic effect on heart, vasoconstriction of vessels of cats upon injection</td>
<td>(Rosell and Samuelsson, 1966)</td>
</tr>
<tr>
<td></td>
<td>Non-random binding to DNA</td>
<td>(Woynarowski and Konapa, 1980)</td>
</tr>
<tr>
<td></td>
<td>Cytotoxic to human KB and HeLa cells <em>in vitro</em></td>
<td>(Konopa et al., 1980)</td>
</tr>
<tr>
<td></td>
<td>Inhibition of translation in hamster BHK-21 cells <em>in vitro</em></td>
<td>(Carrasco et al., 1981)</td>
</tr>
<tr>
<td></td>
<td>Altered ionic permeabilities and depolarisation of the membrane</td>
<td>(Andersson and Johannsson, 1973)</td>
</tr>
<tr>
<td></td>
<td>Enhanced <em>E. coli</em>-stimulated phagocytosis and respiratory burst</td>
<td>(Stein et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Generation of reactive oxygen intermediates and induced expression of mitochondrial Apo2.7 molecules</td>
<td>(Büssing et al., 1999b)</td>
</tr>
<tr>
<td></td>
<td>Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes</td>
<td>(Büssing et al., 1999a)</td>
</tr>
<tr>
<td>Phoratoxins</td>
<td>Bradycardia, negative inotropic effect on heart, vasoconstriction of vessels of cats upon injection</td>
<td>(Rosell and Samuelsson, 1966)</td>
</tr>
<tr>
<td></td>
<td>Toxic to mice upon intraperitoneal injection</td>
<td>(Mellstrand and Samuelsson, 1973)</td>
</tr>
<tr>
<td></td>
<td>Hemolytic to human erythrocyte</td>
<td>(Thunberg, 1983)</td>
</tr>
<tr>
<td></td>
<td>Depolarisation of the membrane on frog skeletal muscle fibres</td>
<td>(Sauviat, 1990)</td>
</tr>
</tbody>
</table>
Protease inhibitors
Protease inhibitor proteins are among the diverse defensive chemicals that are synthesised by plants in response to pathogen attacks. These proteins can inhibit proteases of pests and pathogens, limiting protein digestion, and retarding both growth and development of the attacker (Ryan, 2000). Plant protease inhibitors can have a regulatory role in that, after wounding caused by insect chewing or during chilling-induced oxidative stress, they can block PCD and thereby prevent such death from going beyond what is wanted (Solomon et al., 1999).

Systemin, an 18-amino-acid polypeptide released from wound sites on tomato leaves caused by insects or other mechanical damage, is the most powerful inducer of protease-inhibitor synthesis known. The wound-inducible protease inhibitors have a wide range of specificities that include all four known mechanistic classes of proteases: serine, cysteine, aspartyl, and metallo-carboxyl protease inhibitors (Ryan, 2000). Numerous protein protease inhibitors have been isolated and identified. Many of these are directed towards serine proteases, and are strictly competitive inhibitors, forming 1:1 complexes with the enzyme they inhibit. In these complexes, all activities of the enzyme are completely abolished. Each inhibitor is a substrate for the enzyme it inhibits, at a unique peptide bond called the reactive site peptide bond (Laskowski, 1986).

Squash inhibitors of serine proteases, built of 27-33 amino-acid residues and cross-linked with three disulphide bridges, form a uniform family of small proteins that are highly stable and rigid. The reactive site peptide bond (P1-P1') is between residue 5 (Lys, Arg or Leu) and 6 (always Ile). The major structural motif is a distorted, triple-stranded, antiparallel beta-sheet. A similar folding motif has been recently found in a number of proteins, including conotoxins, carboxypeptidase inhibitor from potato, kalata B1 polypeptide, and proteins in some growth factors (Otlewski and Krowarsch, 1996). Protease inhibitors are of biochemical interests, not only because of their involvement in defence mechanisms, but also because of their involvement in carcinogenesis (Clawson, 1996), virus replication (Weller and Williams, 2001), and inflammation (Konstan, 1998).

Polymorphonuclear Neutrophils
Physiological responses
The polymorphonuclear neutrophil (PMN) is a central component of the inflammatory process, having the ability to migrate to the inflammation site and to release toxic products such as proteolytic enzymes (Travis, 1988), reactive oxygen species (ROS) (Babior, 1984; Nathan et al., 1981; Weiss, 1980), and cationic proteins (e.g., defensins and cathelicidins) capable of killing invading pathogens (Chertov et al., 2000; Clark et al., 1976; Ganz and Lehrer, 1994; Zanetti et al., 1995). The targets of the PMN include bacteria, fungi, viruses, virally infected cells, and tumour cells.

Before entering the tissue, neutrophils must adhere to the endothelium and subsequently migrate through the vessel wall. This process is tightly regulated by adhesion molecules on neutrophils and endothelial cells at sites of inflammation.
Background

(Cohen, 1994; Hiemstra et al., 1998). Within the tissue, neutrophils continue to migrate along a gradient formed by locally produced chemotactic factors, which include the bacterial product N-formyl methionyl-leucyl-phenylalanine (fMLP); secreted products of stimulated phospholipid metabolism, such as the platelet activating factor (PAF) and leukotriene B4 (LTB4); and immunomodulatory molecules, such as the cytokine interleukin 8 (Baggiolini and Dewald, 1986; Curfs et al., 1997; Dewald and Baggiolini, 1986; Goldman and Goetzl, 1982; Marascot et al., 1984; Nardin et al., 1991; Proost et al., 1996; Schiffmann et al., 1975a; Schiffmann et al., 1975b; Williams et al., 1977).

Chemotaxis, the ability to migrate towards an attractant, is essential for the migration of neutrophils and macrophages to sites of tissue damage and infection (Bokoch, 1995). During migration to the site of inflammation, neutrophils may contribute to tissue injury. Tissue injury occurs when neutrophils accumulate in unusually high numbers, when they receive inappropriate stimuli and/or when the activity of their products is not adequately controlled (Dallegri and Ottonello, 1997). Neutrophils are, for example, involved in the pathogenesis of various inflammatory lung disorders, including chronic bronchitis and chronic obstructive pulmonary disease (Hiemstra et al., 1998).

PMNs are equipped with an array of preformed compounds that are stored in the various types of neutrophil granule, and that may be released upon stimulation. These compounds include serine and metalloproteinases, and non-enzymatic polypeptides such as neutrophil defensins (Borregaard and Cowland, 1997). In addition, neutrophil stimulation also results in the synthesis and release of a number of mediators, including reactive oxygen intermediates, lipid mediators, and cytokines that may contribute to injury (Hiemstra et al., 1998).

Phagocytosis
The neutrophil is one of the professional phagocytes in humans. The neutrophil makes tight contact with its target, and its plasma membrane then flows around the surface until the bacterium is completely enclosed. These intracellular compartments are called phagosomes, where digestive and antibacterial compounds are released. The phagosome minimizes the amount of extracellular fluid entering the phagosome with the bacterium, which means that the phagosome is initially a very small space (Hampton et al., 1998) where the bacteria can be subjected to high concentrations of antimicrobial substances. Neutrophils also undergo a burst of oxygen consumption that is caused by an NADPH oxidase complex that assembles at the phagosomal membrane. This oxidative burst has also been shown to be essential for killing a number of microorganisms (Cohen, 1994; Hampton et al., 1998). These agents are strongly anti-microbial but may also cause damage by destructing surrounding tissue and inducing apoptosis in other immune reactive cells.

Exocytosis
The neutrophil has intracellular stores of both membrane proteins and soluble proteins that can be incorporated into the plasma membrane as a means of adhesion to endothelium (secretory vesicles), for migration through basement
membranes (gelatinase granules), and for phagocytosis, killing, and digestion of microorganisms (specific granules and azurophil granules) (Figure 1). One reason for segregating the proteins into different subpopulations of granules is that some proteins cannot exist in the same compartment (Borregaard, 1997). For instance, azurophil granules carry terminally processed serine proteases, antibiotic proteins, and myeloperoxidase (MPO), all in a potentially active state (Gullberg et al., 1997). Specific granules, on the other hand, store metalloproteases and antibiotic peptides as inactive latent proforms, which are allowed after degranulation to become active first (Gullberg et al., 1997). Another reason is that the content of different granules is needed at different times and places (Borregaard, 1997). The exocytosis of granules and vesicles cause not only a release of lytic enzymes, but concurrently, increased cell response by production of reactive oxygen intermediates and chemotactic stimulation. These activities lead to the destruction of invading pathogens (Dahlgren and Karlsson, 1999; Gullberg et al., 1999).

<table>
<thead>
<tr>
<th>Type of granule</th>
<th>Azurophil granules</th>
<th>Specific granules</th>
<th>Gelatinase granules</th>
<th>Secretory vesicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of contents</td>
<td>Myeloperoxidase</td>
<td>Lactoferrin</td>
<td>Gelatinase</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>Antibiotic proteins (e.g., defensins)</td>
<td>Antibiotic proteins (e.g., cathelicidins)</td>
<td>Metallo-proteinases (inactive)</td>
<td>Plasma proteins</td>
<td></td>
</tr>
<tr>
<td>Serine proteases (active)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type of functions</th>
<th>Killing</th>
<th>Killing</th>
<th>Migration</th>
<th>Adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodily functions</td>
<td>Digestion</td>
<td>Migration</td>
<td>Activation</td>
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<td></td>
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</table>

**Figure 1.** Differences in tendency for extracellular release for some typical cell contents, such as azurophil, specific granules, gelatinase granules, and secretory vesicles. Although the granule subtypes have overlapping functions to a great extent, distinct functions associated with the contents of various granules can be recognised.

**Elastase**

Elastases are a group of proteases that possess the ability to cleave the important connective tissue protein elastin. Elastin, which has the unique property of elastic recoil, is widely distributed in vertebrate tissue, and is particularly abundant in the lungs, arteries, skin, and ligaments (Werb et al., 1982).

Neutrophil elastase (NE; EC 3.4.21.37), a 29-kDa glycoprotein and a potent serine protease (Travis, 1988), is stored in primary (azurophilic) granules of neutrophils (Borregaard and Cowland, 1997; Gullberg et al., 1999). Human neutrophil elastase can cleave a variety of substrates, including elastin and all other major connective-tissue proteins (Werb et al., 1982). Neutrophil elastase is essential for phagocytosis and defence against infection by invading microorganisms. The powerful proteolytic activity of neutrophil elastase is essential for migration of neutrophils through connective tissue and for destruction of foreign bacterial...
invaders (Bieth, 1988). Elastase can be extremely destructive if not controlled, because they can destroy many connective tissue proteins. Under normal physiological conditions these proteases are carefully regulated by compartmentalization or by natural circulating plasma inhibitors. Any elastase that reaches the circulation is quickly complexed by the natural inhibitors $\alpha_1$-protease inhibitor ($\alpha_1$-antitrypsin) and $\alpha_2$-macroglobulin (du Bois et al., 1991; Virca and Schnebli, 1984; Virca and Travis, 1984). The complexes are cleared from the plasma and degraded by the liver or by macrophages. When an imbalance occurs because of deficiency, effective $\alpha_1$-protease inhibitors, or abnormally high levels of elastases, can then result in severe permanent tissue damage. Neutrophil elastase has been linked to adult respiratory distress syndrome, cystic fibrosis, rheumatoid arthritis, and other inflammatory disorders (Döring, 1994).

The cytotoxic action of neutrophils
Human neutrophils become capable of lysing tumour cells when activated by a variety of stimuli (Dallegri et al., 1991). PMNs possess at least two mechanisms by which they can lyse mammalian target cells. One of these depends upon the production of toxic oxidative metabolites that are generated from the PMNs respiratory burst (English and Lukens, 1983; Nathan et al., 1979). However a second mechanism, often detected during studies of PMN-induced antibody-dependent cellular cytotoxicity, is independent of the respiratory burst (Dallegri et al., 1984; Katz et al., 1980) and is mediated by one or more toxic proteins released from PMN granules.

At leukaemia (Lichtenstein et al., 1988), pulmonary (Okrent et al., 1990), and endothelial (Okrent et al., 1990) targets for example, the most potent cytolytic proteins of PMN are four small cationic peptides termed defensins, or human neutrophil peptides 1, 2, 3 and 4 (HNP 1-4). The human defensins are secreted by activated PMNs (Ganz, 1987) and could therefore function as cytotoxins during antibody-dependent cellular cytotoxicity. In addition, a synergistic cytotoxic effect occurs when target cells are exposed to a combination of defensins and toxic oxidants or cathelicidins (Lichtenstein et al., 1988; Nagaoka et al., 2000).

Some Host Defence Mechanism in Plants and Animals

Superoxide generation
During phagocytosis of microbial intruders, neutrophils increase their oxygen consumption through the activity of an NADPH-oxidase. This generates superoxide anions ($O_2^-$) and hydrogen peroxide ($H_2O_2$). These oxygen metabolites give rise to other ROS that are strongly anti-microbial (Babior, 1978; Badwey and Karnovsky, 1980), but which also may cause damage by destroying surrounding tissue (Winrow et al., 1993) and induce apoptosis in other immune reactive cells (Dallegri and Ottonello, 1997; Gustafsson and Bengtsson, 1999). ROS have also been shown to induce cell death in plants (Lamb and Dixon, 1997); and (Lamb and Dixon, 1997; Van Camp et al., 1998) speculate that ROS, when involved in activating cell death in plants, may be generated by an NADPH oxidase similar to the ROS signalling system in neutrophils. Recently, plant homologs of NADPH
oxidase components (gp91 phox and Rac) have been cloned (Keller et al., 1998; Torres et al., 1998). Further support for NADPH oxidase involvement in plant cell death is the observation that inhibitors of the NADPH oxidase complex can interfere with the induction of cell death in plants (Lamb and Dixon, 1997). Hydrogen peroxide has also recently been shown to act as a second messenger for the induction of defence genes in response to wounding, systemin, and methyl jasmonate (Orozoxo-Cárdenasa et al., 2001), where the expressions of several defence genes were inhibited by the neutrophil NADPH oxidase inhibitor diphenylene iodonium.

Nitric oxide

Nitric oxide (NO) has previously been shown to serve as key redox-active signal for the activation of various mammalian defence responses, including the inflammatory and innate immune responses (Schmidt and Walter, 1994; Stamler, 1994). In the immune system, ROS often function together with NO, as for example, in macrophage killing of bacteria and tumour cells (Nathan, 1995; Schmidt and Walter, 1994). NO has also been implicated in the activation of plant defences (Delledonne et al., 1998), where it interacts with ROS and salicylic acid to induce PCD and defence gene expression (Delledonne et al., 1998).

Antimicrobial peptides

Among plant antimicrobial peptides, thionins (described above) were the first to be found, whose activity against plant pathogens was demonstrated in vitro. Antimicrobial peptides are cationic and generally not cytotoxic at concentrations where they kill microorganisms (Hoffmann et al., 1999). They appear to kill mammalian target cells and microorganisms using a common mechanism, which involves initial electrostatic interactions with negatively charged target-cell surface molecules (likely the head groups of polar membrane lipids), followed by insertion into the cell membranes that they permeabilize, forming voltage-regulated channels (Hughes et al., 2000; Lehrer et al., 1993).

Among the group of antimicrobial effector molecules, the cysteine-rich defensins are widespread in eukaryotic cells in plants, insects, and mammals. Defensins have wide spectra of activity directed against various bacteria, fungi, and enveloped viruses (Hoffmann et al., 1999). Four defensin families have been reported in eukaryotes: insect defensins, plant defensins, and α- and β-defensins in mammals. Whereas mammalian defensin consists solely of β-sheets linked by disulphide bridges (in a slightly different pattern for α- and β-defensins), insect and plant defensins have an α-helix, stabilized through disulphide bridging to strongly twisted antiparallel β-sheets (Hoffmann et al., 1999).

Plant defensins, which have been isolated from several taxa, are 45-54 amino acids residues in length and probably ubiquitous in the plant kingdom. Based on their amino acid sequences, four defensin groups or subfamilies have been established, with structural differences seeming to correlate with differences in antimicrobial specificity. All known members of the plant defensin family have eight disulphide-linked cysteines. The distribution of defensins in plant tissue is consistent with their putative defence role. Thus, they have been identified in leaves, tubers, flowers, pods and seeds. Although preferentially located in
peripheral cell layers, plant defensins have also been reported in the xylem, and in stomatal cells. All these locations are where first contact and entry of pathogens take place. No toxicity, so far, of plant defensins to animal or plant cells has been found (García-Olmedo et al., 1998).

Human neutrophil defensins are small (29-33 amino acids) cationic peptides that were first identified on the basis of their antimicrobial activity. Four defensins from the human defensin family are known to be expressed in neutrophils, namely, the human neutrophil peptides HNP-1, HNP-2, HNP-3, and HNP–4 (Ganz and Lehrer, 1994; Ganz et al., 1990; Lehrer et al., 1993). Human neutrophil defensins are stored in the azurophil granules of neutrophils (Borregaard and Cowland, 1997; Gullberg et al., 1997), and transferred to the phagolysosome upon phagocytosis (Ganz, 1987). In vitro, these peptides kill a variety of bacteria, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*; many fungi; and some enveloped viruses (Ganz et al., 1985; Lehrer et al., 1989; Lehrer et al., 1993). In addition to their antimicrobial activity, defensins also display cytotoxic activity toward various eukaryotic cells and cell lines (Lehrer et al., 1993). Thus, when released from stimulated neutrophils, defensins may contribute to tissue injury. Various other extracellular activities of defensins have been described (Lehrer et al., 1993). These activities include chemotactic activity for monocytes and lymphocytes (Chertov et al., 1996; Territo et al., 1989) and the ability to stimulate IL-8 synthesis in airway epithelial cells (van Wetering et al., 1997).

Tang et al. (1999) reported the isolation and characterization of a novel cyclic defensin detected in neutrophils and monocytes from macaque rhesus monkeys. This defensin, named rhesus theta defensin 1 (RTD-1), is the product of two homologous genes that generate two highly similar propeptides that are proteolytically cleaved, joined head to tail to form a unique cyclical structure, and targeted to the granules of leukocytes. In contrast to classical defensins, the antimicrobial of RTD-1 is not inhibited by increased salt concentrations.

Recently, Zanetti et al. (1995) identified a novel family of antibacterial proteins, known as cathelicidins, found in mammalian (human, rabbit, bovine, porcine and guinea pig) neutrophils and stored in the specific and gelatinase granules. Cathelicidins-derived antimicrobial peptides range in length from 12 to about 100 residues. These peptides are not inhibited by increased salt concentrations. Both defensins and cathelicidins are released extracellularly (Ganz, 1987; Nagaoka et al., 1997; Yomogida et al., 1997) by activated neutrophils. Although the activities of defensins are completely lost by increasing salt concentrations, it has been shown that they work synergistically with cathelicidins to exert an enhanced antibacterial activity in the extracellular milieu by augmenting the membrane permeabilization of target cells (Nagaoka et al., 2000).

**Programmed cell death or apoptosis**

Apoptosis (or programmed cell death—PCD) describes the physiological process for the killing and removal of unwanted or dangerous cells (Rich et al., 1999; Vaux and Korsmeyer, 1999), which constitutes the main form of cell death in animals, plants, and, other organisms. Numerous stimulants can induce PCD. In
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many systems, oxidative stress was found to be involved either directly or indirectly (Levine et al., 1996; Payne et al., 1995). In plants, it has been shown that H$_2$O$_2$ treatment induces PCD in soybean and Arabidopsis cell cultures (Desikan et al., 1998; Levine et al., 1996; Mazel and Levine, 2001). The conserved similarity of both morphological and biochemical hallmarks suggest a common cell death process in plants and animals (Greenberg, 1996).

The neutrophils are recruited to inflammatory sites in response to infection or tissue injury. However, many defence mechanisms employed by these cells are potentially deleterious to host tissues. For example, excessive release of granule proteases or production of ROS can damage tissue and can amplify the inflammatory response by a variety of mechanisms. Thus, it is important that the neutrophils are effectively and rapidly destroyed concomitant with the removal of an inflammatory stimulus to avoid excessive tissue damage or chronic inflammation (Akgul et al., 2001; Savill and Haslett, 1995). The neutrophils are constitutively programmed to die by apoptosis (Raff, 1992), which is an efficient, non-inflammatory method of removing aging leukocytes from inflammatory sites.

Although apoptotic neutrophils retain membrane integrity, they are non-functional, and lose the ability to degranulate, move by chemotaxis, or generate a respiratory burst. This shutdown in activity and loss of functional capacity, resulting from the disablement of their activation pathways, is aided by decreased expression of surface receptors, which prevents them efficiently binding extracellular ligands. To complete the elimination process, the intact but inactive apoptotic neutrophils are then phagocytosed by macrophages utilizing novel surface recognition mechanisms, which fail to trigger a pro-inflammatory macrophage response (Savill et al., 1993; Savill and Haslett, 1995). By contrast, “accidental” cell death or necrosis, triggered by noxious stimuli such as hypoxia or cell poison, involves a loss of membrane integrity, which leads to the release of potentially toxic intracellular contents (Duvall and Wyllie, 1986), inducing an inflammatory response. Apoptotic cells, if not recognized and removed, eventually undergo secondary necrosis releasing damaging intracellular contents and amplifying the inflammatory response. For example, non-ingested apoptotic neutrophils undergoing secondary necrosis release large quantities of the potent degradative enzyme neutrophil elastase, which eludes endocytic clearance by macrophages. This process might underlie the development of chronic inflammatory conditions in which apoptotic cell load and phagocytic clearance mechanism are mismatched (Ward et al., 1999).

Cytotoxicity

Screening for cytotoxic natural products
The use of crude natural products to treat cancer can be traced back to antiquity. Over 3000 species of plants have been reported to be used in some form of cancer treatment (Cassady et al., 1981). Modern use of pure natural drugs in cancer chemotherapy has a shorter history, tracing back about fifty years. Today there are about 50 commercially available anticancer drugs (excluding endocrines) that have been approved by US Food and Drug Administration (USFDA); and
significantly, the drugs based on natural products represent almost 1/3 of the total approved agents. One example is taxol, a natural product derived from the tree *Taxus brevifolia*, which is used for the treatment of ovarian and breast cancer (Michaud et al., 2000).

**Mechanism-based screening for cytotoxicity**

One of the largest drug discovery efforts in the field of cancer therapy has been performed at US National Cancer Institute (NCI) starting in 1955. After a growing concern with the narrow spectrum of antitumour activity of available drugs, and an increasing dissatisfaction with the clinical results for many of the most promising new investigational drugs (Marsoni et al., 1987), NCI began, in 1985, to phase out its *in vivo* P388 mouse leukaemia screen, and to replace it with a panel of cell lines (currently >60) representing the major forms of human cancer (Alley et al., 1988; Monks et al., 1991). A semiautomated non-clonogenic *in vitro* assay was selected for the analysis of growth inhibition and cytotoxicity. This panel has been shown to generate remarkably reproducible and characteristic profiles of differential *in vitro* sensitivity for cytotoxic agents with different mechanisms of actions (Boyd and Paull, 1995). This fingerprint profile can classify, by the use of correlation analysis or advanced neural network, the agents that are related to specific groups, such as anti-metabolites, alkylators, topo II inhibitors, the P-glycoprotein (P-gp), and the multidrug resistance-associated protein (MRP). By using the same general principles for data treatment, Dhar et al. (1996) showed that prediction of mechanisms of action of anticancer drugs is also possible with a panel of only ten human cell lines, representing defined types of cytotoxic drug resistance.

**Common mechanisms and resistance for cancer drugs**

Multi-drug resistance (MDR) is a phenomenon originally seen in cultured tumour cells that, following selection for resistance to a single anticancer agent, become resistant to a range of chemically diverse anticancer agents. These MDR cells show a decrease in intracellular drug accumulation due to active efflux by transporter proteins. Two proteins have been shown to cause this type of multidrug resistance in human tumour cells, the 170 kDa P-glycoprotein and the 190 kDa multidrug resistance protein. Both proteins belong to the ABC superfamily of efflux pumps. The transporter best characterised is the protein P-gp, which has been identified in many cancers and has been the target for agents able to inhibit its action, thereby reversing resistance (Barrand et al., 1997). More recently, Cole et al. (1992) identified another transporter, MRP, whose gene is overexpressed in a multidrug-resistant variant of the small-cell lung carcinoma cell line NCI-H69. This cell line, unlike most tumour cell lines that are resistant to multiple chemotherapeutic agents, did not overexpress the transmembrane transport protein P-glycoprotein (MDR1). MRP confers resistance to a broad range of natural compounds and mediates the ATP-dependent membrane transport of glutathione S-conjugates of chemotherapeutic drugs (Zaman et al., 1994).

Glutathione conjugation and transport of glutathione conjugates of anticancer drugs out of cells have been shown to work as a system in the detoxification of many anticancer drugs, by changing cellular metabolism. The major components of this system include glutathione (γ-glutamylcysteinyl glycine; GSH), GSH-
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related enzymes, and the glutathione conjugate export pump. GSH can combine with anticancer drugs to form less toxic and more water-soluble GSH conjugates, the conjugation reaction is then catalysed by glutathione S-transferases (GSTs). The GSH conjugates of anticancer drugs can be exported from cells by the glutathione conjugate export pump or by MRP. GSH, glutathione-related enzymes, and the glutathione conjugate export pump (or MRP) have been found to be increased or overexpressed in many drug resistant cells, and in tumour cell lines isolated from patients whose tumours are clinically resistant to drug therapy (Lewis et al., 1988; Russo et al., 1986; Wolf et al., 1987; Zhang et al., 1998). Increased detoxification of anticancer drugs by this system may confer drug resistance, and has particularly been associated with alkylating agents. These agents, which are electrophilic in nature, spontaneously interact with the thiol of reduced GSH (Calvert et al., 1998). Inhibition of this detoxification system is a strategy for modulation of drug resistance.

The nuclear enzymes, topoisomerase I and II (topo I and II), are critical for DNA function and cell survival. Topoisomerase II (topo II), which binds to double-stranded DNA, cleaves both strands, passes a second strand of DNA through the cleaved site, and rejoins the strands at the original site of cleavage. This process produces a species of DNA altered only in its topological configuration. Playing an important role in replicational, recombinational, and transcriptional events, and being the key to cell growth processes, topo II enzymes participate in most aspects of DNA metabolism (Bakshi et al., 2001). Topo II inhibitors (e.g., anthracyclines and epipodophyllotoxins) are active against several types of tumours. However, treatment with these drugs often results in the development of MDR. Because (topo II)-active drugs have several different modes of action, several mechanisms of resistance have been implicated, including decreased activation, and increased detoxification by glutathione-dependent enzymes (Sinha, 1995).

Vinca alkaloids and taxanes, which are known antitubulin agents, are classically perceived as follows: vinca alkaloids depolymerise microtubules, thereby increasing the soluble tubulin pool, whereas taxanes stabilize microtubules and increase the microtubular mass. However, more recent data suggest that both classes of agents have a similar mechanism of action, involving the inhibition of microtubule dynamics (Dumontet and Sikic, 1999). Resistance to vinca alkaloids has been associated with the expression of P-gp, whereas the resistance to the taxanes is supposed to be linked to alterations or mutations in tubulin (Bhalla et al., 1994; Haber et al., 1995).
3. Aims of the Present Investigation

This thesis is a part of a programme at the Division of Pharmacognosy aimed at developing methods for isolation and characterisation of biologically active polypeptides from plant materials. The specific aims of this investigation were

- to develop and validate a fractionation protocol for isolation of polypeptides from plant materials;

- to optimise a human neutrophil model to serve as a multitarget bioassay to investigate the biological activity of the highly purified polypeptide fractions;

- to evaluate polypeptide fractions in the optimised neutrophil bioassay and for the cytotoxicity in a human cell line panel;

- to further characterise, chemically and biologically, the constituents in *Digitalis purpurea* and *Phoradendron tomentosum* responsible for the observed effects in the bioassays.
4. Experimental Bioassay Procedures

**Neutrophil isolation**
PMNs were isolated from heparinized blood samples of healthy volunteers by sequential dextran sedimentation and Ficoll-Paque (Amersham Pharmacia Biotech AB, Uppsala, Sweden) gradient centrifugation at 500 × g for 30 min at 20°C. Contaminating erythrocytes were removed by hypotonic lysis in ice-cold distilled water for 21 s, followed by addition of 9 volumes of Ca\(^{2+}\) and Mg\(^{2+}\) free phosphate-buffered saline (PBS; SVA, Uppsala, Sweden). After centrifugation (500 × g for 10 min, 4°C) the PMNs were resuspended at a concentration of (10 to 30) × 10\(^6\) cells/ml in PBS containing 0.9 mM CaCl\(_2\) and 0.49 mM MgCl\(_2\).

**Elastase release assay**
After the optimisation of the assay as described in Paper II, the following procedure was used. In all experiments, the PMN suspension in each test tube (concentration of 10\(^6\) neutrophils per 970 µl) was pre-incubated for 5 min at 37°C with cytochalasin B (5 µg/ml) (Sigma), 0.8 mM N-succinyl-L-alanyl-L-alanyl-L-valine-L-p-nitroanilide (SAAVNA) (Sigma), and the test solutions (100 µg/ml) or EtOH (0.1%). The challenge was started with 0.1 µM PAF or with 0.1 µM fMLP (all from Sigma). To allow a direct comparison, samples were tested in parallel for both PAF- and fMLP-induced exocytosis, using PMNs from the same preparation. Also, test tubes without the addition of PAF/fMLP were run in parallel. After incubation at 37°C for 10 min, the reaction was stopped by addition of citric acid (2%), and the tubes were then centrifuged at 500 × g for 10 min. The absorbance of each sample was recorded (405 nm) using a Shimadzu UV-VIS double-beam recording spectro-photometer. All samples were run in duplicate. The absorbance of the corresponding background tube (without inducer) was subtracted from that of the sample. The amount of inhibition of fMLP or PAF formation of pNA was, as a percentage, the relative decrease in absorbance compared to that of fMLP or PAF alone (100%), using the formula

\[
\text{% Inhibition} = \left\{ 1 - \left[ \frac{\text{ABS}_{(S+I)} - \text{ABS}_{(S+B)}}{\text{ABS}_{(I)} - \text{ABS}_{(B)}} \right] \right\} \times 100
\]

where, for the subscripts, S is the sample; I, the inducer; and B, the buffer.

**Superoxide production**
Superoxide generation was measured by means of superoxide dismutase-inhibitable reduction of cytochrome C as described by Jones and Hancock (1994). Test tubes containing 100 µM cytochrome C (Sigma) in PBS (Ca\(^{2+}\) and Mg\(^{2+}\)) solution were prewarmed at 37°C in a waterbath. The cell suspension was added to all the test tubes; and, to one of each pair was added the superoxide dismutase (SOD, Sigma) in PBS solution to give a final concentration of 100 µg/ml. After incubation for 5 min at 37°C, 0.1 µM of PAF, fMLP, or the test substance was added to each test tube. After 20 min incubation (37°C) in a shaking water bath, the reaction was stopped by 5 min of centrifugation (500 × g, 4°C). The amount of the cytochrome C reduction was measured by recording the absorbance at 550 nm.
Inhibition assay of the isolated elastase
The PMNs were isolated as described above. The cells were diluted with PBS (having Ca\(^{2+}\) and Mg\(^{2+}\)) and 2.5% BSA to a concentration of \((1.5 \text{ to } 4) \times 10^6\) cells/ml. The PMNs were activated by the addition of cytochalasin B to a final concentration of 5 µg/ml and then incubated for 10 min at 37°C. Addition of PAF to a final concentration of 0.1 µM initiated the release of elastase from the PMNs. After incubation (10 min), the exocytosis was stopped by centrifugation of the solution at 500 \(\times\) g for 10 min at 20°C. After decanting the supernatant, the cell pellet consisting of the PMNs was discarded. The elastase-containing supernatant was added to the test tubes containing either the test solution or 10% EtOH. The background reference samples were inactivated by adding citric acid (2%). The reaction was initiated with the addition of SAAVNA solution to a final concentration of 0.8 mM. After incubation at 37°C for 30 min, the reaction was stopped by adding of citric acid (2%). The test tubes were centrifuged at 500 \(\times\) g for 10 min. The absorbance of all samples, which were run in duplicate, was determined at 405 nm. The inhibition of elastase was then calculated as described above.

Inhibition assay of trypsin
In the method used, described by Shibata et al. (1986), a trypsin containing solution is incubated with the test solution and \(N\)-benzoyl-DL-arginine \(p\)-nitroanilide (BAPNA). The reaction of trypsin with its substrate leads to formation of a coloured product, \(p\)NA, which can be quantified by UV-measurement.

Trypsin was dissolved in 0.001 M HCl and 0.02 M CaCl\(_2\) to a concentration of 200 µg/ml. The trypsin containing solution was added to test tubes containing the test solution, or EtOH (0.1%). After incubation at 37°C for 10 min, 0.01 M BAPNA solution (0.01 M BAPNA in 0.05 M TRIS-HCl adjusted to pH 7.5) was added. The test tubes were incubated at 37°C for 10 min. After incubation, adding 30% HOAc stopped the reaction. The test tubes were centrifuged at 500 \(\times\) g for 2 min. All samples were run in duplicate, and trypsin inhibition was measured and calculated as above.

Haemolytic test
Hessinger and Lenhoff (1973) described this test. In brief, human erythrocytes, obtained from the blood of healthy volunteers, were separated from PMNs by sedimentation in a 10% solution of Dextran T-500 in 0.9% NaCl. The mixture of erythrocytes and dextran was centrifuged for 30 min (500 \(\times\) g, 20°C); and the supernatant, discarded. The remaining erythrocytes were washed with 50 ml of Krebs-Henseleit solution and centrifuged for 10 min (500 \(\times\) g, 4°C). The erythrocyte concentration was adjusted such that the absorbance for total haemolysis, by the addition of a saponin from Quillaja bark (Sigma), was between 1.2 and 1.4 at 570 nm. To each test tube, the test substance in Krebs-Henseleit solution and the erythrocyte test solution were added. After incubation for 15 min at 37°C, the reaction was terminated by centrifugation for 10 min at 1000 \(\times\) g and 20°C. The absorbance of each sample was compared with a sample that was without blood (as a blank), and with saponin for total haemolysis, and with water for no haemolysis. The polypeptide fractions, which were run in duplicate, were tested at a concentration of 100 µg/ml.
Experimental bioassay procedures

Cell line panel
The cell line panel consisted of four sensitive parental cell lines, five drug-resistant sublines, and one cell line with primary resistance (see Table 2).

Table 2. The cell lines, their origins, and their mechanisms of resistance.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Mechanism of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 8226-S</td>
<td>Myeloma</td>
<td>Parental</td>
</tr>
<tr>
<td>RPMI 8226-LR5</td>
<td>Myeloma</td>
<td>*GSH-associated</td>
</tr>
<tr>
<td>RPMI 8226-Dox40</td>
<td>Myeloma</td>
<td>*Pgp-associated</td>
</tr>
<tr>
<td>U-937 GTB</td>
<td>Histiocytic lymphoma</td>
<td>Parental</td>
</tr>
<tr>
<td>U-937 Vcr</td>
<td>Histiocytic lymphoma</td>
<td>*Tubulin-associated</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>Small Cell Lung Cancer</td>
<td>Parental</td>
</tr>
<tr>
<td>NCI-H69AR</td>
<td>Small Cell Lung Cancer</td>
<td>*MRP-associated</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>Leukaemia</td>
<td>Parental</td>
</tr>
<tr>
<td>CEM-VM-1</td>
<td>Leukaemia</td>
<td>*Topo II-associated</td>
</tr>
<tr>
<td>ACHN</td>
<td>Renal</td>
<td>Primary resistant</td>
</tr>
</tbody>
</table>

*= subline. Abbreviations: topo II (topoisomerase II), MRP (multidrug-associated protein), P-gp (P-glycoprotein), GSH (glutathione).

Choice of cell lines in Table 2 proceeded as follows: for melphalan resistance, the RPMI-8226-LR5 cell line, proposed to be associated with increased levels of glutathione (Bellamy et al., 1991; Mulcahy et al., 1994), was selected; for doxorubicin (Dox) resistance, the RPMI 8226-Dox 40 cell line, which shows the classical MDR phenotype with overexpression of P-gp 170 (Dalton et al., 1986; Dalton et al., 1989), was selected; for vincristine (Vcr) resistance, the U-937 Vcr cell line, suggested to be tubulin-associated (Botling et al., 1994), was selected; for Dox resistance, the NCI-H69AR cell line, expressing a MDR phenotype proposed to be mediated by MRP (Mirsks et al., 1987; Slovak et al., 1993), was selected; and finally, for teniposide resistance, the CEM-VM-1 cell line, suggested to be topo II associated, and expressing the atypical MDR phenotype (Danks et al., 1988; Danks et al., 1987), was selected. The drug resistance of the primary resistant ACHN cell line is probably multifactorial (Nygren and Larsson, 1991).

Measurement and data analysis of cytotoxic activity
Substances were tested for cytotoxicity using the non-clonogenic 72-hour fluorometric microculture cytotoxicity assay (FMCA) (Larsson and Nygren, 1989; Larsson and Nygren, 1990). The FMCA is based on measurement of fluorescence generated by hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes. Experiments with cell lines were repeated 2-3 times. Quality criteria for a successful analysis included a fluorescence signal in the control wells of more than ten times mean blank value, and a mean coefficient of variation (CV) in the control and blank wells of less than 30%.

V-shaped 96-well microtitre plates were prepared with 20 µl per well of test solution at ten times the desired concentration. Each plate contained three substances, using triplicate wells for each concentration and compound, six control wells, six blank wells, and three wells for each positive (0,1% Triton X-100) and negative (PBS) control.

To measure cell survival, a survival index (SI) was defined as the ratio of fluorescence in experimental wells, and that in control wells (with blank values subtracted)—multiplied by 100 to reflect a percentage. The IC_{50} value, calculated for each compound, was defined as the concentration of the compound at SI equal
to 50%. A procedure similar to the COMPARE analysis described by Paull et al., (1989) (using Pearson´s correlation coefficients) was used for comparing compounds and rank-ordering them for their similarity to a “mean” profile as described in Paper IV. For pairs of compounds (x and y), amount of linear correlation of log IC$_{50}$ values was determined using Excel (Microsoft). In addition, the resistance factor (RF) was calculated for each compound, defined as the ratio of IC$_{50}$ values, that for the resistant subline and that for its (sensitive) parental cell line. The pairs of resistant/parental cell lines used for RF calculations of P-gp, GSH, MRP, topo II, and tubulin associated resistance were RPMI 8226-Dox40/RPMI 8226-S, RPMI 8226LR5/RPMI 8226-S, NCI-H69AR/NCI-H69, CEM-VM-1/CCRF-CEM, and U-937 Vcr/U-937 GTB, respectively (Dhar et al., 1996).
5. Development of Methods for Isolation of Polypeptides from Plant Material

The use of pre-fractionated plant extracts instead of crude extracts conceivably offers advantages in finding bioactive compounds present only in small amounts in the plant, and can be utilised to exclude or include activity from certain classes of substances. Although peptides in plants are often present only in small amounts (e.g., 1 µg systemin was isolated from 28 kg tomato leaves) (Marx, 1996), yet convenient assays for purifying peptides in low concentrations have been lacking. In this study a fractionation protocol for isolating peptides from a small amount of plant material was developed (Paper I).

Plant material

The plants were selected to represent a major part of the angiosperms with emphasis on medicinal plants, reputed Swedish anti-inflammatory plant taxa and plants known to contain peptides. The plant materials were mainly collected in Uppland, Sweden, including the Uppsala University Botanical Garden. Voucher specimens have been deposited at the Uppsala University Herbarium (UPS). Fresh plant material was dried at 55-60°C to preserve it, and to prevent enzymatic degradation of peptides. Previously reported plant peptides, containing several disulphide bonds, appear to be largely heat stable (Gran, 1973; Samuelsson, 1973), and several are reportedly stable to aqueous boiling. The dried, powdered plant material was stored at –20°C until used.

Fractionation of polypeptides

Fractionation of plant materials was done in five steps to obtain a highly purified fraction (fraction P). Before applying the plant material to the protocol, each step was optimised and analysed. Figure 2 shows the flowchart of the fractionation procedure, applied to 100 plant materials.

Step 1 and 2: Extraction

Plant material was first extracted in dichloromethane; and then, in 50% EtOH. Although polypeptides are insoluble in dichloromethane, yet ubiquitous lipophilic substances, such as chlorophyll, lipids, and other low-molecular-weight substances (e.g., terpenoids, phenylpropanoids, etc.) are extracted and thus removed. The extraction with 50% EtOH was chosen because it does not extract most polysaccharides (Beutler et al., 1993; Thunberg and Samuelsson, 1982) or enzymes, and the solubility of polypeptides is generally better in the 50% mixture than in pure water or alcohol. Furthermore, such extractions need no preservation from microbial growth.

Step 3: Removal of tannins by filtration through polyamide

After being acidified by HOAc to a final concentration of 2%, the 50% EtOH extract was passed through a column of polyamide, to remove the contained polyphenols (tannins). The binding of tannins to the polyamide is highly pH-dependent, so lowering the pH from 7.5 to 3 leads to strong hydrogen bonding, which occurs between polyphenolics and polyamide. Thus, the tannins are
Development of methods for isolation of polypeptides from plant material

practically irreversibly bound to the column. The polypeptides were eluted with EtOH 50%/HOAc 2%.

Step 4: Size-exclusion chromatography on Sephadex G-10
For this gel filtration step, several experiments were performed to establish a suitable mobile phase. The solution EtOH 50%/HOAc 2%/NaCl 0.2 M was chosen. The high-molecular substances (>700 Da) appeared in the void volume, and the basic amino acids eluted in the low-molecular fraction by including the 0.2M NaCl. The plant extracts were readily dissolved by the combination EtOH (50%)
and HOAc (2%), and the non-specific interactions between plant pigments and the gel were largely suppressed.

**Step 5: Desalting by solid-phase extraction on C\textsubscript{18} material**

The high-molecular-weight fraction described above contained, besides polypeptides, a large amount of NaCl and polysaccharides. The salt and the polysaccharides were removed by solid-phase extraction (SPE) using a C\textsubscript{18} reversed-phase column. Experiments with commercially available polypeptides (oxytocin, gramicidin, bacitracin, and insulin) showed that they are not eluted in 50 mM NH\textsubscript{4}HCO\textsubscript{3}, but salt and polysaccharides were easily removed. The polypeptides were then eluted from the column in three steps by adding 20%, 50%, and 80% EtOH in 50 mM NH\textsubscript{4}HCO\textsubscript{3}, respectively. None of the reference peptides required higher concentrations of ethanol to be eluted. Evaporating in vacuo and then lyophilising the combined eluates yielded a polypeptide fraction (fraction P).

**Isolation of varv peptide A**

To further validate the fractionation protocol, the procedure was applied to plant material of *Viola arvensis* (Violaceae), which had been reported to contain a cyclic 29-residue polypeptide, viola peptide-I (Schöpke et al., 1993). From the hydrolysis of fraction P of *Viola arvensis*, free amino acids were obtained; and from the RP-HPLC chromatogram of the fraction, several peaks with UV spectra consistent with tryptophan-containing peptides was shown.

![Figure 3](image)

**Figure 3.** Semipreparative RP-HPLC of the polypeptide fraction obtained from *Viola arvensis*. The peak labeled A in the chromatogram corresponds to varv peptide A. The semipreparative HPLC was performed with a Shimadzu system, equipped with an SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan), and a 250 × 10 (i.d.) mm Dynamax column (C\textsubscript{18}, 5µm, pore size 300Å). Mobile phase: 40% CH\textsubscript{3}CN-i-PrOH (6:4)/ 0.1% TFA (adjusted to pH=2.25 by addition of NH\textsubscript{4}OH). UV detection at 215 nm. Flow rate 4 ml/min.

The same isolation strategy was applied to a larger amount of plant material (as described in Paper I). The substance corresponding to the major peak in the chromatogram at 215 nm was collected and designated varv (*Viola arvensis*) peptide A (Figure 3). Quantitative amino acid analysis of the hydrolysate of varv peptide A indicated the amino acid composition shown in Table 3. The calculated average mass of a linear peptide with this composition would be between 2901.3 Da (2Asn; 1 Gln) and 2904.3 Da (2 Asp; 1 Glu). Mass spectrometry of varv peptide A, however, provided a lower molecular weight of 2879.4 Da, suggesting the
peptide to be macrocyclic, like the previously reported viola peptide-I (Schöpke et al., 1993).

Varv peptide A was then reduced with mercaptoethanol and subsequently alkylated with 4-vinylpyridine. Cleavage of the alkylated peptide with endoproteinase Glu-C resulted in a single linear product, consistent with the opening of a macrocyclic ring. The linear product was subjected to automated Edman degradation, defining 2 Asx and 1 Glx from the quantitative amino acid analysis as 2 Asn and 1 Glu (Table 3, Paper I). Consistent with the amino acid sequence obtained for the linear product, varv peptide A had the following structure:

cyclo- (-Thr-Cys-Val-Gly-Gly_5-Thr-Cys-Asn-Thr-Pro_{10}-Gly-Cys-Ser-Cys-Ser_{15}-Trp-Pro-Val-Cys-Thr_{20}-Arg-Asn-Gly-Leu-Pro_{25}-Val-Cys-Gly-Glu-)

With this amino acid composition, the calculated average mass of a linear peptide is 2902.3 Da. Assuming the peptide to be macrocyclic (-18 Da), and its six cysteine residues to be engaged in intramolecular disulphide bonds (-6 Da), its molecular weight is 2878.3 Da. This value agrees with the molecular weight 2879.4 Da for the varv peptide A, experimentally determined by means of MALDI-TOF MS analysis.

Varv peptide A and the previously reported viola peptide-I share a very high degree of sequence homology. They differ only at two amino acid positions. The Trp and the Arg residues in varv peptide A are substituted for an Arg residue and an X (unidentified amino acid) residue, respectively, in the published sequence of viola peptide-I (Schöpke et al., 1993).

### Table 3. Amino acid analysis of varv peptide A isolated from *Viola arvensis*.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues from amino acid analysis</th>
<th>Residues from sequencing$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>2,0</td>
<td>2 (2 Asn)</td>
</tr>
<tr>
<td>Thr</td>
<td>3,7</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
<td>2,0</td>
<td>2</td>
</tr>
<tr>
<td>Glx</td>
<td>1,0</td>
<td>1 (Glu)</td>
</tr>
<tr>
<td>Pro</td>
<td>2,9</td>
<td>3</td>
</tr>
<tr>
<td>Gly</td>
<td>4,9</td>
<td>5</td>
</tr>
<tr>
<td>Cys</td>
<td>5,2$^b$</td>
<td>6$^c$</td>
</tr>
<tr>
<td>Val</td>
<td>3,0</td>
<td>3</td>
</tr>
<tr>
<td>Leu</td>
<td>1,0</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>1,0</td>
<td>1</td>
</tr>
<tr>
<td>Trp</td>
<td>1,0$^d$</td>
<td>1</td>
</tr>
</tbody>
</table>

$^a$ A total of 29 residues were determined by sequencing.

$^b$ Half-cystine was determined as cysteic acid, based on a separate sample, following oxidation with performic acid.

$^c$ Cys was determined as (pyridylethyl)cysteine, following alkylation with vinylpyridine.

$^d$ Trp was determined photometrically.
6. A Multitarget Bioassay on the Neutrophil

The multitarget functional bioassays, in which observed effects cannot be attributed directly to a specific mode of action, encompasses assays on whole animals, isolated organs, and intact cells. For example, an observed relaxation of the isolated guinea pig ileum can be due to mechanisms involving cell membrane receptors, second messengers, or ion channels, or due to other mechanisms. Single target bioassays, on the other hand, are typically tests run on an isolated enzyme or a receptor (receptor binding). For such assays, which are highly specific, the observed effect can be directly attributed to a specific mechanism, such as enzyme inhibition or receptor affinity.

Dewald and Baggiolini (1987) developed an assay to detect PAF-antagonists. In that assay, neutrophils were incubated with the antagonist under evaluation, PAF, and with a chromogenic substrate SAAVNA for released elastase. Elastase reacts with SAAVNA, which leads to formation of a coloured product, p-nitroanilide (pNA), which can be quantified photometrically (Bieth and Wermuth, 1973; Wenzel et al., 1980). This detection of pNA formed by elastase released from a chemoattractant-stimulated neutrophil can be described as a multitarget functional bioassay (Claeson and Bohlin, 1997), where both known and previously unknown potential drug targets are present. An observed inhibition of formation of pNA can be due to elastase inhibition, for example, or due to cytotoxic effects on the cell, or affinity for a receptor on the neutrophil (Figure 4). By the same method, chemoattractant activity of a test compound is also possible, seen as increased amount of pNA formed. In Paper II, the evaluation of the originally described assay conditions is described, with the aim of optimising the bioassay for multitarget functional screening of natural products. Polypeptide fractions (fraction P) of 100 plant materials were investigated in the, thus optimised, neutrophil multitarget bioassay (Paper II).

![Figure 4](image-url) Schematic picture of the neutrophil, viewed as a multitarget functional bioassay with various possible modes of action for test substances. Both inhibiting and stimulating effects can be detected.
Optimisation of a multitarget bioassay on the neutrophil

The bioassay method was optimised with respect to the formation of pNA. Throughout the optimisation procedure, the aim was to select conditions to facilitate the comparison of activities of polypeptide fractions and test compounds towards PAF or fMLP induced release of pNA. As described in Paper II, the following parameters were optimised:

- cytochalasin B concentration
- SAAVNA concentration
- PAF and fMLP concentration

The formation of pNA in PAF and fMLP-induced PMNs required cytochalasin B. The established optimal concentration of cytochalasin B for priming was 5 µg/ml (Figure 3a, Paper II). Concentration-response curves for the inducers PAF and fMLP were recorded and tested in parallel using PMNs from the same preparation. Equal biological responses for PAF and fMLP, reaching approximately 80% of maximum, were obtained at a concentration of 0.1 µM (Figure 3c, Paper II), which was chosen as the assay condition. Amount of formation of pNA depended on the SAAVNA concentration (Figure 3b, Paper II). For 0.1 µM PAF and fMLP, pNA reached a maximum at a concentration of 0.8 mM of SAAVNA, which was chosen as the assay condition (Figure 3d, Paper II). To validate the assay, thus optimised, one additional agonist, interleukin-8 (IL-8), was evaluated under the test conditions described. The same concentration as that for PAF and fMLP (0.1 µM) can be used to induce elastase release from neutrophils by IL-8 (Figure 4, Paper II).

Validation

When reference compounds were evaluated in the optimised assay; different modes of action were clearly detected (Table 4). The PAF-antagonist ginkgolide BN 52021 (Földes-Filep et al., 1987) exhibited a selective inhibitory effect on PAF, as expected, with the IC$_{50}$ value (Table 4) being 72 µg/ml, which is consistent with previously reported values for inhibition of PAF by the ginkgolide BN 52021 (Dewald and Baggiolini, 1987). At the highest tested concentration (100 µg/ml), ginkgolide was unable to inhibit the fMLP inducer. The elastase inhibitor α$_1$-antitrypsin (Virca and Schnebli, 1984) [IC$_{50}$; 10 µM (PAF), 11 µM (fMLP)] had a non-selective inhibitory effect.

<table>
<thead>
<tr>
<th>Substances</th>
<th>PAF IC$_{50}$ µg/ml</th>
<th>n</th>
<th>fMLP IC$_{50}$ µg/ml</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>α$_1$-antitrypsin</td>
<td>10</td>
<td>9</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>Trypsin-inhibitor</td>
<td>10</td>
<td>9</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>BN 52021</td>
<td>72</td>
<td>12</td>
<td>-</td>
<td>11</td>
</tr>
</tbody>
</table>
Identification of the extracellular release of superoxide, elastase and defensins

Superoxide generation by the neutrophils

The generation of the superoxide anion $O_2^-$ in PAF (or fMLP) activated PMNs was measured. Comparing the two agonists, fMLP induced a higher increase in the production of $O_2^-$ than did PAF (Figure 5). This result agrees with reports in the literature, indicating that PAF only weakly stimulates superoxide generation ($EC_{50}$ below 10 µM) of neutrophils, whereas fMLP is more potent ($EC_{50}$: 48 nM) (Turner et al., 1994).

![Figure 5. Superoxide production by PMNs when stimulated by the addition of different concentrations of PAF (□) and fMLP (●). Each point is the mean absorbance value ± SEM of three independent experiments run in duplicate.](image)

SDS-PAGE of exocytosed proteins from stimulated neutrophils

The PMNs were isolated and stimulated as described above. The PMNs were pelleted by centrifugation, and the supernatant was saved. The supernatant was diluted 50% with 2X treatment buffer (0.125 M tris-Cl, 4% SDS, 20% v/v glycerol, 0.2 M DTT, 0.02% bromophenol blue, pH 8.8), boiled for 2 min, and stored in a freezer. SDS-PAGE was run (180V, 40mA and 1.5h) on a Hoefer miniVE (Amersham Pharmacia Biotech, Uppsala, Sweden). The running buffer was SDS (sodium dodecyl sulphate) containing tris-glycine. Amersham Pharmacia Biotech Low Molecular Weight Calibration Kit, containing proteins of 94, 67, 43, 30, 20, and 14 kDa, was used as the molecular weight marker.

The sample was run on a 10%T gel with ReadySol 40% (Amersham Pharmacia Biotech, Uppsala, Sweden) and polymerised with ammonium-persulfate, catalysed by TEMED. The gels were stained by means of a silver staining kit protein (Amersham Pharmacia Biotech, Uppsala, Sweden). As the results show (Figure 6), the sample prepared from the neutrophils contains enough proteins to be visualised.

The biggest spot is albumin (at 67 kDa), which is shown to be present in all samples. In the sample preparations, albumin is added to the assay, but also
A multitarget bioassay on the neutrophil

The release of defensins from fMLP-stimulated neutrophils

The human PMNs were isolated, as described above in experimental procedures, from 100 ml heparinized blood samples. The cells were activated by adding cytochalasin B to a final concentration of 5 µg/ml, and then were incubated for 10 min. To initiate release, the PMNs were stimulated by the addition of fMLP to a final concentration of 0.1 µM. After incubation (10 min), the exocytosis was stopped by the addition of 30% acetic acid, and then the solution was centrifuged at 500 × g for 5 min. The supernatant, after centrifugation, was diluted and lyophilised. After being lyophilised, it was extracted with 80% MeOH on ice at a room temperature of 8°C for 8h.

Figure 6. SDS-PAGE of the exocytosed proteins from the PMNs. The lanes are from the left: weight marker, proteins exocytosed from PAF-stimulated neutrophils, unstimulated neutrophils, human elastase (Sigma).

Figure 7. RP-HPLC of fMLP-stimulated extract of neutrophils. A linear gradient from 10% CH₃CN/0.1% TFA to 60% CH₃CN/0.1% TFA was used. UV detection, at 215nm; flow rate, 0.3 ml/min.
Table 5. Comparison of measured masses (MH⁺) from an enriched defensin fraction from human neutrophils and calculated masses of HNP-1, -2, -3, and 4.

<table>
<thead>
<tr>
<th>Collect</th>
<th>Measured masses MALDI-TOF MS (MH⁺)</th>
<th>Calculated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>3443.1</td>
<td>3442.1 (HNP 1)</td>
</tr>
<tr>
<td></td>
<td>3372.2</td>
<td>3371.0 (HNP 2)</td>
</tr>
<tr>
<td></td>
<td>3487.3</td>
<td>3486.1 (HNP 3)</td>
</tr>
<tr>
<td>D</td>
<td>3711.1</td>
<td>3709.5 (HNP 4)</td>
</tr>
</tbody>
</table>

The RP-HPLC chromatogram of the enriched defensin fraction (Figure 7) included several peaks, with UV spectra consistent with tryptophan-containing peptides. Fractions were collected and analysed by MALDI-TOF MS. By MALDI-TOF MS, fraction B was shown to contain three peptides with masses 3443.1, 3372.2, and 3487.3 MH⁺ (Table 5), in agreement with masses calculated for the neutrophil defensins HNP-1, -2, and -3. In fraction D, the mass 3711.1 MH⁺ was found, in agreement with the calculated for HNP 4.

The evaluation of the activity
This study includes an introductory classification of polypeptide fractions, sorting them into mechanistic classes that can facilitate prioritising the fractions P for future studies. Figure 8 provides an outline of the classification procedure. The 100 polypeptide fractions (fraction P) according to the fractionation protocol, were first tested for inhibition of PAF- and fMLP-induced pNA formation, and for ability to induce elastase release in the absence of challenge with PAF or fMLP. Inhibitory polypeptide fractions were classified as being either selective inhibitors (of PAF or fMLP) or non-selective inhibitors (of pNA formation).

Figure 8. Schematic representation of the evaluation of the activity of the polypeptide fraction.
Polypeptide fractions that were potent non-selective inhibitors of pNA formation (>80%) were further evaluated for direct inhibitory effect on isolated elastase enzyme. To roughly evaluate selectivity of elastase inhibition, polypeptide fractions were also tested for ability to inhibit the enzyme trypsin. In addition, each fractions P was tested for haemolytic activity to determine whether it could damage cells.

The activity of fraction P

After optimisation, the assay was operated successfully for 100 polypeptide fractions (listed in Table 2, Paper II) from 96 species, representing 46 families of 26 orders, where both PAF and fMLP were used to induce exocytosis.

The results from the screening (Paper II) are presented in a manner that links recorded biological activity to phylogenetic information (see Figure 9). Of the 100 tested polypeptide fractions, 41% inhibited pNA formation more than 60%; 18% non-selectively inhibited pNA formation less than 30%; and 3% stimulated formation of pNA, without the challenge of PAF or fMLP. In the order Malpighiales, 4 of the 8 tested species inhibited pNA formation less than 30%; whereas in the family Violaceae, 3 of 4 tested species inhibited formation less than 30%, and one, Viola patrinii, stimulated formation (Table 2, Paper II).

In other orders, such as Lamiales and Brassicales, a comparably high proportion of the tested species showed prominent inhibitory activity. For Lamiales, 11 out of 17 polypeptide fractions inhibited pNA formation by more than 60%, and 2 enhanced formation. The fraction P of Nymphoides peltata of Menyanthaceae inhibited pNA formation of PAF-induced elastase release 36 ± 14%, and fMLP-induced elastase release 90 ± 3%, which may indicate selective inhibition by fMLP receptors. However, since all the polypeptide fractions contain a mixture of substances, more than one active compound is possible, and therefore the presence of selective inhibitors in fractions, which can influence and modify non-selective inhibition, cannot be ruled out.

Haemolytic test

A lysis test on erythrocytes was used to determine whether the polypeptide fractions at a concentration of 100 µg/ml could damage the cells, which may affect the release of elastase from the neutrophil. The cell-damaging effect on erythrocytes is not connected to elastase release by PMNs, but might, as a first step, facilitate determining whether the fraction or substance is damaging cells at the concentration used in the neutrophil assay. At extract concentration 100 µg/ml, haemolysis occurred in 7 of the polypeptide fractions tested (Table 2, Paper II).

Inhibition of the isolated elastase

The polypeptide fractions that non-selectively inhibited pNA formation more than 80%, were tested for ability to inhibit, in a single target bioassay, the isolated enzyme elastase. For comparison, other polypeptide fractions were included as negative controls. The polypeptide fractions inhibited the isolated elastase in the same concentration range as they inhibited pNA formation (Table 2, Paper II), which indicates that the observed effects in the whole-cell assay are due to inhibition of the isolated enzyme elastase.
A multitarget bioassay on the neutrophil

Figure 9. The biological activity of fraction P (•) in the neutrophil elastase release assay. The activity is linked to the systematic position and distribution of taxa included in the study. The phylogenetic relationships are according to APG (1998) (Bremer et al., 1998) and the NCBI/GenBank taxonomy section. A numbers following an ordinal name indicates the approximate number of included families; a number following a familial name indicates the approximate number of genera and species, respectively. An ordinal name in bold typeface indicates an order represented in the study.

*These fractions stimulated pNA formation.
Inhibition of trypsin
Some of the plant materials were also tested against the enzyme trypsin, to give an indication of the enzyme selectivity of the observed inhibition of elastase. Inhibition of trypsin by polypeptide fractions of Reseda luteola was high (97%). The fraction P of Rubus idaeus and Tabernaemontana dichotoma inhibited elastase (80% and 77%), significantly more than they inhibited trypsin (8% and 11%). These results suggest that some of the polypeptide fractions studied might contain selective protease inhibitors.

Stimulating activity
Examination of the fraction P of Digitalis lanata, Digitalis purpurea, and Viola patrinii revealed an unusual and unexpected effect on the formation of pNA. The fraction P from these plants significantly increased formation of pNA, in a concentration-dependent manner (Figure 8, Paper II), independent of challenge with PAF or fMLP. Furthermore, these polypeptide fractions enhanced the pNA formation as much or more than exocytosis induced by either agonist, PAF or fMLP. The formation of pNA was dependent in these cases on the presence of cytochalasin B, and was not inhibited by the PAF-antagonist ginkgolide BN 52021; but instead the presence of α₁-antitrypsin decreased the amount of pNA formed. Bioassay-guided fractionation of the specific fraction P (substances with molecular weights >700 Da) of the leaves of Digitalis purpurea Ehrl. led to the isolation of digitoxin, as described in chapter 7.
7. Isolation and Characterisation of Substances with Cytotoxic and Neutrophil Elastase Releasing Activity

After a primary fractionation and activity evaluation, the constituents responsible for the observed effects in *Phoradendron tomentosum* (Paper III) and *Digitalis purpurea* (Paper IV) were further characterised (chemically and biologically).

**Phoratoxins from *Phoradendron Tomentosum***

The first phoratoxin was isolated in 1967 by Samuelsson and Ekblad from *Phoradendron tomentosum* (DC) Engelm. subsp. *macrophyllum*. This peptide was later renamed phoratoxin A. In 1974 the amino acid sequence was determined. Phoratoxin A consists of 46 amino acids (Mellstrand and Samuelsson, 1974a) tightly bound together by three disulphide bridges in the same way as for the viscotoxins (Mellstrand and Samuelsson, 1974b).

Thunberg (1983) isolated phoratoxin B from *P. tomentosum*. Three lyophilised extracts were investigated. The leaves from *P. tomentosum* subsp. *macrophyllum* (Cockerell) Wiens grown on *Juglans hindsii* Jepson extracts (extract A), and the leaves (extract B) and twigs and stems (extract C) from *P. tomentosum* subsp. *macrophyllum* grown on *Populus fremontii* S. Wats. These three plant materials from *P. tomentosum* L. were homogenised and extracted in 2% acetic acid. The concentrated extract was evaporated in vacuo, lyophilised, and stored at −20°C (Mellstrand and Samuelsson, 1973; Samuelsson and Ekblad, 1967). The dried extract was diluted with distilled water, with the pH set to 5.0. The solution was diluted with water until the conductivity was slightly lower than the acetate buffer employed. The solution was passed through a cellulose phosphate column and washed with 20 column volumes of 0.1 M NaOAc (pH 5.0). The adsorbed substances were eluted with 0.1 M NaOAc containing 0.8 M NaCl (pH 5.0).

**Figure 10.** Separation of basic proteins on a column of SP-Sephadex C-25 eluted with a linear gradient of sodium phosphate buffer. A, B, and C represent separation of basic proteins from extracts A, B, and C respectively. Reproduced from Thunberg (1983).
Fractions were collected and concentrated in vacuo. To remove the salt, the fraction was applied to a column (Sephadex G-25). The high molecular fraction was collected, evaporated in vacuo, and passed through a cation-exchange column (SP-Sephadex C25), and then eluted in a linear gradient from 0.05 M sodium phosphate (pH 5.05) to 0.3 M sodium phosphate (pH 6.0). Fractions I-V were collected from the extract A-C (Figure 9), desalted and lyophilised (Thunberg, 1983). In extract A, from which phoratoxin A was previously isolated, was shown to be a mixture of at least two peptides, since a half valine residue was found. Phoratoxin B was isolated from peak III in extract B (Thunberg, 1983). The sequences of phoratoxins A and B are shown in Figure 12.

Recent progress in the chromatography and sequence analysis of peptides has markedly improved the separation of peptides. One standard method today for separating peptide mixtures is RP-HPLC with gradients of increasing concentrations of acetonitrile in the presence of trifluoroacetic acid. With modern instruments and columns, complex peptide mixtures can be separated. Difficult separations are addressed by modifying the gradient slope or organic eluant composition. Further improvements in resolution are often needed, requiring fundamental changes in mobile phase composition or selection of complementary chromatographic separation mechanisms.

**Isolation and characterisation of peptides from *Phoradendron tomentosum***

The fractions IV and V from extract B (see Figure 10) (Thunberg 1983) were further fractionated. The peptides isolated from fraction IV were named phoratoxin C and phoratoxin D. The molecular weights of phoratoxins C and D, experimentally determined by mass spectrometry, were 4879.3 MH\(^+\) and 4311.2 MH\(^+\), respectively.

The peptides from fraction V were the previously described phoratoxin B, and two novel peptides named phoratoxins E and F. The molecular weights were 4893.0 MH\(^+\) (phoratoxin B), 4879.2 MH\(^+\) (phoratoxin E), and 4873.2 MH\(^+\) (phoratoxin F).

The results from the amino acid analyses of the isolated peptides are shown in Table 6. The primary sequences of phoratoxins B-F were determined unambiguously by means of a combination of procedures: Edman degradation, trypsin enzymatic digestion, and electrospray ionization MS/MS sequencing. To gain information on the C-terminal part of the molecule, the tryptic fragments of the pyridylethylated peptide (Table 7), or a fragment of the native peptide, was analysed by MS/MS (illustrated in Figure 11). Cysteine was identified as carbamidomethyl cysteine after alkylation with iodoacetamide. The MH\(^+\) of the carbamidomethylated peptides (Table 6) exceeded that of the native peptides by 343.2 Da. This corresponds to the mass of six carbamidomethylated cysteines, 57.03 Da for each derivatized cysteine, which indicates the presence of six cysteine residues in the native peptides. The previously isolated phoratoxin B and the novel phoratoxins C, E, and F consist of 46 amino acid residues; and phoratoxin D has 41 amino acid residues, all with six cysteines, similar with the earlier described phoratoxins A and B, which belong to the group of thionins. The sequences of the isolated peptides are shown in Figure 12, where in addition, alignment with other known thionins is shown.
Table 6. The amino acid composition and molecular weights of the phoratoxin peptides. For each peptide, the residues from amino acid analysis are listed to the left and the residues from sequencing to the right.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Phoratoxin B</th>
<th>Phoratoxin C</th>
<th>Phoratoxin D</th>
<th>Phoratoxin E</th>
<th>Phoratoxin F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asn (D/N)</td>
<td>4.0</td>
<td>3.2 (2 N)</td>
<td>3.1</td>
<td>4.18</td>
<td>4.4</td>
</tr>
<tr>
<td>Thr (T)</td>
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<td>6.0</td>
<td>5.0</td>
<td>5.09</td>
<td>4.4</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>4.9</td>
<td>5.0</td>
<td>4.2</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>1.9</td>
<td>2.2</td>
<td>6.4</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Gly (G)</td>
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<td>2.1</td>
<td>5.3</td>
<td>6.4</td>
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</tr>
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<td>Ala (A)</td>
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<td>2.0</td>
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<td>2.9</td>
</tr>
<tr>
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<td>5.2</td>
<td>5.2</td>
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</tr>
<tr>
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<td>-</td>
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</tr>
<tr>
<td>Ile (I)</td>
<td>3.5</td>
<td>3.6</td>
<td>2.7</td>
<td>3.8</td>
<td>4.0</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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</tr>
<tr>
<td>His (H)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>3.9</td>
<td>4.2</td>
<td>4.3</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>2.9</td>
<td>3.0</td>
<td>3.1</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fragments</th>
<th>Phoratoxin B</th>
<th>Phoratoxin C</th>
<th>Phoratoxin D</th>
<th>Phoratoxin E</th>
<th>Phoratoxin F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-10</td>
<td>4893.0</td>
<td>4879.3</td>
<td>4311.2</td>
<td>4879.2</td>
<td>4873.2</td>
</tr>
<tr>
<td>2-10</td>
<td>4892.3</td>
<td>4878.3</td>
<td>4310.1</td>
<td>4878.3</td>
<td>4872.3</td>
</tr>
<tr>
<td>11-17</td>
<td>5235.4</td>
<td>5221.1</td>
<td>4653.3</td>
<td>5221.6</td>
<td>5215.8</td>
</tr>
<tr>
<td>29-33</td>
<td>5234.5</td>
<td>5220.5</td>
<td>4652.2</td>
<td>5220.5</td>
<td>5214.5</td>
</tr>
</tbody>
</table>

Table 7. Masses of theoretical trypsin digests of phoratoxins B-F are compared with experimentally observed MS fragments. For each peptide, the calculated masses are listed to the left and the measured masses (MH') to the right.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Phoratoxin B</th>
<th>Phoratoxin C</th>
<th>Phoratoxin D</th>
<th>Phoratoxin E</th>
<th>Phoratoxin F</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-28</td>
<td>1092.6</td>
<td>1092.6</td>
<td>1093.6</td>
<td>1092.6</td>
<td>1079.6</td>
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<tr>
<td>2-10</td>
<td>939.4</td>
<td>940.4</td>
<td>939.4</td>
<td>940.3</td>
<td>939.4</td>
</tr>
<tr>
<td>11-17</td>
<td>883.4</td>
<td>884.5</td>
<td>883.4</td>
<td>884.4</td>
<td>883.4</td>
</tr>
<tr>
<td>40-46</td>
<td>819.3</td>
<td>805.3</td>
<td>805.3</td>
<td>819.3</td>
<td>820.3</td>
</tr>
<tr>
<td>34-39</td>
<td>618.4</td>
<td>618.4</td>
<td>618.4</td>
<td>618.4</td>
<td>618.4</td>
</tr>
<tr>
<td>29-33</td>
<td>507.3</td>
<td>507.3</td>
<td>507.3</td>
<td>507.3</td>
<td>507.3</td>
</tr>
</tbody>
</table>

1 The tryptic fragments, in order of decreasing molecular weights.
Isolation and characterisation of substances with cytotoxic and neutrophil elastase releasing activity

Analysis of the reference materials of phoratoxins A and B
Analyses were made on the previously isolated phoratoxins A and B that had been deponated in a deep freezer of the Division of Pharmacognosy by Eva Thunberg and Gunnar Samuelsson. According to the HPLC and MS analyses, both reference materials contained several substances, and had very similar chromatogram/spectra. The C-terminal of the reference substances was also examined using MS/MS. Both phoratoxins A and B were shown to have the C-terminal sequence DSGWDH. Thunberg (1983) reported that phoratoxin A probably was a mixture of at least two peptides based on the low value of valine obtained in the amino acid analyses. Here, the reference material of phoratoxin B was further fractionated on RP-HPLC, with a linear gradient from 20% CH$_3$CN/0.2% TFA to 40% CH$_3$CN/0.2% TFA. The two main peaks isolated. The native peptides (3 nmol) were subjected to reduction and alkylation with iodoacetamide. The reaction products were sequenced by automated Edman degradation, and the amino acid sequences, confirmed by sequencing using MS/MS. The results from the amino acid analysis and Edman degradation are shown in Table 8.

The molecular weights of the major peptides isolated from reference material of phoratoxin B were determined, and found to be 4879.1 MH$^+$ (peak 1) and 4892.7 MH$^+$ (peak 2). Sequencing by means of Edman degradation of the alkylated peak 1 yielded 41 residues from the N-terminal end: KSC*C*P TTTAR NIYNT C*RFGG GSR PVC*AK LSGC*KIISGTKC*D. A tryptic fragment gave the C-terminal sequence DSGWDH at m/z 438.6 [M+2H]$^{2+}$ after MS/MS analysis.

Peak 2 yielded an N-terminal sequence of 41 residues, KSC*C*PTTAR NIYNT C*RFGG GSRPI C*AKLS GC*KI SGTKC*D, by Edman degradation. The native peptide fragment gave the partial C-terminal sequence KIISGTKC*DSGWDH at m/z 873.5 [M+6H]$^{6+}$ by MS/MS analysis.
Table 8. The amino acid composition and molecular weights of the phoratoxin peptides isolated from the reference material of phoratoxin B. For each peptide, the residues from amino acid analysis are listed to the left and the residues from sequencing to the right.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp/Asn (D/N)</td>
<td>4.0</td>
<td>4 (2 N)</td>
</tr>
<tr>
<td>Thr (T)</td>
<td>4.6</td>
<td>5</td>
</tr>
<tr>
<td>Ser (S)</td>
<td>5.0</td>
<td>5</td>
</tr>
<tr>
<td>Pro (P)</td>
<td>1.9</td>
<td>2</td>
</tr>
<tr>
<td>Gly (G)</td>
<td>6.0</td>
<td>6</td>
</tr>
<tr>
<td>Ala (A)</td>
<td>2.0</td>
<td>2</td>
</tr>
<tr>
<td>Cys (C)</td>
<td>5.6</td>
<td>6</td>
</tr>
<tr>
<td>Val (V)</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Ile (I)</td>
<td>2.6</td>
<td>3</td>
</tr>
<tr>
<td>Leu (L)</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>Tyr (Y)</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>Phe (F)</td>
<td>0.9</td>
<td>1</td>
</tr>
<tr>
<td>His (H)</td>
<td>1.1</td>
<td>1</td>
</tr>
<tr>
<td>Lys (K)</td>
<td>4.0</td>
<td>4</td>
</tr>
<tr>
<td>Arg (R)</td>
<td>3.0</td>
<td>3</td>
</tr>
<tr>
<td>Trp (W)</td>
<td>1.0</td>
<td>1</td>
</tr>
<tr>
<td>No aa:s</td>
<td>46</td>
<td>46</td>
</tr>
</tbody>
</table>

Mass, native  
(MH⁺ measured)  
4879.1  
4892.7

Mass, native  
(calculated)  
4878.3  
4892.3

Mass, alkylated  
(MH⁺ measured)  
5221.5  
5236.9

Mass, alkylated  
(calculated)  
5221.4  
5235.4

---

a Half-cystine was determined as cysteic acid, using a separate sample following oxidation with performic acid.  
b Cysteine was determined as carbamidomethyl(cysteine), following alkylation with iodoacetamide.  
c Tryptophan was determined photometrically  
d Measured mass of peptide alkylated by iodoacetamide in excess.  
e Calculated mass of fully C-carbamidomethylated peptide.

Peak 1 is very similar to the reported sequence of phoratoxin A, but peak 1 contains Asp instead of Asn at position 45. Peak 1 has the same sequence as phoratoxin E, which was isolated in the present study. Peak 2 has the same sequence as the previously reported phoratoxin B.

So in conclusion, the reference material of phoratoxin B, isolated by Thunberg (1983), was shown to be a mixture of peptides with the sequences given below in Figure 12 as phoratoxin B (peak 2 of Table 8) and phoratoxin E (peak 1 of Table 8). The reference material of phoratoxin A contained a mixture of peptides very similar to those of the phoratoxin B reference material, according to the MS analysis. The relative amount of peak 1 (phoratoxin E) was higher in the reference material of phoratoxin A than in that of phoratoxin B. Phoratoxin E is probably identical to the previously reported phoratoxin A, since the reference material of phoratoxin A contained the same C-terminal sequence as phoratoxin E. However, this remains unclear since the original reference material of phoratoxin A (Mellstrand and Samuelsson, 1973) is no longer available.
Alignment of Phoratoxins with Other Thionins

For several members of the thionin group, amino acid sequences were aligned (Figure 12). The sequences are currently available in literature (EMBL and SWISS-PROT). The primary structures of the phoratoxins share a high degree of homology with the viscotoxins and the α- and β-thionins.

Figure 12. Alignment of amino acid sequences of the members of the four different types of thionins currently available from literature (i.e., the EMBL, and SWISS-PROT databases). The one-letter code for amino acids is used. The sequences were derived from phoratoxin A and B (Mellstrand and Samuelsson, 1974a; Thunberg, 1983); viscotoxin A1, A2, A3, B and 1-Ps (Olson and Samuelsson, 1972; Orrù et al., 1997; Samuelsson, 1973; Samuelsson and Pettersson, 1971); ligatoxin (Thunberg and Samuelsson, 1982); denclatoxin (Samuelsson and Pettersson, 1977); α1-, α2- and β-purothionin (Jones and Mak, 1976; Mak and Jones, 1976; Ohtani et al., 1975; Ohtani et al., 1977); α- and β-hordothionin (Hernández-Lucas et al., 1986); secalethionin (Békés et al., 1982); and crambin A and B (Teeter et al., 1981; Vermeulen et al., 1987).

The neutrophil challenging activity of phoratoxins

The phoratoxins fractions II, III, and IV from Thunberg, 1983, were tested in the neutrophil elastase release assay. The fraction V showed 48% and 38% inhibition (PAF and fMLP induced, respectively) of the pNA formation at the concentration 100 µg/ml (Table 9). The isolated peptides from this fraction V (phoratoxins B, E, and F) were further evaluated in the neutrophil elastase release assay. Phoratoxin C was included for comparison.
Table 9. Inhibiting activity (%) of the different fractions and phoratoxin B (isolated by Thunberg, 1983) on the pNA formation in the neutrophil elastase release assay at a concentration of 100 µg/ml.

<table>
<thead>
<tr>
<th></th>
<th>PAF±SEM</th>
<th>fMLP±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phoratoxin B</td>
<td>-1.5± 9</td>
<td>8.2± 8</td>
</tr>
<tr>
<td>Fraction II</td>
<td>7.2± 6</td>
<td>-1.7± 3</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>23± 3</td>
<td>22.5± 2</td>
</tr>
<tr>
<td>Fraction V</td>
<td>48± 5</td>
<td>38± 7</td>
</tr>
</tbody>
</table>

The phoratoxins B, C, E, and F were tested in the neutrophil elastase release assay at three different concentrations. Phoratoxin D was not tested due to the limited amount available. In this assay, the phoratoxins showed an enhanced formation of pNA in a concentration-dependent manner as seen in Figure 13. At the highest tested concentration, 20 µM, they all increased the pNA formation in the assay. The initial observed inhibition of the formation of pNA in fraction V may be caused by the presence of several phoratoxins. This excess of “inducers” might influence the formation of pNA by desensitising the receptors, which is often seen for agonists (Venter, 1994), or might in some other way give false indication of inhibition. In an earlier study (Mellstrand, 1974), phoratoxin A did not inhibit the enzyme trypsin; and in this study, the phoratoxins did not inhibit the elastase. Because the closely related viscotoxins affect the exocytosis of neutrophils by inducing the generation of ROS (Stein et al., 1999), the release of elastase from neutrophils was therefore expected.

Parallel samples of PMNs were pretreated with phoratoxins in several concentrations, and then assayed for fMLP induced elastase release. PMNs pretreated with phoratoxins were still able to increase pNA formation after challenge with the inducers PAF or fMLP.

The cytotoxic evaluation of the fractions and the isolated peptides

Cytotoxic activity in the cell line panel

Three fractions II, IV, and V, and the five isolated peptides, phoratoxins B–F, from P. tomentosum, induced a concentration-dependent decrease in cell viability. The IC<sub>50</sub>-values for the three fractions ranged from 0.43 µg/ml to 41.4 µg/ml (Table 10), with fraction II being the least potent (IC<sub>50</sub> ranging from 2.2 to 41.4 µg/ml) and fraction IV (IC<sub>50</sub> ranging from 0.43 to 3.9 µg/ml) being the most potent. From fraction IV, phoratoxins C and D were isolated; and from fraction V, phoratoxins B, E, and F were isolated. The IC<sub>50</sub>-values for these isolated mistletoe toxins (Table 10) ranged from 0.038-0.83 µM where phoratoxin C (mean IC<sub>50</sub>: 0.16 µM),
being the most potent, and phoratoxin F (mean IC50: 0.4 µM), the least. In the small-cell lung cancer cell lines NCI-H69 and NCI H69AR, the phoratoxins were equipotent to each other. The phoratoxins showed high potency towards the cell line NCI-H69, whereas the potency for NCI-H69AR was much lower.

Table 10. IC50 values in the cell line panel for the fractions and the isolated peptides.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Fraction II µg/ml</th>
<th>Fraction IV µg/ml</th>
<th>Fraction V µg/ml</th>
<th>PTX B µM</th>
<th>PTX C µM</th>
<th>PTX D µM</th>
<th>PTX E µM</th>
<th>PTX F µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 8226-S</td>
<td>19.0</td>
<td>1.7</td>
<td>3.6</td>
<td>0.35</td>
<td>0.18</td>
<td>0.27</td>
<td>0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>RPMI 8226-LR5</td>
<td>8.4</td>
<td>1.0</td>
<td>2.2</td>
<td>0.30</td>
<td>0.18</td>
<td>0.27</td>
<td>0.38</td>
<td>0.36</td>
</tr>
<tr>
<td>RPMI 8226-Dox 40</td>
<td>41.4</td>
<td>3.9</td>
<td>7.6</td>
<td>0.37</td>
<td>0.13</td>
<td>0.23</td>
<td>0.43</td>
<td>0.51</td>
</tr>
<tr>
<td>U-937 GTB</td>
<td>9.8</td>
<td>1.4</td>
<td>2.2</td>
<td>0.21</td>
<td>0.092</td>
<td>0.15</td>
<td>0.35</td>
<td>0.31</td>
</tr>
<tr>
<td>U-937 Vcr</td>
<td>8.2</td>
<td>1.0</td>
<td>2.1</td>
<td>0.18</td>
<td>0.10</td>
<td>0.14</td>
<td>0.29</td>
<td>0.23</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>2.2</td>
<td>0.43</td>
<td>1.8</td>
<td>0.038</td>
<td>0.040</td>
<td>0.045</td>
<td>0.038</td>
<td>0.038</td>
</tr>
<tr>
<td>NCI-H69AR</td>
<td>10.9</td>
<td>2.3</td>
<td>2.9</td>
<td>0.23</td>
<td>0.23</td>
<td>0.24</td>
<td>0.26</td>
<td>0.30</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>14.6</td>
<td>2.1</td>
<td>4.6</td>
<td>0.33</td>
<td>0.20</td>
<td>0.38</td>
<td>0.45</td>
<td>0.50</td>
</tr>
<tr>
<td>CEM-VM-1</td>
<td>12.0</td>
<td>2.0</td>
<td>3.1</td>
<td>0.32</td>
<td>0.15</td>
<td>0.22</td>
<td>0.34</td>
<td>0.42</td>
</tr>
<tr>
<td>ACHN</td>
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<td>3.1</td>
<td>7.5</td>
<td>0.59</td>
<td>0.34</td>
<td>0.64</td>
<td>0.58</td>
<td>0.83</td>
</tr>
<tr>
<td>mean</td>
<td>16.8</td>
<td>1.9</td>
<td>3.8</td>
<td>0.29</td>
<td>0.16</td>
<td>0.26</td>
<td>0.36</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Abbreviations: PTX (phoratoxin).

Correlation analysis of the phoratoxins

The fractions and the isolated peptides were tested in the cell line panel and compared with one another (Table 11, part a). Correlations between isolated peptide with isolated peptide were high (0.82-0.99), whereas correlations between fraction and peptide were moderately high (0.55-0.86). The highest correlation was between phoratoxin B and phoratoxin F (0.99), and the lowest correlation was between phoratoxin E and the fraction V, from which it was isolated (0.55). This shows that the correlation between fraction and pure substances is lower when many compounds are present in the fraction. Fraction V, which contained three peptides, was shown to exhibit the lowest correlation to the isolated peptides (0.55-0.69), whereas fraction IV (containing two peptides) was shown to have a slightly higher correlation with the pure peptides (0.73-0.85). This may complicate the predication of a known resistance mechanism during the isolation procedure. However, the pure substance will show if a known resistance mechanism is involved (Dhar et al., 1996). The correlation of the phoratoxins with standard drugs (Table 11, part b) was low (R<0.35). Further, the 10 highest correlations (R) with cytotoxic standard drugs and experimental agents with known targets, previously tested in the cell line panel, were ranked (Table VI, Paper III). Surprisingly, digitoxin gave overall the highest correlation to the phoratoxins.

Resistance factors in the mechanism-based cell line evaluation

The resistance factors (RFs) were calculated for the Pgp, topo II, MRP, GSH, and tubulin resistance mechanisms (Table 12). The isolated phoratoxins were assigned the highest RF values (ranging from 5.3 to 7.8) for the MRP-associated mechanism, whereas the fraction V obtain the lowest value (1.6). The overall low RFs for the other resistance mechanisms examined (<3) indicate minimal dependence on these mechanisms.
Table 11. Correlation coefficients (R), obtained in the cell line panel, for analysing relationships of the log IC$_{50}$ values of the fractions and the isolated phoratoxins (a) among themselves and (b) with standard drugs.

<table>
<thead>
<tr>
<th></th>
<th>PTX B</th>
<th>PTX C</th>
<th>PTX D</th>
<th>PTX E</th>
<th>PTX F</th>
<th>Fraction IV</th>
<th>Fraction V</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTXB</td>
<td>1.00</td>
<td>0.88</td>
<td>0.94</td>
<td>0.97</td>
<td>0.99</td>
<td>0.85</td>
<td>0.69</td>
</tr>
<tr>
<td>PTXC</td>
<td>0.88</td>
<td>1.00</td>
<td>0.96</td>
<td>0.82</td>
<td>0.87</td>
<td>0.73</td>
<td>0.56</td>
</tr>
<tr>
<td>PTXD</td>
<td>0.94</td>
<td>0.96</td>
<td>1.00</td>
<td>0.89</td>
<td>0.94</td>
<td>0.77</td>
<td>0.64</td>
</tr>
<tr>
<td>PTXE</td>
<td>0.97</td>
<td>0.82</td>
<td>0.89</td>
<td>1.00</td>
<td>0.98</td>
<td>0.78</td>
<td>0.55</td>
</tr>
<tr>
<td>PTXF</td>
<td>0.99</td>
<td>0.87</td>
<td>0.94</td>
<td>0.98</td>
<td>1.00</td>
<td>0.85</td>
<td>0.68</td>
</tr>
<tr>
<td>Fraction IV</td>
<td>0.85</td>
<td>0.73</td>
<td>0.77</td>
<td>0.78</td>
<td>0.85</td>
<td>1.00</td>
<td>0.86</td>
</tr>
<tr>
<td>Fraction V</td>
<td>0.69</td>
<td>0.56</td>
<td>0.64</td>
<td>0.55</td>
<td>0.68</td>
<td>0.86</td>
<td>1.00</td>
</tr>
<tr>
<td>b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>0.11</td>
<td>0.34</td>
<td>0.28</td>
<td>-0.02</td>
<td>0.09</td>
<td>0.39</td>
<td>0.54</td>
</tr>
<tr>
<td>Vincristine</td>
<td>-0.26</td>
<td>0.03</td>
<td>-0.10</td>
<td>-0.41</td>
<td>-0.29</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Cytoxarin</td>
<td>-0.07</td>
<td>0.10</td>
<td>0.01</td>
<td>-0.18</td>
<td>-0.12</td>
<td>-0.01</td>
<td>0.20</td>
</tr>
<tr>
<td>Melphalan</td>
<td>-0.01</td>
<td>0.18</td>
<td>0.05</td>
<td>-0.07</td>
<td>-0.05</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Topotecan</td>
<td>0.00</td>
<td>0.32</td>
<td>0.15</td>
<td>-0.14</td>
<td>-0.04</td>
<td>0.20</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Abbreviations: PTX (phoratoxin).

Table 12. Mechanistic resistance factors (RFs) for the fractions and the isolated phoratoxins.

<table>
<thead>
<tr>
<th></th>
<th>Fraction II</th>
<th>Fraction IV</th>
<th>Fraction V</th>
<th>PTX B</th>
<th>PTX C</th>
<th>PTX D</th>
<th>PTX E</th>
<th>PTX F</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-pg</td>
<td>2.2</td>
<td>2.4</td>
<td>2.1</td>
<td>1.1</td>
<td>0.7</td>
<td>0.8</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>GSH-associated</td>
<td>0.4</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Topo II-associated</td>
<td>1.2</td>
<td>1.1</td>
<td>1.5</td>
<td>1.0</td>
<td>1.3</td>
<td>1.8</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Tubulin</td>
<td>0.8</td>
<td>0.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>MRP</td>
<td>4.9</td>
<td>5.4</td>
<td>1.6</td>
<td>6.1</td>
<td>5.7</td>
<td>5.3</td>
<td>7.0</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Resistance factor = (IC$_{50}$ resistant cell line)/ (IC$_{50}$ parental cell line). Abbreviations: PTX (phoratoxin), P-gp (P-glycoprotein), GSH (glutathione), topo II (topoisomerase II), MRP (multidrug-resistance associated protein).

The activity in primary tumour cells from patients

The IC$_{50}$ values obtained in primary tumour cells ranged from 0.58 to 38.2 µg/ml for the fractions (Table 13). As in the cell line panel, fraction IV was the most potent fraction, and fraction II, the least potent.

Table 13. Comparison of the IC$_{50}$-values (µg/ml) of the fractions for tumour cells from patients.

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
<th>CLL</th>
<th>BC</th>
<th>Ratio</th>
<th>CLL/BC</th>
<th>Ratio</th>
<th>PBMC/CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction II µg/ml</td>
<td>25.5</td>
<td>38.2</td>
<td>4.4</td>
<td>8.7</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction IV µg/ml</td>
<td>4.5</td>
<td>4.4</td>
<td>0.58</td>
<td>7.6</td>
<td>1.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction V µg/ml</td>
<td>7.3</td>
<td>4.8</td>
<td>2.2</td>
<td>2.2</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

PBMC (peripheral blood mononuclear cells), CLL (chronic lymphocytic leukaemia), BC (breast carcinoma).

The activity of phoratoxin C was further characterised in 25 human tumour samples, 16 solid and 9 haematological. The IC50-values of phoratoxin C (Table VIII, Paper III) ranged from 87 nM to 2.1 µM. Interestingly, phoratoxin C was assigned the highest activity in the four breast-carcinoma samples with an IC50 of 87 nM. Phoratoxin C was selectively toxic to the solid tumour samples relative to haematological tumour samples, shown in Table VIII by the ratio H/S of the mean IC50 value for the haematological tumours and that mean for the solid tumours.
Isolation and characterisation of substances with cytotoxic and neutrophil elastase releasing activity

Phoratoxin C was pronouncedly selectively toxic to the solid tumour samples from breast carcinoma (BC) relative to the haematological (CLL) tumour samples (CLL/BC=18.4), and selectively toxic to the breast cancer cells relative to the normal peripheral blood mononuclear cells (PBMCs) (PBMC/BC=8.0).

Table 14. Comparison of the IC₅₀-values (µM) of phoratoxin C and D for tumour cells from patients.

<table>
<thead>
<tr>
<th></th>
<th>PBMC</th>
<th>CLL</th>
<th>BC</th>
<th>Ovca</th>
<th>Ratio CLL/BC</th>
<th>Ratio CLL/Ovca</th>
<th>Ratio PBMC/CLL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTX C µM</td>
<td>0.7 (n=4)</td>
<td>1.6 (n=5)</td>
<td>0.087 (n=4)</td>
<td>0.3 (n=5)</td>
<td>18.4</td>
<td>5.3</td>
<td>0.44</td>
</tr>
<tr>
<td>PTX D µM</td>
<td>0.7 (n=2)</td>
<td>0.86 (n=2)</td>
<td>0.36 (n=2)</td>
<td>0.56 (n=3)</td>
<td>2.4</td>
<td>1.5</td>
<td>0.81</td>
</tr>
</tbody>
</table>

Abbreviations: PTX (phoratoxin), PBMC (peripheral blood mononuclear cells), CLL (chronic lymphocytic leukaemia), BC (breast carcinoma), Ovca (ovarian carcinoma).

Phoratoxin D was preliminarily tested against some of the tumour samples (Table 14). Comparison of the IC₅₀s (IC₅₀ values) for phoratoxin C and D shows that they are equipotent against PBMC. For the tumour cells, phoratoxin D is twice as potent as phoratoxins C against CLL, whereas in reverse, phoratoxin C is twice as potent against ovarian carcinoma, and four times as potent against breast carcinoma. Comparing the ratios CLL/BC, CLL/Ovca, and PBMC/CLL, phoratoxin C was more selectivity toxic to solid tumour patient samples than was phoratoxin D. The ratio CLL/BC is 18.4 for phoratoxin C and 2.4 for phoratoxin D. Phoratoxin C and D consist of 46 and 41 amino acid residues. Except for having a longer sequence, the amino acid sequence for phoratoxin D is the same as that for phoratoxin C, all the way through amino acid residue 41. This indicates that the C-terminal sequence is important to the higher selectivity observed for phoratoxin C. However, this should be further investigated.

Comparison with the other 46 amino-acid-residue phoratoxins tested in the cell line panel, phoratoxin C is the only one that differed in the C-terminal sequence by having a Thr at position 45 instead of an Asp. Whether or not the Thr45 was important for the higher selectivity against the solid tumour cells remains unclear. For pharmacological activity, the loop in the amino acid sequence formed by the Cys16–Cys26 bridge is important. By modifying the conserved arginine residues at positions 17 and 23 within this loop, Thunberg and Samuelsson (1983) (Thunberg et al., 1983) showed that these arginines are essential to the toxicity of the phoratoxins. Within this loop, phoratoxin F has the amino acid residues Leu18 and Ala19, whereas phoratoxins B-E have Phe18 and Gly19. In the cell line panel, potencies of the phoratoxins vary only little, but may still vary considerably in selectivity, as seen for phoratoxin D compared to phoratoxin C. However, this also must be further investigated.
Digitoxin and Related Cardiac Glycosides

Cardiac glycosides

Long ago William Withering (1741-1799) recognised the usefulness of foxglove (Digitalis) extracts, particularly for the treatment of congestive heart failure. But, perhaps less well-known and surprisingly, the drug prior to Withering was generally applied by inunction as a plaster or ointment in treating headaches and swellings, as well as cancerous skin conditions (Groves and Bisset, 1991).

Cardenolides and bufadienolides are regarded cardiac glycosides because both increase the contractile force of the heart by inhibiting the enzyme Na+, K+-ATPase. The molecular basis for the increased force of contraction is largely due to an increase in cytosolic Ca\(^{2+}\) during systole, which increases the velocity and extent of muscle shortening. The toxicity associated with cardiac glycosides may result from excessive intracellular Ca\(^{2+}\) that causes a transient late depolarisation followed by an after contraction. Both cardenolides and bufadienolides have previously been tested for cytotoxicity in a variety of tumour models (Cassady et al., 1981; Hembree et al., 1979; Inada et al., 1993; Kelly et al., 1965; Koike et al., 1980; Repke et al., 1995; Shiratori, 1967).

A bioassay guided isolation of digitoxin from *Digitalis purpurea*

Examination of the fractionated plant extracts of *Digitalis lanata*, *Digitalis purpurea*, and *Viola patrinii* (Figure 7, Paper II) revealed an unusual and unexpected effect of increased formation of pNA, as mentioned in chapter 6 and described in Paper II. For further investigation of this effect, *D. purpurea* was chosen due to the limited amount of *V. patrinii* and *D. lanata* available. The fraction P of *D. purpurea* was dissolved in a mobile phase of aqueous 25% CH\(_3\)CN and 0.1% TFA, and then was fractionated on a semipreparative HPLC system as described in Paper IV. The column was eluted for 30 minutes using a linear gradient from 25% CH\(_3\)CN/0.1% TFA to 75% CH\(_3\)CN/0.1% TFA. Ten fractions were collected and tested in eight concentrations in the neutrophil elastase release assay. Two of the fractions, F6 and F8, were capable of enhancing the formation of pNA, as seen in Figure 14, which also highlights the importance of testing in several concentrations in the bioassay-guided isolation procedure. At the highest tested concentration (100 µg/ml), F6 was the most active compound. Testing at several concentrations, F8 was the most potent fraction, and was then further purified by HPLC. The most potent fraction, which was homogeneous, was unambiguously identified as the cardiac glycoside digitoxin, using spectroscopic methods. The \(^1\text{H}\) NMR spectrum (recorded at 600 MHz) and mass spectrum (obtained by the Electro Spray Ionization technique) were in agreement with spectrums of an authentic sample of digitoxin (Sigma).

The neutrophil challenging activity of digitoxin

Digitoxin’s concentration-response for enhancement of pNA formation was bell shaped (Figure 15). In the assay, the maximal pNA-formation enhancement by isolated digitoxin was lower than that of the inducers PAF, fMLP, and the original extract of *D. purpurea*. The substantial enhancement by the fraction P was possibly a synergistic effect involving more than one cardiac glycoside.
Isolation and characterisation of substances with cytotoxic and neutrophil elastase releasing activity

Figure 14. The fraction P of *Digitalis purpurea* was separated into ten fractions F1-F10 on a RP-column. Each fraction was tested for activity in several concentrations of neutrophil elastase release. Of these ten fractions, two showed a dose-dependent activity, F6 (■) and F8 (○). Fraction F8 was shown to be the most potent fraction in the elastase release. Digitoxin, the most potent compound in the elastase release, was later isolated from F8.

Figure 15. Amount of pNA formation from neutrophils treated with several concentrations of the isolated digitoxin. Each point is the mean absorbance value ± SEM of three independent experiments.

The cytotoxic evaluation of digitoxin and related compounds

Cytotoxic activity in the cell line panel

The fractions P of *D. lanata* and *D. purpurea* were tested in the cell line panel. The IC_{50} values for these fractions ranged from 1.1 to 5.8 µg/ml (*D. lanata*) and from 0.63 to 1.5 µg/ml (*D. purpurea*). *D. purpurea* was further separated into two neutrophil challenging fractions, F6 and F8, which were also tested in the cell line panel. The potencies for these fractions were higher than the potency of fraction P. The IC_{50} values for the fraction F6 ranged from 0.20 to 1.4 g/ml; and for fraction F8, from 0.20 to 0.81 µg/ml. Digitoxin was isolated from the fraction F8.

The unexpected finding that digitoxin expressed potent cytotoxicity prompted us to investigate the structure-activity relationship of cardiac glycosides. For this, the cytotoxicities of digitoxin and six related compounds were compared. All seven of the related compounds (Figure 1, Paper IV)—five cardenolides (digoxin, digitoxin, lanatoside C, ouabain and digitoxigenin), one bufadienolide (proscillaridin A), and one saponin (digitonin)—induced a dose-dependent decrease in cell viability. The IC_{50} values for these compounds (Table 15) ranged from 6.4 to 7000 nM, with digitonin, (IC_{50} ranging from 1070 to 7000 nM; Table 15) being the least potent, and proscillaridin A (IC_{50} values ranging from 6.4 to 76 nM), the most potent. These results agree with literature data showing that the cytotoxic activity of cardenolides is generally weaker than the corresponding bufadienolides having the same steroidal skeletons (Kamano et al., 1998).
Isolation and characterisation of substances with cytotoxic and neutrophil elastase releasing activity

Table 15. IC<sub>50</sub>-values (nM) of cardiac glycosides and digitonin for the human tumour cell line panel.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Digitoxin</th>
<th>Digitoxigenin</th>
<th>Digoxin</th>
<th>Lanatoside C</th>
<th>Ouabain</th>
<th>Proscillaridin A</th>
<th>Digitonin</th>
<th>mean IC&lt;sub&gt;50&lt;/sub&gt; cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI 8226-S</td>
<td>34</td>
<td>242</td>
<td>84</td>
<td>220</td>
<td>73</td>
<td>13</td>
<td>1280</td>
<td>278</td>
</tr>
<tr>
<td>RPMI 8226-LR5</td>
<td>25</td>
<td>201</td>
<td>64</td>
<td>150</td>
<td>57</td>
<td>15</td>
<td>1310</td>
<td>260</td>
</tr>
<tr>
<td>RPMI8226-Dox 40</td>
<td>59</td>
<td>395</td>
<td>172</td>
<td>339</td>
<td>148</td>
<td>20</td>
<td>1070</td>
<td>315</td>
</tr>
<tr>
<td>U-937 GTB</td>
<td>32</td>
<td>251</td>
<td>68</td>
<td>142</td>
<td>66</td>
<td>6.4</td>
<td>1880</td>
<td>349</td>
</tr>
<tr>
<td>U-937 Vcr</td>
<td>36</td>
<td>344</td>
<td>74</td>
<td>133</td>
<td>63</td>
<td>6.4</td>
<td>1100</td>
<td>251</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>12</td>
<td>635</td>
<td>56</td>
<td>137</td>
<td>70</td>
<td>66</td>
<td>2600</td>
<td>511</td>
</tr>
<tr>
<td>NCI-H69AR</td>
<td>31</td>
<td>200</td>
<td>41</td>
<td>154</td>
<td>45</td>
<td>10</td>
<td>4730</td>
<td>744</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>25</td>
<td>283</td>
<td>49</td>
<td>127</td>
<td>57</td>
<td>&lt;6.4*</td>
<td>2650</td>
<td>457</td>
</tr>
<tr>
<td>CCRF-VM-1</td>
<td>40</td>
<td>441</td>
<td>63</td>
<td>190</td>
<td>74</td>
<td>9</td>
<td>4430</td>
<td>750</td>
</tr>
<tr>
<td>ACHN</td>
<td>76</td>
<td>658</td>
<td>125</td>
<td>602</td>
<td>126</td>
<td>76</td>
<td>7000</td>
<td>1237</td>
</tr>
</tbody>
</table>

mean IC<sub>50</sub> compounds: 37 365 80 219 78 23 2805

*Since the IC<sub>50</sub> value is below the tested concentrations, the lowest tested concentration is used (Boyd and Paull, 1995). Each IC<sub>50</sub> value is calculated from the mean of 2-3 experiments, all performed in triplicate.

In most cases, the order of potency (high to low) of these substances on the cell line panel was proscillaridin A, digitoxin, ouabain, digoxin, lanatoside C, digitoxigenin, and digitonin. An exception to this was observed in the cell line NCI-H69, where digitoxin was more potent than proscillaridin A. In the renal adenocarcinoma cell line ACHN, digitoxin was equipotent with proscillaridin A. Also, in the cell lines NCI-H69, CCRF-CEM, and its sublines H69AR and CEM-VM-1, digoxin showed a lower IC<sub>50</sub> value than that of ouabain. The order of cytotoxic potency of the cardiac glycosides found in this study virtually parallels the inhibitory potency of the cardiac glycosides on the Na<sup>+</sup>/K<sup>+</sup>- transporting ATPase from human cardiac muscle from data in the literature (Schönfeld et al., 1986). The cytotoxic concentrations are higher than the concentrations required to inhibit Na<sup>+</sup>/K<sup>+</sup>-ATPase activity in human cardiac muscle; but for digitoxin, the average IC<sub>50</sub> (37 nM; Table 15) is still within the therapeutic concentration used for cardiac congestion (13-45 nM) (Gilman et al., 1985). For the bufadienolide proscillaridin A, which was found to be of even higher potency (average IC<sub>50</sub> 23 nM), the cytotoxic concentration is not within the range of therapeutic plasma concentration (0.9-1.1 nM) used for this drug in the treatment of heart congestion.

Correlation analysis of the cardiac glycosides

The correlation analysis of the seven natural compounds with one another (Table 3, part a, Paper IV) showed that the correlation of digitoxin with digoxin, ouabain, and lanatoside C was moderately high, whereas correlation with digitoxigenin, proscillaridin A, and digitonin was much lower. The highest correlation was between digoxin and ouabain (0.946). The correlation of those natural compounds with standard drugs (Table 3, part b, Paper IV) was very low to moderately high (–0.3 to 0.7). Digitoxin and digoxin were less correlated with standard drugs than proscillaridin A. The low correlations to standard drugs in our database (Dhar et al., 1996) support the idea that the cardiac glycosides may act by cytotoxic mechanisms other than those of standard drugs.

Resistance factors of the cardiac glycosides in the mechanism-based cell line evaluation

The RFs were calculated for the P-gp, topo II, MRP, GSH, and tubulin resistance mechanisms (Table 4, Paper IV). The overall low RFs for the substances (<3)
indicate minimal dependence on the resistance mechanisms examined here. Proscillaridin A was more active against the MRP-expressing resistant subline NCI-H69AR than to its parental cell line NCI-H69 (RF=0.15).

*The structure-activity relationship for the digitoxin and related cardiac glycosides*

The structure activity relationship was investigated for digitoxin and the related cardiac glycosides. Digitoxigenin is the aglycone of digitoxin. This structural difference caused marked differences in the cytotoxicity patterns of digitoxin and digitoxigenin. Virtually no significant correlations were found between the activity profiles of these two compounds. Moreover, for the cell line panel tests, digitoxin’s additional three digitoxose sugar units increased its mean potency 6.5 to 50 times compared to that of digitoxigenin, suggesting that an intact glycoside is essential for potent cytotoxic activity. The correlation of digitoxin with other intact cardenolides (i.e., digoxin, ouabain, and lanatoside C) was moderately high, which may indicate (as might be expected) a similar mode of action for these compounds. The highest correlation occurred for digoxin and ouabain. Only low correlation of the activity patterns of digitoxin with that of proscillaridin A, digitoxigenin, and digitonin was found.

Lanatoside C and digoxin are structurally closely related in that digoxin can be obtained from lanatoside C by hydrolytic removal of the acetyl and glucose moieties. This structural difference in the sugar part of the compounds was manifested in a three–times-higher mean potency of digoxin compared to lanatoside C, approximately.

*The activity of the cardiac glycosides in primary tumour cells from patients*

The cardiac glycosides were tested against tumour cells from patients. The IC\(_{50}\) values obtained in primary tumour cells ranged from 17 to 7050 nM (Table 16). As in the cell line panel, proscillaridin A was the most potent compound, and digitonin, the least potent. The ratios between the haematological and the solid tumour samples (CLL/Ovca; CLL/BC) were calculated (Table 16). In most cases, the solid tumour samples (Ovca, BC) were more sensitive than the haematological (CLL) sample, which in turn was, in several cases, more sensitive than PBMC, its normal counterpart. The solid tumour toxicity was most pronounced for digitoxin and digoxin, while proscillaridin A, lanatoside C, and digitoxigenin showed no selectivity for haematological or solid tumour samples.

**Table 16.** Comparison of the IC\(_{50}\) values (nM) of cardiac glycosides and digitonin for tumour cells from patients.

<table>
<thead>
<tr>
<th>Patient samples</th>
<th>Digitoxin</th>
<th>Digitoxigenin</th>
<th>Digoxin</th>
<th>Lanatoside C</th>
<th>Ouabain</th>
<th>Proscillaridin A</th>
<th>Digitonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC (n=3)</td>
<td>106</td>
<td>1000</td>
<td>327</td>
<td>740</td>
<td>277</td>
<td>26</td>
<td>4640</td>
</tr>
<tr>
<td>Ovca (n=2)</td>
<td>55</td>
<td>339</td>
<td>232</td>
<td>335</td>
<td>229</td>
<td>17</td>
<td>2637</td>
</tr>
<tr>
<td>BC (n=2)</td>
<td>65</td>
<td>489</td>
<td>127</td>
<td>316</td>
<td>147</td>
<td>21</td>
<td>4786</td>
</tr>
<tr>
<td>CLL (n=3)</td>
<td>150</td>
<td>527</td>
<td>520</td>
<td>424</td>
<td>358</td>
<td>18</td>
<td>7050</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ratio</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMC/CLL</td>
<td>0.71</td>
<td>1.90</td>
<td>0.63</td>
<td>1.75</td>
<td>0.77</td>
<td>1.44</td>
<td>0.66</td>
</tr>
<tr>
<td>CLL/Ovca</td>
<td>2.73</td>
<td>1.55</td>
<td>2.24</td>
<td>1.27</td>
<td>1.56</td>
<td>1.06</td>
<td>2.67</td>
</tr>
<tr>
<td>CLL/BC</td>
<td>2.31</td>
<td>1.08</td>
<td>4.09</td>
<td>1.34</td>
<td>2.44</td>
<td>0.86</td>
<td>1.47</td>
</tr>
</tbody>
</table>

PBMC (peripheral blood mononuclear cells), Ovca (ovarian carcinoma), BC (breast carcinoma), CLL (chronic lymphocytic leukaemia). Each IC\(_{50}\) value is calculated from the mean of 2-3 experiments, all performed in triplicate.

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Early epidemiological studies show that congestive heart-failure patients who receive cardiac glycosides have a tendency to develop breast tumours of lower growth potential than untreated patients (Stenkvist et al., 1980; Stenkvist et al., 1979). The observed activity is proposed to be associated with an oestrogen-like effect (Stenkvist, 1999; Stenkvist et al., 1982; Stenkvist et al., 1980; Stenkvist et al., 1979) of cardiac glycosides. This hypothesis conflicts with our result in which cardiac glycosides inhibited the growth not only of the cells of breast carcinoma, but of all tumour cells tested (Table 15 and 16). Other papers also report (Cove and Barker, 1979; Falconer et al., 1983) that it seems unlikely that oestrogen-like properties of digoxin are of major importance in the growth suppression of breast tumours. Digitoxin has also recently been shown to induce apoptosis (Haux et al., 1999a), and to inhibit prostate (Haux et al., 2000) and breast cancer cell lines in therapeutic concentrations used in the treatment of heart congestion (Haux et al., 1999a). Recent data on the potential of cardiac glycosides as anticancer agents (Haux, 1999; Haux et al., 1999a; Haux et al., 1999b; Haux et al., 2000; Stenkvist, 1999) show that digitoxin inhibits proliferation and induces apoptosis in various cell lines. In another recent study, the cardiac glycosides digoxin, ouabain, and oleandrin were shown to induce apoptosis in metastatic human prostate adenocarcinoma cells, the effect of which was linked to their abilities to induce sustained Ca\textsuperscript{2+} increases in the cells (McConkey et al., 2000).
8. Summary and Concluding Discussion

The fractionation protocol
When this study was initiated, the overall aim was to develop methods for isolation and characterisation of biologically active polypeptides from plant material. The fractionation protocol was developed to detect biologically active polypeptides present in small amounts in plants, and was validated by the isolation of the macrocyclic varv peptide A. Today, we have isolated varv peptides B-H from Viola arvensis (Göransson et al., 1999), and recently Craik et al., (1999) reported the discovery of several peptides from Viola odorata, Viola hederaceae, and Oldenlandia affinis. There is approximately 40% sequence conservation throughout the known sequences.

The bioassay-guided isolation of the cardiac glycoside digitoxin (765 Da) showed that some modifications of the fractionation protocol would be advantageous. Further complementary analysis (e.g., LC-MS) and chromatographic mechanisms (e.g., Sephadex LH-20, cation exchange chromatography) may be included in the isolation of peptides to avoid the isolation of other high molecular weight substances (>700 Da) with both polar and lipophilic characters that are able to elute in the void volume in the gel filtration step with Sephadex G-10.

A multitarget bioassay on the neutrophil
The utility of a neutrophil multitarget functional bioassay may provide important hints on how substances from plants interact with the neutrophil. From the assay, it is possible to make several predictions. First, an inhibition at the receptor level will be by different biological responses in the elastase release, if different inducers are used. In that case, receptor inhibition may be worth investigating. Second, if non-selective inhibition is observed, enzyme inhibition can be investigated. Last, if a stimulating activity is observed, receptor binding studies or functional pharmacological experiments with various known receptor binding antagonists can be performed. Thus, a multitarget bioassay might provide opportunities to find new agents, with known as well as new mechanisms of action, involved in neutrophil interaction.

Biological activity linked to phylogenetic information
Although screening of natural products remains a major method of discovering new drugs, newer techniques of rational drug design, computer-aided drug design, and combinatorial synthesis promise to broaden the scope of compounds available for screening. To address this, the results were presented in a manner that links recorded biological activity to phylogenetic information. With increased acceptance that the distribution of chemical substances and substance classes in plants is not purely random, a logical consequence is that, in order to efficiently explore the chemical diversity of plants, one must also explore the biological and evolutionary diversity. Furthermore, the results will be useful if sampling is expanded, in order, for example, to select groups of plants with comparably good potential for the discovery of additional active substances (e.g., macrocyclic peptides), or to select groups of plants that have not been fully investigated.
The activity of fraction P

There has been growing interest in isolation and characterisation of properties of leukocyte elastase inhibitors because the inhibitors may be used as therapeutic agents to prevent chronic inflammatory diseases (Barnes, 1999). Plants are known to produce protease inhibitors in their defence against pathogens, and may therefore serve as an important source of protease inhibitors. In this study, the fractionation protocol was applied to 100 plant materials, which were tested in a neutrophil multitarget functional bioassay. Of these 100 polypeptide fractions (fraction P), 41% showed more than 60% inhibition, and most of them were shown to be a form of inhibition on the enzyme elastase.

The fraction P of *Crambe maritima* (Brassicaceae), *Momordica charantia* (Curcurbitaceae), and *Viscum heyneanum* (Santalaceae) showed high activity in the neutrophil elastase release assay. *M. charantia* and *V. heyneanum* also highly inhibited isolated elastase, 85% and 99% respectively. These polypeptide fractions most likely contain peptides. For example, the thionin crambin has been isolated from another species in Brassicaceae, *Crambe abyssinica*. The trypsin inhibitors (MCTI) and elastase inhibitors (MCEI) have been isolated from *M. charantia* (Hamato et al., 1995; Hara et al., 1989). Recently, two new macrocyclic peptides derived from *Momordica cochinchinensis* have been reported (Felizmenio-Quimio et al., 2001; Hernandez et al., 2000), MCoTI-I and MCoTI-II, which are trypsin inhibitors comprising 34 amino acids. This suggests that, besides linear peptides, macrocyclic peptides are also common in plants. The extensive data set for the neutrophil elastase release assay, and especially for inhibition of isolated elastase, shows that plants may be an important source of new protease inhibitors.

Further characterisation of phoratoxins and digitoxin, biological and chemical

The mistletoes are known to produce peptides. In the neutrophil elastase release assay, *Viscum heyneanum* and *Phoradendron tomentosum* were tested, and shown to inhibit the formation of pNA. In previous research within this department, mistletoe peptides have been isolated, including isolation of phoratoxins A and B from *Phoradendron tomentosum*. In this study we reinforced and extended this previous work (Lecomte et al., 1987; Mellstrand, 1974a; Mellstrand, 1974b; Mellstrand and Samuelsson, 1973; Mellstrand and Samuelsson, 1974a; Mellstrand and Samuelsson, 1974b; Rosell and Samuelsson, 1966; Sauviat, 1990; Thunberg et al., 1983) on peptides from *Phoradendron tomentosum*. Methods for isolation of peptides have much improved during the past years. In this study, the structurally closely related phoratoxins, in some cases only differing in one amino acid, were isolated. The isolated phoratoxins B-F were all basic, containing 6 cysteine residues; and except phoratoxin D, all were shown to have 46 amino acids. Phoratoxin D, which had 41 amino acids, might be an artifact or a truncated peptide.

Cytotoxic and neutrophil challenging activity

Serendipitous discoveries have always played an important role in science, especially in the search for new drugs or new targets (Kaul, 1998; Kubinyi, 1999; Mueller and Scheidt, 1994), and recently played a role in the following discoveries. By bioassay-guided fractionation, the cardiac glycoside digitoxin was isolated as the most potent compound, both in cytotoxicity and in neutrophil
challenging activity. Digitoxin was shown to be cytotoxic in therapeutic concentrations used for cardiac congestion (13-45 nM) (Gilman et al., 1985). Phoratoxins are also known to be cardiac active (Rosell and Samuelsson, 1966), and excessive intracellular Ca\(^{2+}\) is known to have a potential role for both cardiac glycosides and thionins cytotoxicity (Beeler, 1977; Evans et al., 1989). Interestingly, when a database of drug response patterns of more than 100 additional investigational agents was searched for similarities with the phoratoxins, digitoxin, had the highest correlation coefficients. Notably, in this respect, the unusual stimulatory effects of phoratoxins on granulocyte exocytosis are found as well for the cardiac glycoside digitoxin. Both digitoxin and the phoratin C were here shown to act selectively against solid breast carcinoma cells from patients, by ratios 2.3 and 18, respectively, and thus to be less toxic to the normal peripheral blood mononuclear cells (PBMCs) than to the breast cancer cells.

The discussion above raises the question of whether the observed effect on the neutrophil could be a cytotoxic activity. Necrotic neutrophils release elastase extracellularly while apoptotic cells retain the toxic products within the granules. Further, neutrophils undergoing cell death are incapable of responding to further stimuli (Savill, 1997; Savill et al., 1993; Savill and Haslett, 1995). However, in this study, neutrophils pretreated with digitoxin or phoratoxins were shown to be still capable of responding when the inducer fMLP was added. In contrast, the neutrophils pretreated instead with digitonin, which is known to lyse membranes, were shown to be unable to respond to the added inducers fMLP, suggesting that cardiac glycosides possess a mechanism of action other than just the cell-membrane lysing effect of digitonin. In addition, there is a large difference in the incubation time between the neutrophil elastase release assay (15 min) and the cytotoxic assay (72 hour).

As mentioned above, phoratin C (Paper III) and digitoxin (Paper IV) were shown to act selectively against solid breast carcinoma. This observation is encouraging, since drugs with high solid tumour activity are rare (Fridborg et al., 1996; Larsson et al., 1994; Nygren et al., 1994). The main reason for the failure of cytotoxic therapies is their insufficient selectivity for tumours. For example, treatments with radiation or alkylating agents perturb many functions that are common to all cells. The more selective cytotoxic drugs, for instance, methotrexate, taxol, and etoposide, perturb the functions of specific macromolecular targets (dihydrofolate reductase, microtubules, and topoisomerase II), but these targets are present in both normal and malignant cells (Gilman et al., 1985). In our cytotoxic assay, established chemotherapeutic agents are generally more active against haematological compared to solid tumours (Csoka et al., 1995a; Csoka et al., 1995b; Dhar et al., 1998; Nygren et al., 1994). Most standard and experimental agents show a ratio well below unity. For example, one FMCA-based study with eighteen clinically used cytotoxic drugs showed that among those drugs, only cisplatinum reached a ratio >1 (approx 1.3) of responders, solid relative to haematological tumors in vitro (Fridborg et al., 1999). Furthermore, this estimate was shown to positively correlate with solid tumour activity. The observed selectivity ratios, far above 1 for digitoxin and phoratin C, are notable. If this antitumour activity can be translated into the in vivo situation, these compounds may serve as lead prototypes for developing new classes of anticancer agents with improved activity against solid tumour malignancies.
9. Concluding Remarks

The recent field of innate immunity shows that nature, despite its enormous diversity of species, very often uses the same basic principles for solving related problems in defence against micro-organisms. Certainly, plants and mammals are organised very differently for carrying out their essential functions, but many similarities exist in the way they resist attacks by micro-organisms.

A variety of bioactive secondary compounds, as well as defensive proteins, are usually considered to be a plant’s front line defence against all but the most specialised herbivores and pathogens. These products, which are directly toxic to the consumer, serve as antitherbivore or antimicrobial defence compounds. These substances include the cardiac glycosides, saponins, protease inhibitors, cyclic peptides, and thionins (Howe and Westley, 1988; Ryan et al., 1993; Ryan, 2000; Tam et al., 1999; Wink et al., 1993). In human, the neutrophils are considered to be the front line in defence against the micro-organisms by releasing different toxic products. In this thesis, a large number of plant extracts, including those of Digitalis purpurea and Phoradendron tomentosum, are shown to interact with the neutrophil.

Digitoxin and the phoratoxins induced the exocytotic release of elastase from the neutrophils, and most likely the release of other antimicrobial substances as well. Beside the exocytosis inducing effect, these natural products have been shown to have cytotoxic action on cancer cells. The cytotoxic mechanism of the isolated neutrophil stimulating substances in this study is unclear.

Digitoxin is cytotoxic in vitro at a therapeutic plasma concentration used for cardiac congestion, a concentration that seems to produce no bad side effects in persons without cardiac disease (Grossmann et al., 1998). For the final answer as to whether digitoxin has a place in medical oncology, clinical studies must be done. Because the safety profile of the drug is well known, the threshold for implementing new evaluations of the drug should be low.

Future perspectives
This thesis has shown that substances involved in plant defence may be potentially useful in the development of new drugs in the treatment of inflammatory diseases and cancer. To conclude this thesis, I propose as future studies the following:

• to further fractionate plant extracts, which were shown to have high inhibition on the neutrophil elastase release assay (This would probably results in isolation of protease inhibitors. However, some modifications in the isolation procedure may be necessary.)

• to further investigate the defensins in the neutrophils (The preliminary result indicates a large number of peptides in the neutrophils, and so far only four human neutrophil defensins have been reported in the literature.)
• to further investigate the fraction P of Viola patrinii, which belongs to the family Violaceae that has been shown to contain a large number of macrocyclic peptides (The fraction P of Viola patrinii was shown to induce the release of elastase.)

• to investigate the mechanism behind the neutrophil challenging activity of phoratoxins and digitoxin

• to investigate if there is a relationship between the neutrophil elastase releasing properties and the cytotoxic activity

• to further investigate the mechanism of action for phoratoxins cytotoxic activity, and to investigate if the phoratoxins are capable of inducing the release of defensins from neutrophils, and whether this release differs from fMLP or PAF-induced exocytosis

• to further investigate the clinical potential of phoratoxins

• to investigate digitoxin in clinical studies on patients with breast cancer
10. Acknowledgement

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Stockholm, August, 2001
11. References


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**Appendix**

**Abbreviations used in this thesis**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>BAPNA</td>
<td>$N$-benzoyl-DL-arginine p-nitroanilide</td>
</tr>
<tr>
<td>BC</td>
<td>Breast carcinoma</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>Dox</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>fMLP</td>
<td>N-formyl methionyl-leucyl-phenylalanine</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Concentration that gives 50% inhibition</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
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<tr>
<td>LTB$_4$</td>
<td>Leukotriene B$_4$</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
</tr>
<tr>
<td>Mel</td>
<td>Melphalan</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>Ovca</td>
<td>Ovarian carcinoma</td>
</tr>
<tr>
<td>PAF</td>
<td>Platelet activating factor</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear neutrophils</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroanilide</td>
</tr>
<tr>
<td>R</td>
<td>Correlation</td>
</tr>
<tr>
<td>RF</td>
<td>Resistance factor</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>SAAVNA</td>
<td>N-succinyl-L-alanyl-L-alanyl-L-valine-L-p-nitroanilide</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SI</td>
<td>Survival index</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>Topo II</td>
<td>Topoisomerase II</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet spectrometry</td>
</tr>
<tr>
<td>Vcr</td>
<td>Vincristine</td>
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