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EVALUATION OF NATURAL PRODUCTS IN APOPTOSIS, PROTEIN KINASE C ACTIVATION AND CACO-2 CELL PERMEABILITY

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ACADEMIC DISSERTATION

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Abstract

Nature provides a great diversity of different kinds of health-promoting compounds and is an important source of new biologically active molecules for drug discovery. These compounds may help to cure or alleviate serious diseases, among them cancer. In addition various polyphenolic compounds, affecting wellbeing, are gained from daily nutrition and from concentrated health promoting products. The discovery of new molecules and determination of the effects of natural compounds require the development of new, high-capacity assays. In the studies that comprise this thesis, methods for evaluating drug effects on clinically important targets (apoptosis and protein kinase C), were developed, the intestinal absorption of various natural compounds was evaluated, and an accelerated method for enhancing these laborious studies was devised.

The apoptosis assay for screening new potential drug compounds was optimized with natural compounds and then used to study the apoptosis-inducing effects of derivatives of 5-(hydroxymethyl)isophthalic acid. Two of the compounds seemed to be potent and selective apoptosis inducers and should be studied further for their possible use in cancer therapy. An automated protein kinase C (PKC) assay was developed and used to screen the PKC inhibiting effects of extracts from plants growing in Finland. Twenty-one extracts from 7 plants significantly inhibited PKC. The inhibition was usually induced by all parts of the plants. Fractionation of an active extract showed that the method is suitable for identifying compounds from natural product extracts and provides a quick and low-volume non-radioactive alternative to PKC experiments.

To make permeation studies more effective and more suitable for screening purposes, an automated 7-day, 96-well plate Caco-2 permeability model was developed to provide preliminary information on the permeability of pharmacologically active compounds. The results showed that proper monolayers were formed and that the permeation of the model compounds was comparable to that in the traditionally used method. Automation of the protocol made it more accurate and suitable for screening purposes.

The absorption studies of flavonoids and alkyl gallates showed that the degree of hydroxylation, molecular configuration and the length of the side chain governed the ability of the molecules to cross the Caco-2 cell monolayer and to be retained on the phospholipid membrane. As the number of OH-groups in the structure or the number of carbons in alkyl chain increased the permeability decreased and the membrane affinity increased. A glycosidic flavonoid could not permeate the Caco-2 membrane at all, while the aglyconic form of the same molecule could. The permeation study on coumarins using the new Caco-2 permeability model showed that the compounds permeated the monolayer rapidly and more in the apical to basolateral direction than the basolateral to apical direction. The results suggest that all the coumarins studied have high passive permeability in the gut lumen and that P-glycoprotein efflux does not limit absorption. The type and position of substituents affected permeability more than the number of substituents.

In order to evaluate the effect of concentrated natural products on concomitant medication, the influence of raspberry extract and its fractions on the permeability of commonly used drugs was studied with the Caco-2 cell line. Raspberry samples affected the permeability of some model drugs, probably by interactions with the cell membranes. Raspberry extract and ellagitannin fraction had very similar effects on the permeation of the model drugs studied, whereas the effects of anthocyanin fraction were smaller and opposite.

The findings of this thesis show that nature is an excellent source for the discovery of new drugs when effective methods are used. Such methods make the initial screening of natural products fast and straightforward, even from complex matrices.

List of original publications

This thesis is based on the following publications:

I Galkin, A., Surakka, A., Boije af Gennäs, G., Ruotsalainen, T., Kreander, K., Tammela, P., Sivonen, K., Yli-Kauhaluoma, J., Vuorela, P. (2008). Hydrophobic derivatives of 5-(hydroxymethyl)isophthalic acid that selectively induce apoptosis in leukemia cells but not in fibroblasts. *Drug Development Research* 69:185-195.

II Galkin, A., Jokela, J., Wahlsten, M., Tammela, P., Sivonen, K., Vuorela, P. (2008). Discovering protein kinase C active plants growing in Finland utilizing automated bioassay combined to LC/MS. *Natural Product Communications*, Submitted.

III Galkin, A., Pakkanen, J., Vuorela, P. (2008). Development of an automated 7-day 96-well Caco-2 cell culture model. *Die Pharmazie* 63:464-469.

IV Tammela, P., Laitinen, L., Galkin, A., Wennberg, T., Heczko, R., Vuorela, H., Slotte, P., Vuorela, P. (2004). Permeability characteristics and membrane affinity of flavonoids and alkyl gallates in Caco-2 cells and in phospholipid vesicles. *Archives of Biochemistry and Biophysics* 425:193-199.

V Galkin, A., Fallarero, A., Vuorela, P. (2008). Coumarins permeability in Caco-2 cell model. *Journal of Pharmacy and Pharmacology,* Accepted.

VI Kreander, K., Galkin, A., Vuorela, S., Tammela, P., Laitinen, L., Heinonen, M., Vuorela, P. (2006). In vitro mutagenic potential and effect on permeability of co-administered drugs across Caco-2 cell monolayers of *Rubus idaeus* and its fortified fractions. *Journal of Pharmacy and Pharmacology* 58:1545-1552.

The publications are referred to in the text by their roman numerals.

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Abbreviations

ADME	absorption, distribution, metabolism, excretion
Apaf-1	apoptosis protease activating factor
AP-BL	apical to basolateral
ATCC	American Type Culture Collection
BCRP	breast cancer resistance protein
BL-AP	basolateral to apical
Ca^{2+}	calcium ion
Caco-2	human colon adenocarcinoma cell line
Caspases	family of cysteine proteases
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
$\Delta\psi m$	mitochondrial membrane potential
ER	efflux ratio
F	flower
FasL	Fas ligand
FDA	US Food and Drug Administration
FLIP	FLICE inhibitory protein
HBSS	Hank's balanced salt solution
HL-60	human promyelocytic leukemia cell line
HOECHST 33342	2'-[4-ethoxyphenyl]-5'-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole DNA binding stain
HPLC	high-performance liquid chromatography
HTS	high-throughput screening
IAP	inhibitor of apoptosis proteins
IC_{50}	concentration yielding 50% inhibition
JC-1	5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propen-1-yl]-1,3-diethyl-iodide stain to evaluete mitochondrial membrane poptential
K_d	partition coefficient
L	leaf
LC/MS	liquid chromatography coupled with mass spectrometry
LDH	lactate dehydrogenase
LY	Lucifer Yellow
MCT	monocarboxylic acid transporter
MDR1	multidrug resistance protein 1 = P-gp
MRP	multidrug resistance associated protein
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide stain used to detect mitochondrial function
MS	mass spectrometry
NF-κB	nuclear factor κB
NMR	nuclear magnetic resonance spectrometry
NO	nitric oxide

OATP	organic anion transporter polypeptide
OCT	organic cation transporter
P_{app}	apparent permeability coefficient
PEPT	oligopeptide transporter
P-gp	P-glycoprotein = MDR1
PKC	protein kinase C
POPC	1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine membrane forming agent
PS	phosphatidylserine
R	root
S	stem
TEER	transepithelial electrical resistance
TNF α	Tumor necrosis factor α
TNFR-1	Tumor necrosis factor receptor 1
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate stain used to detect mitochondrial function

1. INTRODUCTION

The extensive chemical diversity of nature provides models and ideas for modern drug design (Strohl 2000, Gullo et al. 2006). Naturally occurring compounds consist of structures selected by evolutionary processes to interact with a wide variety of proteins and other biological targets for specific purposes (Koehn and Carter 2005). Although natural products are not developed to bind to human proteins, many human proteins consist of the same building blocks and contain similar structural domains to the targets for which natural products have evolved (Zhang and Wilkinson 2007). Many potential molecules are still yet to be found, since natural product resources are largely unexplored (Lam 2007). Natural compounds do not only serve new therapeutic molecules or templates for new molecules, but they can also lead to the discovery and improved understanding of the targets and pathways involved in disease processes (Tulp and Bohlin 2005).

However, natural products are not the easiest source for drug discovery (Strohl 2000, Gullo et al. 2006). The amount of any pharmacologically active compound is often small and in a mixture with a number of interfering compounds. The methods currently used to find new molecules or to explore the properties of new compounds are often not suitable for higher throughput screening purposes (Lam 2007). Thus sensitive techniques are needed that can detect small quantities of compounds from complex mixtures and handle large number of samples in an automated environment. This also applies to studies of the first-hand toxicity and permeability of active natural compounds.

Cancer is a major public health threatening disease in developed countries (Balunas and Kinghorn 2005, Tan et al. 2006). Natural products play a significant role in cancer since many anticancer compounds are either natural products or are derived from natural products (Newman and Cragg 2007). These compounds have also led to significant discoveries related to their mode of action. The etiology of major cancers is still largely unknown and there is a need for more effective and less toxic chemotherapeutic agents. Apoptosis plays a central role in cancer, since induction of apoptosis in cancer cells is critical to successful therapy (Bunz 2001). Cancer therapy aims to trigger apoptosis and resistance development is usually associated with apoptosis resistance. The efficacy of cancer chemotherapy is often impaired due to a phenomenon called multidrug resistance (Gottesman et al. 1996, Stromskaya et al. 2008). Cells respond to the treatment at first, but then become resistant to it due to accelerated expression of efflux transporters such as P-glycoprotein (Pgp) (Gottesman et al. 1996,

Stromskaya et al. 2008). Due to its high substrate specificity P-gp significantly affects the treatment of cancer and other diseases as well as drug absorption through intestinal mucosa after oral administration (Schinkel 1997, Ambudkar et al. 1999). Thus knowledge about the substrate activity of drug candidates for P-gp has become an integral part of drug discovery (Gao et al. 2001, Polli et al. 2001, Perloff at al. 2003).

The role of dietary polyphenols in the prevention of cancer is emerging (Manach et al. 2004, Fresco et al. 2006). Phenolic compounds, abundant in fruits and vegetables, could modulate signaling pathways, inhibit kinase activity and induce apoptosis in precancerous or malignant cells, resulting in the inhibition of cancer development or progression. They could also be used as sensitizers to radiation and anticancer drugs, resulting in lower doses and less adverse effects with similar efficacy (Singh and Avgarwal 2006).

The current intake of dietary phenolics is often insufficient to protect against mutagens (Manach et al. 2004, Fresco et al. 2006). Functional foods and other concentrated dietary supplements are used to enhance the intake of such beneficial compounds. The effective compound, or combination of compounds, in such products should be well characterized and tested to ensure there are no toxic effects (Skalli et al. 2007). The absorption of these compounds and their effects on the absorption of different concomitant medicines should also be evaluated prior to use to avoid interactions and adverse effects in the human body (Laitinen et al. 2004). The more concentrated the product the more important this evaluation is.

Nature provides diverse health-promoting compounds that we only have to identify. These compounds may help to cure or alleviate serious diseases, including cancer. However, in order to find new molecules and determine their effects we need to develop assays that are suitable for use with natural products.

2. REVIEW OF THE LITERATURE

2.1. DRUGS FROM NATURE

2.1.1. Natural compounds as a source of drugs

A huge variety of pharmacologically active compounds are just waiting to be discovered in nature. In general these compounds are secondary metabolites produced by organisms in response to some external stimuli such as infection, wounding, dryness or nutritional changes. The compounds are also used for attraction and protection. Research into plant secondary compounds can be considered to have started when Friedrich Wilhelm Sertürner isolated morphine ("principium somniferum") from opium poppies in 1806 (Hartman 2007). This was followed by rapid advances in this field. However the chemical structure of morphine was not elucidated until 1923 and complete synthesis of its complex structure was not achieved until 1950. In those days the time-span from natural product to pure compounds was far longer than that of modern drug discovery with its rapid analytical methods. Table 1 lists some natural derived compounds that have been used as medicines for several years and that have different modes of action. These compounds play major roles in the treatment of various disorders.

Table 1. Some natural derived compounds used as therapeutic agents (da Rocha et al. 2001, Balunas and Kinghorn 2005).

Compound	Mode of action	Therapeutic use
Acetylsalicylic acid	Cyclo-ocxygenase inhibition	Analgesic, anti-inflammatory, antipyretic
Atropine	Muscarine receptor antagonist	Pupil dilator, anticholinergic in anesthesiology
Caffeine	Adenosine receptor antagonist, phosphodiesterase inhibitor	Stimulant
Codeine	Opioid receptor agonist	Analgesic, antitussive
Cyclosporine	Inhibits lymphokine production - prevention of colonial proliferation of lymphocytes	Immunosupressant
Digoxin	Na^+/K^+ ATPase inhibitor- intracellular Ca^{2+} increase	Congestive heart failure, atrial fibrillation
Ephedrine	α- and β-adrenergic receptor stimulator	Bronchodilation, cardiac muscle stimulant
Ergotamine	α-adrenergic receptor stimulator - vasoconstriction	Migraine treatment
Eugenol	Reduces Ca^{2+} influx and increases K^+ efflux - reduction of excitability of sensory nerves	Toothache
Galanthamine	Acetylcholine esterase inhibitor, nicotinic acetylcholine receptor sensitizer	Alzheimer's disease
Morphine	Opioid receptor agonist	Analgesic
Noscapine	Bronchodilation, smooth muscle relaxation	Antitussive
Penicillin	Inhibition of synthesis of bacterial cell wall peptidoglycan	Antibiotic
Pilocarpine	Muscarine receptor agonist	Glaucoma
Quinine	Inhibition of protein synthesis in the malaria parasite	Anti-malarial
Scopolamine	Muscarine receptor antagonist	Nausea
Tetracyclin	Binding to the bacterial ribosome 30S subunit - inhibition of protein synthesis	Antibiotic
Theophylline	Elevates intracellular cAMP and decreases Ca^{2+} levels, phosphodiesterase inhibitor	Bronchial smooth muscle relaxant

The formation of natural products represents a selected evolutionary advantage to the producing organism (Koehn and Carter 2005, Lam 2007). Over 100,000 secondary metabolites are known but only a few species have been studied for the presence of secondary metabolites (Vuorela et al. 2004). Marine resources in particular are still poorly explored despite the increasing popularity of marine research (Newman and Cragg 2006, Lam 2007). The marine environment is an extremely rich source of both novel chemistry and novel biology. Microbial natural products are both interesting and worth screening since in addition

to their potential therapeutic activities they frequently possess desirable pharmacokinetic properties for clinical use. It has also been suggested that exploring the full biodiversity of nature is not necessary to be successful in lead discovery (Tulp and Bohlin 2002). This is based on the idea that it is unlikely that the molecular targets in the human body are restricted to one species and that important molecular mechanisms are likely to be ubiquitous.

Historical inequity in the use of biodiversity prompted the establishment of the United Nations Convention on Biological Diversity (Tan et al. 2006). Its objectives are the conservation of biological diversity, the sustainable use of its components, and the fair and equitable sharing of the benefits arising out of the utilization of these resources. It should be recognized that countries have the right of ownership of their biological property, both marine and terrestrial, within their legal boundaries (Cordell 2000). Indigenous peoples also have a right to protect and seek compensation for the knowledge which they have developed based on their local biodiversity. Some countries have very strict rules to control access to their biome, but most countries encourage interested parties to work with local people. Working together is the wise choice both for those who hold the resources and for those who are seeking them (Boyd 1996). It is important to respect laws and national conventions, to work together, and to share knowledge to make discoveries.

2.1.2. New molecules from nature

In the past, when no drug industry existed, the only cure for illness had to be found in nature. The use of a particular herb or mixture of compounds was passed on from one generation to another through the knowledge of healers. This knowledge is still valuable, though most of the drugs used today are now obtained from industry. Different approaches can be used to find active substances (Harvey 1999, Houghton 2000, Vuorela et al. 2004). In the random approach samples are collected randomly in one or more places. The collection of material may be limited to certain botanically related species with known active compounds or to species with closely related secondary metabolisms. The traditional ethnopharmacolological approach relies on knowledge gained over the years in the use of certain natural products. The epidemiological approach focuses on the manifestation of a disease and the factors affecting it. The empirical approach tries to understand the physical process within the disease and then to find a cure, while in the molecular approach, the molecular target is known and a

compound specific to that target has to be found. Genomics and metabolomics provide new tools for drug discovery by generating structural information about genomes and biosynthetic pathways.

The chemical diversity of nature provides many advantages, models and ideas for modern drug design even though nature is not the easiest source for drug discovery (Strohl 2000, Gullo et al. 2006). The average volumes of natural products correlate with the average volumes of protein cavities (Zhang and Wilkinson 2007). Although natural products have not developed to interact with human proteins, many human proteins consist of the same building blocks and contain similar structural domains to the targets for which natural products have coevolved. It has been estimated that the hit rate is ~100 fold higher for natural products than for synthetic compounds (Bérdy 2005). Despite the facts, the majority of large pharmaceutical companies have down-regulated or even terminated their natural product research programs during the past 15 years (Lam 2007). The introduction of new high-throughput screening (HTS) techniques and combinatorial chemistry persuaded pharmaceutical companies that these are the keys to success (McChesney et al. 2007). Unfortunately, combinatorial libraries with low hit rates have been disappointing or at least not as promising as expected.

There are several reasons why a synthetic chemistry library is preferable to a natural products library in modern screening methods (Lam 2007). The creation and maintenance of a natural product library needs skills that the industry often lacks. Finding a novel chemical entity from a natural source is much more difficult than from a synthetic library of pure compounds. Natural product extracts are difficult or time-consuming to assay due to the large number of interfering compounds such as chlorophyll, sugars and color (McChesney et al. 2007). Individual active compounds are often produced in such small amounts that there may not be enough compound to produce any effect. Also, the heterogeneity of natural product samples complicates sample preparation and the analysis or extraction of active compounds.

Rediscovery of already known substances is said to be a major drawback in short time-spans (Koehn and Carter 2005). The time from hit to knowing the chemical structure responsible for the action is long and thus not desirable for an industry seeking ever-greater cost effectiveness. On the other hand, the finding that a known compound exists in unrelated species could lead to identification of a new molecular target (Tulp and Bohlin 2005). When a natural compound is synthesized in unrelated species, it is not likely that it is synthesized for

no reason at all; it must have some biological function. Studying this kind of compound could lead to important discoveries.

In addition, the complexity of compounds, their multiple hydroxyl moieties and chiral centers may cause problems in drug development. On the other hand this could be an advantage. A compound may have a surprising new chemical structure and provide new ideas for alternative synthesis of derivatives. Discovery of a novel natural compound could also lead to a better understanding of the targets or pathways of diseases (Gullo et al. 2006).

Figures relating to the sources of new drugs in the last 25 years show that natural products still play an important role in drug discovery despite the current low level of natural product-based drug discovery programs in the pharmaceutical industry (Newman and Cragg 2007). Infectious diseases (microbial, viral and parasitic), cancer, antihypertensive and anti-inflammatory disorders are the most common areas for drug research. In the area of cancer, natural products or natural derived products cover almost 50 % of the medicines. Several top-selling pharmaceuticals are natural derived products (Myles 2003), like one of the best-selling drugs (natural derived atorvastatin) for treating hypercholesterolemia (Newman and Cragg 2007).

Natural products are good sources of novel structures but not necessarily the final drug entity itself. Minor differences in molecular structure can dramatically change the effect of certain compounds (Calixto et al. 2000). For example, morphine is a powerful painkiller with addictive properties. One natural analog of morphine – codeine – differs from morphine by just one methyl group but is a much less effective analgesic. On the other hand acetylation of the two hydroxyls in the morphine structure leads to heroin, which can readily cross the blood-brain barrier and have dramatic and undesirable effects on the central nervous system. Also a study of the effects of flavonoids on Ca^{2+} fluxes showed that a change of one functional group led not only to lost activity, but changed an inhibitor into an activator (Summanen et al. 2001). This shows how important modification of the structure can be in producing the desired effect.

A multidisciplinary approach to drug discovery, involving the generation of novel molecular diversity from natural product sources, combined with total and combinatorial synthetic methods, including the manipulation of biosynthetic pathways, could provide the best route to successful drug discovery and development processes (Myles 2003, Vuorela et al. 2004, Lam

2007, Newman and Cragg 2007). In combinatorial engineering the genes responsible for particular metabolic pathway steps from different source organisms are combined to generate novel branches in metabolic pathways and to biosynthesize products that were previously inaccessible (Goossens et al. 2003, Mijts and Schmidt-Dannert 2003). The benefits of analogs are that they can be used to maintain or improve the active parts of molecules while getting rid of unnecessary complexity.

The question is not whether we should screen natural products for drug discovery but whether we can afford not to (Lam et al. 2007). As Aristotle said: "Nature does nothing without purpose or uselessly" (McChesney et al. 2007).

2.1.3. Bioassay methods for screening purposes

The objective in drug discovery is to find new molecules with desired actions or new modes of action. In order to achieve these goals different bioassay methods are needed. Bioassay can be defined as the tests used to detect the biological activity of an extract or pure substance obtained from a living organism (Houghton 2000). Finding an activity from plant material follows subsequent procedures (Claeson and Bohlin 1997, Vuorela et al. 2004). The process starts with source selection and extraction. The extraction procedure should be planned carefully, since the choice of method and chemicals affects the final composition of the compound obtained. The material and extraction method used depend greatly on the assay target chosen. The selected assay is then used to evaluate the bioactivity of extracts. When activity is detected, the active extracts are fractionated to isolate the bioactive molecules. Fractionation can be achieved using chromatographic methods. The active molecules are then identified using, for example, MS (mass spectrometry) or NMR (nuclear magnetic resonance spectrometry) techniques, which provide rapid and reliable information about the structure in question (Wolf and Siems 2007). However, it is not unusual for the activity of the initial extract to be higher than that of any of the fractions (Houghton 2000). This could be due to synergy between compounds in the extract. It may also be due to breakdown or transformation of the active component during fractionation. For example compound may be hydrolyzed in aqueous medium and alcoholic or acid groups may be esterified. The other approach is pure compound screening, which starts with the isolation and structure

elucidation of most of the secondary metabolites in the crude extract and continues with screening of the purified compounds (Bindseil et al. 2001, Wolf and Siems 2007).

For an assay to be suitable for high-throughput screening (HTS) the sample volume has to be small and the results should be obtained quickly (Houghton 2000). Microtiter plates, in which large numbers of samples can be tested simultaneously, are generally used. The assay process should also be suitable for automation. Detection should be rapid and enable measurement or sampling straight from the plates. When the assay target has been decided, it should be checked that it is sensitive enough to give a quantifiable response with the amounts and concentrations used (Vuorela et al. 2004). Positive and negative controls should be used to ensure that the method works as it should. With biological material the variation is much higher than when pure synthetic compounds are used. Thus an adequate number of replicates should be included in the experiments to get reliable results.

An activity assay can be a specific assay measuring a single target like receptor binding or enzyme inhibition (Claeson and Bohlin 1997). The assays are well characterized and the observed effect can be directly assigned to a specific mechanism of action. These methods are often suitable for rapid screening purposes and for automation. Multiple target assays are broader in scope and an observed action cannot directly be assigned to a specific mode of action. These bioassays include assays on whole animals, isolated organs and intact cells. The assays are time consuming and not suitable for screening purposes except the assays with established cell lines.

Microarrays, detecting gene expression after exposure to some substance, are providing new possibilities for drug discovery (Gerhold et al. 2002). The arrays can be used in drug discovery to find targets or compounds and later on to optimize the selection of lead compounds. The use of the technique can also help to understand diseases and lead to better treatments (Olson 2004, Winnepeninckx et al. 2006).

The choice of assay depends on what we are aiming to find. Is it an agent that exerts its biological action by an established mechanism but with a new chemical structure (structural novelty) or do we want to find a new mechanism or a new biological target for an already known compound (biological novelty) (Claeson and Bohlin 1997). No matter what method is used, efficient reviewing of bioassay data is one of the most important factors leading to a successful outcome in natural product drug discovery.

2.1.4. Evaluation of intestinal absorption

In order to have a therapeutic effect, the drug must get into the body. One of the limiting factors is the ability of a drug to cross biological membranes (Ungell 2004). Drug metabolism in the gut lumen can diminish the absorption even more. The metabolism of compounds is divided to two groups phase I and phase II. In Phase I the molecule is oxidized, reduced and hydrolyzed. The reactions mainly mediated by cytochrome P450 (CYP) enzyme family members. In phase II the molecules conjugate with sulfatate, glucuronidate, glutathione or amino acid, or are methylated or acetylated. By these modifications molecules are converted to more soluble form, which the can be more easily excreted. In many cases the metabolism of the compound and efflux transporters can act synergistically diminishing the fraction absorbed furthermore (Ho and Kim 2005). For example, many lipophilic compounds conjugated with glutathione, glucuronate or sulfate are substrates for efflux transporters of MRP (multidrug resistance associated protein) family (Keppler 1999, Homolya et al. 2003).

Pharmaceutical companies spend a lot of money developing drugs that can be administered orally and it is unacceptable for a compound to fail in clinical trials due to low oral availability. Thus it is important to determine ADMET (absorption, distribution, metabolism, excretion, toxicity) parameters as early as possible to avoid unnecessary work and to save money and time (Bleicher et al. 2003, Flaten et al. 2006). The use of cell lines in the early phase of drug discovery studies reduces the number of animals being exposed to compounds unnecessarily and at the same time enables screening with live material (Allen et al. 2005).

Caco-2 cell monolayers are widely used and regulatory accepted in *in vitro* model system for predicting the absorption and permeability of compounds in humans (Bailey et al. 1996, Artursson et al. 1996b, FDA 2000). Caco-2 cells, originating from a human colonic adenocarcinoma, differentiate spontaneously morphologically and functionally to resemble intestinal enterocytes, and the model is useful in determining the roles of various physical and biochemical barriers to drug absorption (Artursson 1990, Artursson et al. 1996a, Gan and Thakker 1997). When Caco-2 cells are cultured on permeable membranes the cells differentiate to form tight junctions and brush border membrane with microvilli on the apical (luminal) side (Miret et al. 2004). Since the cells originate from the colon, the tight junctions are tighter than in the small intestine (Bohets et al. 2001). Also the distinct mucus layer that covers enterocytes is absent in Caco-2 cells (Crespi et al. 2000). Nevertheless, a comparison of drug transport in Caco-2 cells with *in vivo* intestinal absorption indicates that Caco-2 cell

monolayers can be used to predict drug transport by different pathways across the intestinal epithelium.

The absorption of drugs may occur by one or more of four different routes: the passive transcellular (over the cell membrane) and paracellular (between cells) routes, the carrier mediated route and the transcytosis route (Artursson et al. 1996b). Permeation can also be hindered by efflux proteins. The best correlation is obtained for compounds permeating passively trough the transcellular route, which is the most common drug permeation route in the human intestine**.** It has been reported that 80-95% of commercial drugs are absorbed primarily via passive diffusion (Mandagere et al. 2002). Active transport can also be studied, although the abundance of each transporter must be characterized if more detailed mechanistic studies are needed (Yee 1997). In a recent study with two different Caco-2 cell clones the profiles of transporter expression in the two clones correlated significantly with those in human small intestine, which support the use of Caco-2 cells for investigating intestinal drug transport (Maubon et al. 2007). However, the multidrug resistance associated proteins MRP2, MRP3, MRP4, MRP5 and MRP6, organic anion transporter polypeptides OATP-A and OATP-B, organic cation transporter OCT1, and monocarboxylic acid transporter MCT1 were quantitatively expressed at higher levels, and breast cancer resistance protein BCRP at lower levels in Caco-2 cells compared to small intestine. The relative expression of mRNA of multidrug resistance protein 1 MDR1, MRP2, OATP-A and oligopeptide transporter PEPT1 was found to differ in the two Caco-2 cell clones analyzed. This highlights the fact that, depending on cell origin, some variations in transporter expression may occur in Caco-2 cells, which in turn underscores the importance of carefully characterizing transporter levels in Caco-2 cells. Also, the level of metabolizing CYP3A4 enzyme is generally low, which may lead to overestimation of the absorption of its substrates (Schmiedlin-Ren et al. 2001, Ungell 2004). Thus, if needed, enzyme expression may be induced (Anderle et al. 1998). Expression levels of the transporters also vary in different parts of the intestine (Englund et al. 2006). Transporter function may vary with the prevailing drug concentration, pH and nutritional constituents. Thus, we cannot mimic the exact situation in the lumen.

Variations in Caco-2 cell permeabilities between different laboratories are often quite large while intralaboratory variation is small, which is normal for measurements on living material. This emphasizes the importance of using standard compounds with known permeabilities, which can then be compared. When the characteristics of Caco-2 cells are known, the cell line

is well suited for permeation and uptake experiments as well as for determination of absorption mechanisms and toxicity studies.

2.2. CANCER

For a cell to function normally it must be attached to other cells and to the cell matrix and continuously receive signals, mediated by growth factors, hormones and cytokines from the surroundings (Vermes et al. 2000). If cells get detached from their surroundings, the apoptotic process starts. However, in many malignancies apoptosis is not initiated and cells can survive in nonphysiological sites (Thompson 1995). This is crucial in tumor infiltration and metastasis formation. Tumors can remain "silent" for years, endure low oxygen levels, and then start to proliferate rapidly (Varner and Cheresh 1996).

The etiology of major cancers remains largely unknown (Tan et al. 2006). Cancer is a multifunctional disease that requires modulation of multiple pathways and targets. Although a great number of cancer drugs and cancer treatments exist, there is an urgent need for more effective and less toxic chemotherapeutic agents.

2.2.1. Dietary polyphenols and cancer prevention

It has been suggested that cancer can largely be prevented by following the appropriate diet (Fresco et al. 2006, Kandaswami et al. 2005). Asians, who consume more fruits and vegetables and tea in their diet than western populations, have lower incidences of breast, colon and prostate cancers, which are among the most common cancers in the world (Tan et al. 2006). Dietary phenolics can work in a way that arrests or reverses carcinogenic changes before the appearance of malignant disease (Singh and Agarwal 2006). Cell proliferation and differentiation are disturbed in cancer cases. Dietary polyphenolic compounds have been shown to have anticancer effects such as ability to induce apoptosis and inhibit cell growth and kinase activity (Kandaswami et al. 2005). Flavonoids can also act as sensitizers when used in combination with radiation and anticancer drugs (Singh and Agarwal 2006). This

allows lower doses of radiation or chemotherapeutic drugs to be used resulting in fewer adverse effects without loss of efficacy.

Extensive studies are needed to determine the doses, efficacy and mechanism of dietary flavonoids in relation to the growth and progression of different types of cancer (Graf et al. 2005, Singh and Agarwal 2006). Knowing the exact mechanism of action of flavonoids would help: not only their use as chemopreventive agents, but also as models in the development of semi-synthetic or synthetic anticancer drugs with fewer side-effects.

2.2.2. Natural compounds as anticancer agents

A number of important commercial drugs have been obtained straight from nature or by structural modification of a natural product or by synthesis of a new compound using a natural compound as a model (Gordaliza 2007). Paclitaxel and related taxanes exert their anticancer effects by promoting tubulin polymerization and stabilizing microtubules, leading to mitotic G2/M arrest and apoptosis (Gallagher 2007). Docetaxel, a second-generation taxane, is one of the most powerful drugs against breast cancer (Kim and Noguchi 2008). Vinca alkaloids (vincristine, vinblastine) and their derivatives (e.g. vinorelbine) block mitosis through metaphase arrest by binding specifically to tubulin and leading to its depolymerization (Okouneva et al. 2003). These alkaloids are used to treat certain kinds of cancer, including non-small cell lung cancer, breast cancer, testicular cancer and Hodgkin's lymphoma. Camptothecin and its derivatives (e.g. irinotecan and topotecan) inhibit topoisomerase I, which is involved in the cleavage and reassembly of DNA (Lorence and Nessler 2004). These compounds are traditionally used to treat ovarian, colon and small cell lung cancer, but recent studies suggest they can also be used in the treatment of gastric cancer, cervical cancer and malignant gliomas. Podophyllotoxin is the most abundant lignan isolated from podophyllin, a resin produced by Podophyllum species, but it was found to be too toxic for clinical use (Gordaliza et al. 2004). Etiposide and teniposide, two semi-synthetic derivatives of podophyllotoxin, inhibit topoisomerase II and have shown good clinical efficacy against several types of neoplasms including testicular and small cell lung cancers, lymphoma and leukemia. All these natural products have led to significant biological discoveries concerning their mechanism of action and have an important influence on current cancer therapy (Balunas and Kinghorn 2005). Another important group is the products of

Table 2. List of some natural derived and semisynthetic compounds used as anticancer agents (da Rocha et al. 2001, Gordaliza et al. 2004, Balunas and Kinghorn 2005, Tan et al. 2006, Newman and Cragg 2007).

Compound	Natural source	Family	Year introduced
Bleomycin	*Streptomyces verticillus*	Actinomycetales	1962
Vincristine	*Cantharanthus roseus* (L.) G. Don	Apocynaceae	1963
Vinblastine	*Cantharanthus roseus* (L.) G. Don	Apocynaceae	1965
Doxorubicin	*Streptomyces peucetius*	Actinomycetales	1974
Mitomycin	*Streptomyces caespitosus*	Actinomycetales	1974
Paclitaxel	*Taxus brevifolia* Nutt.	Taxaceae	1992
Derivatives			
Epirubicin	*Streptomyces spp.*	Actinomycetales	1984
Vinorelbine	*Cantharanthus roseus* (L.) G. Don	Apocynaceae	1989
Idarubicin	*Streptomyces spp.*	Actinomycetales	1990
Docetaxel	*Taxus baccata* L.	Taxaceae	1995
Irinotecan	*Camptotheca acuminata* Decne.	Nyssaseae	1994
Topotecan	*Camptotheca acuminata* Decne.	Nyssaseae	1996
Teniposide	*Podophyllum peltatum* L.	Berberidaceae	1992
Etoposide	*Podophyllum peltatum* L.	Berberidaceae	1996

Streptomyces species (e.g. bleomycin, doxorubicin, mitomycin) and their many semi-synthetic derivatives (e.g. epirubicin, idarubicin). Table 2 presents some cancer-preventing compounds and their natural sources together with their year of introduction.

The sea has also proved to be a good source of potential anticancer agents. The novel antitumor agent trabectedin was originally isolated from the marine tunicate Ecteinascidia turbinata althogh it is now used as the synthesized molecule (van Kesteren et al. 2003, Carter and Keam 2007). Trabectedin interferes with DNA-binding proteins and transcription factors and blocks the cell cycle at the G2 phase. It also inhibits over-expression of the multidrug resistance-1 gene (MDR1) (Anonymous 2006). In 2001 trabectedin received orphan drug status for the treatment of soft tissue sarcoma. In addition many other compounds derived

from marine species have shown potency in cancer treatment (Newman and Cragg 2006). For example, aplidine from the Mediterranean tunicate Aplidium albicans has shown broad spectrum activity against various types of cancers including colorectal, thyroid and renal cancers (Russo et al. 2008) by interfering with several pathways, including cell cycle arrest, inhibition of protein synthesis and antiangiogenic activity (Le Tourneau et al. 2007).

2.2.3. P-glycoprotein (P-gp) and multidrug resistance

Multidrug resistance is a phenomenon in which cells treated with a drug become resistant to the cytotoxic effect of a drug and a variety of other structurally and functionally unrelated drugs (Raguz et al. 2008). It is often associated with the expression of P-glycoprotein, an efflux membrane pump coded by the MDR1 gene. Expression of P-gp is associated with resistance to chemotherapy in cancer and the phenomenon was first observed in cancerous tumors (Callaghan et al. 2008). In cancer, cells initially respond to treatment but then become resistant to it due to enhanced expression of efflux transporters (Gottesman et al. 1996, Stromskaya et al. 2008). P-gp substrates include vinblastine, vincristine, actinomycin, doxorubicin, imatinib, and paclitaxel.

P-gp is a membrane protein, which consists of two similar transmembrane domains joined by a linker region (Idriss et al. 2000). Each transmembrane domain is followed by a cytoplasmic nucleotide domain, which is responsible for the ATP hydrolysis associated with drug efflux. P-glycoprotein efflux transporters are expressed in various tissues (Cordon-Cardo et al. 1990) and affect the absorption, distribution and excretion of a number of clinically important drugs (Hunter and Hirst 1997, Stromskaya et al. 2008). P-gp can transport a variety of structurally unrelated compounds from the interior of plasma membranes out of the cells, thereby limiting access by xenobiotics into cells (Schinkel 1997, Ambudkar et al. 1999).

Because of its high substrate specificity P-gp may significantly affect drug absorption through intestinal mucosa after oral administration. Moreover it can indirectly enhance intestinal CYP3A4 metabolism by increasing the intestinal residence time of the drug and by preventing CYP3A4 product inhibition by the removal of primary metabolites (Gan et al. 1996, Watkins 1997, Hochman et al. 2000). The influence of P-gp in drug-drug interactions is increasingly being recognized (Marchetti et al. 2007). Inhibition or induction of P-gp by coadministered

drugs, food or herbal products may result in pharmacokinetic interactions leading to unexpected toxicities or undertreatment. On the other hand, modulation of P-gp expression and/or activity may be a useful strategy to improve the pharmacological profile of P-gp substrate drugs. Thus knowledge of the substrate activity of drug candidates for P-gp has become an integral part of drug discovery (Gao et al. 2001, Polli et al. 2001, Perloff at al. 2003). P-gp is the most studied efflux transporter, but recently the meaning of other efflux transporters, such as multridrug associated proteins (MRPs) and breast cancer resistance protein (BCRP), is also noticed (Pal and Mitra 2006).

2.2.4. Apoptosis

Programmed cell death, apoptosis, is a genetically coded mechanism that rids the body of unwanted cells. Apoptosis is the normal physiological response to various stimuli, including irreparable DNA damage. It plays a crucial role in development, in tissue homeostasis maintenance and in defense against viral infections and mutations. It is a rapid process and causes no tissue damage, since apoptotic cells are rapidly phagocytosed by other cells (Kerr et al. 1972, Reed 2000).

2.2.4.1. Apoptosis induction

Apoptosis is induced by two major pathways (Hengartner 2000): by the cell's own signals or by external stimuli. Shortly, in the first, intrinsic pathway, cell damage stimulates the activity of p53, which in turn activates the proapoptotic members of Bcl-family (Bax, Bak, Bad) (Bunz 2001). The activation leads to the release of cytochrome C from mitochondria. Cytochrome C then interacts with Apaf-1 protein (apoptosis protease activating factor). Apaf-1 and cytochrome C interact with caspase 9-protease to produce a complex called apoptosome. Activated caspase 9 cleaves and activates other caspases i.e. caspase cascades (Schneider and Tschopp 2000). The reaction leads to the DNA degradation and formation of the "apoptotic bodies", the cellular fragments that are covered with the cell membrane. Apoptosis can also be induced by an extrinsic death-receptor pathway (Schneider and Tschopp 2000, Bunz 2001, Kaufman and Hengartner 2001). Death receptors like Fas/CD95

and TNFR-1 (tumor necrosis factor receptor-1) are transmembrane proteins. Their active site is situated on the outer membrane of the cells. Binding of ligands like FasL (Fas ligand) activator and TNF-α (tumor necrosis factor α) cytokine multimerizise and activate the receptors. The activation signal is transduced to the cytoplasm and initiates the activation of caspase 8 which activates the caspase cascade. The apoptotic process has started. The two main pahtways of apoptosis are illustrated in Figure 1.

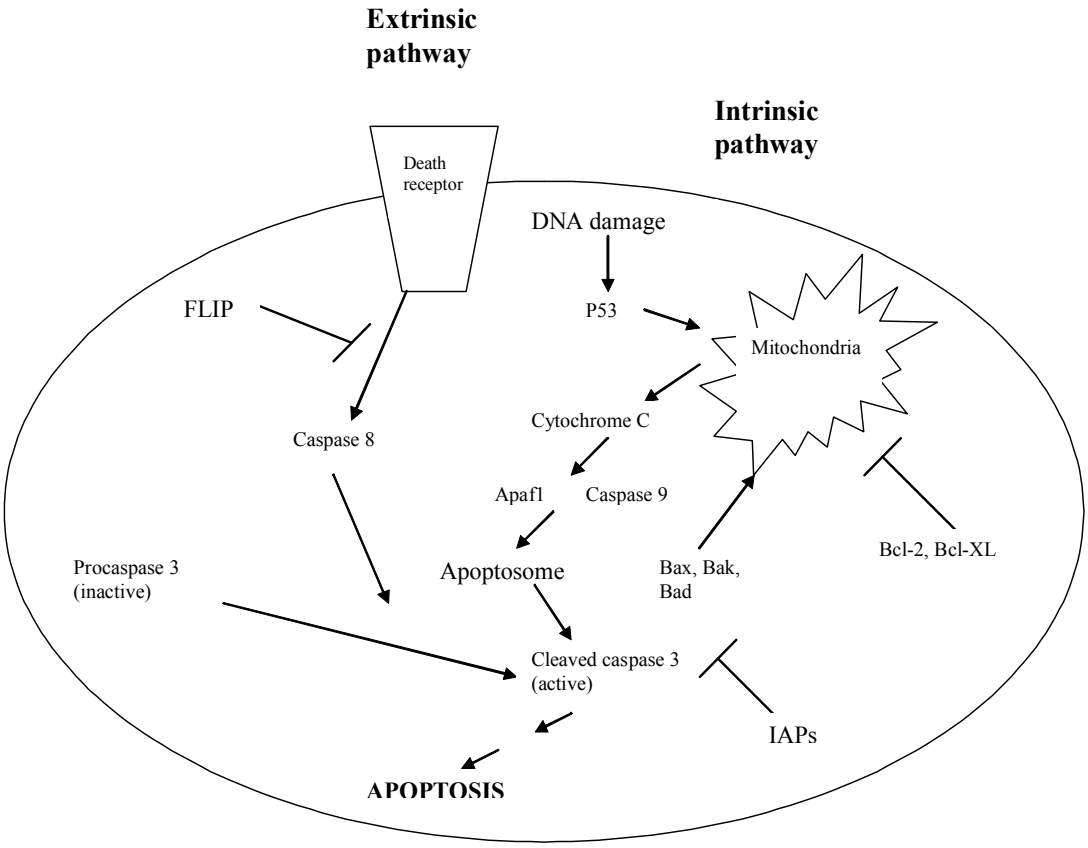


Figure 1. A simplified scheme of the two main pathways, the extrinsic and the intrinsic pathway, of apoptosis. Activation of caspases is negatively regulated by FLIP-proteins, antiapoptotic Bcl-2 members (Bcl-2, Bcl-XL) and IAP-proteins. FLIP=FLICE inhibitory protein, IAP=inhibitor of apoptosis protein.

Central to the apoptotic program is activation of caspases, a family of cysteine proteases that cleave cellular proteins at the carboxyl site of aspartate residues (Kaufman and Hengartner 2001). Caspases are present in cells as inactive procaspases, which can be rapidly activated. After a pro-apoptotic signal, initiator caspases (caspase-2, -8, -9 and -10) are first activated, and initiator caspases then activate precursor forms of effector caspases (caspase-3, -6, and -7). Effector caspases cleave several cellular proteins with essential functions leading to the apoptotic phenotype (Thornberry and Lazebnik 1998).

2.2.4.2. Apoptotic genes

Defects in apoptosis pathways allow cells with genetic abnormalities to survive. The most important carcinogenesis regulating genes are p53 and bcl-2 (Kaplan and Fisher 2002). p53 is a tumor-suppressing gene, named by the protein product of the gene which weights 53 kd (Samali et al. 1996). It is also called antioncogene, because it inhibits oncogene actions. Mutational inactivation is observed in more than 50% of human cancers (Sun 2006). p53 arrests the cell cycle at the G1/S phase if DNA is not properly replicated (Canman and Kastan 1997). Because p53 is the main mediator of the apoptotic response to chromosomal damage, disturbed function of p53 can lead to the development of serious tumor promotion (Webb et al. 1997, Bunz 2001).

Bcl-2 family members are the key regulators that control cytochrome C release from mitochondria (Kaufman and Hengartner 2001). Some of them are apoptosis supressors (e.g. Bcl-2, Bcl-x_L) some are apoptosis inducers (e.g. Bax, Bak and Bad) (Reed 2000). In healthy cells both subfamilies are in equilibrium (Los et al. 2003). The Bcl-2 gene is situated in human chromosome 18 and has been named after leukemic B-cell. Overexpression of Bcl-2 in these cells results in down-regulation of apoptosis and colonial growth of B-cells.

2.2.4.3. Apoptosis association with diseases

Defective apoptosis regulation is associated with many diseases; for example, insufficient apoptosis occurs in cancer and autoimmunity diseases (Los et al. 2003, Rashedi et al. 2007,

Howley and Fearnhead 2008). In contrast, excessive apoptosis is associated with heart failure, neurodegenerative disorders and AIDS (Thompson 1995, Nicholson 2000, Robertson et al. 2000, Reed 2002). Thus new apoptosis-modulating molecules are widely being sought from natural products (e.g. Chen et al. 2007, Gallagher 2007, Sarath et al. 2007, Ishibashi and Ohtsuki 2008, Ohtsuki et al. 2008, Sasiela at al. 2008) and from synthetic compounds (e.g. Khazanov et al. 2002, Nesterenko et al. 2003, Ikeda et al. 2004, Padron 2006).

Apoptosis plays a central role in cancer. A successful approach in cancer therapy is to trigger apoptosis but it is often complicated by development of resistance mechanisms. Tumor heterogeneity and clonal variability will provide further challenges for apoptosis-based therapies (Sjöström and Bergh 2001). The rate of apoptosis has also been shown to be faster in adenomas than in malignant carcinomas (Kaufmann and Gores 2000).

In *in vivo* experiments elimination of p53 increased the aggressive behavior of the tumor and decreased apoptosis (Canman and Kastan 1997). p53 gene mutation correlates with the incidence of cancer cases (Shah et al. 2008, Verkler et al. 2008). Tumor incidence is also elevated in Li-Fraumen syndrome, where another p53 allele is inactivated (Izawa et al. 2008). Bcl-2 transgenic mice have been shown to suffer from lymphatic hyperplasia (Strasser et al. 1990). Elevated antiapoptotic Bcl-2 levels are also associated with melanoma and bladder cancer (Hameed et al. 2008, Triozzi et al. 2008). Bcl-2 can attenuate apoptosis induced by inhibiting caspase-3 activity (Park et al. 2001, Moon et al. 2008). In cases in which Bcl-2 is elevated, cancer has a poor prognosis (Thompson 1995). Reducing levels of antiapoptotic Bcl-2 proteins with antisense molecules has produced encouraging experimental and clinical results (Gardner 2004). The ratios of pro- and antiapoptotic Bcl-2 family proteins affect the sensitivity or resistance of the cells to anticancer drugs, and manipulation of these will have an impact on future cancer treatment (Reed 2000, Gardner 2004, Adams et al. 2005, Basu et al. 2006, Adams and Cory 2007).

2.2.5. Protein kinase C as a drug target

Protein kinase C (PKC) is a family of closely related serine/threonine kinases involved in the transduction of signals, mediated by phospholipid hydrolysis, for cell proliferation, differentiation and regulation of apoptosis (Nishizuka 1984, Nishizuka 1988, Clemens et al.

1992, Newton 1997, Jiang et al. 2002). At least 12 isoforms with different tissue expressions, substrate specificities and subcellular localizations have been described (Jaken 1996, Dempsey et al. 2000, Serova et al. 2006, Verma et al. 2006). PKC consists of a single polypeptide chain containing an amino terminal regulatory domain and a carboxy terminal catalytic domain (Nishizuka 1988, Newton 2003). Different isoforms are divided into subclasses based on their diacylglycerol, phosphatidylserine, calcium and phorbol ester requirements for activation. Conventional (α, βI, βII, γ) PKCs are regulated by diacylglycerol, phosphatidylserine and calcium. Novel PKCs (δ, ε, η, θ) are regulated by diacylglycerol and phosphatidylserine but not calcium. Both of these groups are also activated by phorbolesters, which function as analogs of diacylglycerol. The functions of atypical isoforms (ζ, ι/λ) depend on phosphatidylserine but not calcium, diacylglycerol or phorboelsters. In addition, two PKC related kinases PKC μ and PKC ν has been described, but their mode of action is distinct from another PKC isoforms (Ron and Kazanietz 1999, Battaini 2001, Parker and Murray-Rust 2004). The activators and tissue distributions of PKCs have presented in Table3.

Table 3. The activators and tissue distributions of protein kinases (Baitani 2001, Idris et al. 2001). Ca^{2+}= calcium, DAG= diacylglycerol, PS= phosphatidylserine.

		Tissue	Co-factor requirements		
	Isoenzyme	**distribution**	**Ca^{2+}**	**DAG**	**PS**
Classical	α	widespread	+	+	+
	βI	widespread (low levels)	+	+	+
	βII	widespread	+	+	+
	γ	brain, spinal cord	+	+	+
New	δ	widespread	-	+	+
	ε	brain, heart	-	+	+
	η	heart, skin, lung	-	+	+
	θ	muscle, brain, blood, lung	-	+	+
Atypical	ζ,	widespread	-	-	+
	ι/λ	kidney, brain, pancreas, lung	-	-	+

Protein kinases are thought to play a role in various diseases including cancer, autoimmune disorders, diabetes, vascular diseases and degenerative brain diseases (Carter and Kane 2004, Murphy and Frishman 2005, Serova et al. 2006, Alkon et al. 2007, Das Evcimen and King 2007, Hayashi and Altman 2007, Podar et al. 2007). PKCs with multiple functions are an interesting target for drug discovery. The finding that PKC function is altered in some neoplasias and that this dysfunction is related to uncontrolled proliferation aroused interest in PKC as a potential target in anticancer drug design (Gesher 2000). PKCs are also known to modulate multidrug resistance, and thus the use of PKC modulators in combination with classical cytotoxic drugs could be beneficial (Serova et al. 2006, Martiny-Baron and Fabbro 2007). PKC activity targeting is challenging because of the opposite roles of different isoforms, broad and partly overlapping substrate specificity, and the existence of multiple isoforms in a single cell type (Liyanage et al. 1992, Hug and Sarre 1993, Cacace et al. 1993, Gutcher et al. 2003). So far, no specific PKC inhibitors have proven to be effective and safe enough to get into the market (Mackay and Twelves 2007). Thus research in this field and new selective inhibitors are inevitably needed.

Natural products have been shown to regulate PKC activity and interest in them as PKC modulators has increased (Gamet-Payrastre et al. 1999, Carter and Kane 2004, Williams et al. 2004). For example, the well known PKC inhibitor staurosporine is an alkaloid from *Streptomyces* sp. (Tamaoki et al. 1986) and its derivatives have been shown to have potential in cancer therapy (Shao et al. 1997, Gescher 2000). Flavonoids could interfere with signaling molecules and have various outcomes depending on the cell type and disease in question (Williams et al. 2004, Weldon et al. 2005). Sphingolipid breakdown products, sphingosine and lysosphingolipids, isolated from Euphorbiaceae, have been shown to inhibit protein kinase C and to possess antitumor activities (Cateni et al. 2003). Many PKC-modulating compounds, such as quercetin, myricetin, epigallocatechin gallate, ellagic acid and curcumin could be obtained from the daily diet – from vegetables, berries, spices, tea and wine – and are thus easily available (Häkkinen et al. 1999, Arts et al. 2000, Varadkar et al. 2001, Kandaswami et al. 2005).

2.2.5.1. The role of PKC in apoptosis

The actions of both pro- and antiapoptotic factors are often regulated by modulation of the phosphorylation status of key elements of the apoptotic process (Cross et al. 2000). Protein

kinases have been implicated in both the up-stream induction phase of apoptosis and the down-stream execution stage. The role of PKC in apoptosis is controversial since activation of PKC can either induce or prevent apoptosis (Jiang et al. 2002). Conventional and atypical PKCs (α, βI, βII, γ and ζ, ι/λ) are in general considered mainly antiapoptotic as they promote cell survival and proliferation (Gutcher et al. 2003). Most of the novel PKCs (δ, η, θ), on the other hand, are regarded as having tumor-suppressing and apoptosis-promoting activity. Two isoforms have been characterized in more detail: isoform PKC α, belonging to the conventional PKC isoforms, has been shown to prevent apoptosis, while PKC δ, a novel PKC isoform, has been found to promote it. Protein kinase C inhibitors have been reported to be effective in cancer therapy and thus lot of research is concentrated to this area (Schwartz 1996, Harkin et al. 1998, Jarvis and Grant 1999, Cross et al. 2000, Serova et al. 2006, Podar et al. 2007). Selective targeting of PKC seems to be useful in improving the efficacy of anticancer agents (Jarvis and Grant 1999, Kaubish and Schwartz 2000). Reduction of PKC activity in cancerous cells can either directly induce apoptosis or enhance the influence of other pro-apoptotic stimuli. PKC inhibitors can exert their activity for example by inhibiting cell proliferation, by blocking angiogenesis and by regulating cell cycle proteins.

3. AIMS OF THE STUDY

The overall aim of this study was to evaluate natural products in apoptosis, protein kinase C activation and Caco-2 cell permeability and to develop tools for these purposes.

In pursuit of this aim the following studies were conducted:

1) Assays measuring apoptosis induction [**I**] and protein kinase C inhibition [**II**] were developed for screening new potential drug compounds. The apoptosis assay was optimized using Swiss 3T3 fibroblasts and HL-60 promyelocytic leukemia cells and natural product compounds [**I**]. The method was then used to screen a set of derivatives of 5-(hydroxymethyl)isophthalic acid designed to bind PKC [**I**]. An automated PKC assay was used to screen the protein kinase C inhibitory effects of Finnish plant extracts [**II**].

2) To support drug discovery, an accelerated Caco-2 permeability model was developed to make drug permeability evaluation more effective and more suitable for screening purposes [**III**]. The permeation characteristic of set of flavonoids and alkyl gallates were evaluated using a Caco-2 cell line and phospholipid vesicles [**IV**] and a set of coumarins with different substituents was studied using the accelerated Caco-2 cell model [**V**].

3) In order to evaluate the effect of concentrated natural products on concomitant medication, the influence of raspberry extract and fractions on the permeability of commonly used drugs was studied with the Caco-2 cell line [**VI**].

4) Cytotoxicity studies were included in the experiments with cells to eliminate the possibility of false positives caused by cytotoxicity [**I, IV-VI**].

4. EXPERIMENTAL

Detailed descriptions of materials and methods can be found in the original publications **I-VI**.

4.1. REAGENTS AND STANDARD COMPOUNDS [I, III-VI]

The EnzChek Caspase-3 Assay Kit was obtained from Molecular Probes (Leiden, The Netherlands) to evaluate apoptosis induction [**I**]. Commercially available drug compounds were used as reference substances in Caco-2 permeation studies [**III, IV, VI**]. Metoprolol [**III, VI**], ketoprofen [**III, IV, VI**], and naproxen [**III**] were purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA), paracetamol [**IV, VI**] was obtained from Orion Pharma (Espoo, Finland) and verapamil [**III, VI**] and hydrochlorthiazide [**III**] from Sigma Chemical Co (St. Louis, MO, USA). Permeation marker D-[1-^{14}C]-mannitol [**III, IV**] was bought from Amersham Pharmacia Biotech UK Ltd. (Amersham, England), while Lucifer Yellow [**III, V**] and rhodamine 123 [**III, V**] were obtained from Fluka Chemie AG (Buchs, Switzerland). The CytoTox96® kit [**I**] obtained from Promega (Madison, WI, USA), MTT reagent (3-[4,5-dimethylthiezol-2yl]-2,5-diphenyltetrazolium bromide) [**IV, VI**] purchased from Sigma-Aldrich Co. (St. Louis, USA) and cell proliferation reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) [**V**] obtained from Roche Diagnostics GmbH (Mannheim, Germany) were used for the cytotoxicity tests.

4.2. NATURAL COMPOUNDS AND DERIVATIVES [I-III, VI]

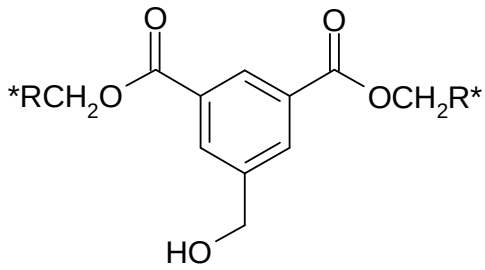
A set of natural compounds and their derivatives were used in the experiments as model compounds in assay development [**I, II**] or to evaluate their permeation characteristics [**IV, V**]. Flavonoids were used as reference substances in apoptosis [**I**] and in PKC [**II**] assay development. The permeation and membrane affinity of a selected group of flavonoids and alkyl gallates were studied with Caco-2 cell monolayers and in phospholipid vesicles [**IV**]. Permeation experiments with the new accelerated Caco-2 cell model were conducted with a set of coumarins [**V**]. The classification and sources of the natural compounds used in these studies are presented in Table 4. All compounds were highest commercial quality and highly.

Table 4. Sources of natural compounds and their derivatives used in publications **I-II** and **IV-V.**

Compound	Publication	Source
Flavonoids		
Apigenin	**I**	Fluka Chemie (Buchs, Switzerland)
(+)-Catechin	**IV**	Sigma Chemical Co (St.Louis, MO, USA)
(-)-Epicatechin	**IV**	Sigma Chemical Co (St.Louis, MO, USA)
Flavone	**IV**	Carl Roth GmbH (Karlsruhe, Germany)
Kaempherol	**I**	Extrasynthese (Genay, France)
Luteolin	**II, IV**	Extrasynthese (Genay, France)
Morin	**IV**	Carl Roth GmbH (Karlsruhe, Germany)
Myricetin	**I, II**	Extrasynthese (Genay, France)
Naringenin	**IV**	Carl Roth GmbH (Karlsruhe, Germany)
Naringin	**IV**	Extrasynthese (Genay, France)
Quercetin	**I, II, IV**	Merck (Darmstadt, Germany)
Alkyl gallates		
Methyl gallate	**IV**	Fluka Chemie (Buchs, Switzerland)
Octyl gallate	**IV**	Fluka Chemie (Buchs, Switzerland)
Propyl gallate	**IV**	Sigma Chemical Co (St.Louis, MO, USA)
Coumarins		
Citropten	**V**	Extrasynthese (Genay, France)
Coumarin	**V**	Sigma Chemical Co (St.Louis, MO, USA)
Daphnetin-7-methylether	**V**	Extrasynthese (Genay, France)
5,7-Dihydroxy-4-methylcoumarin	**V**	Extrasynthese (Genay, France)
6,7-Dihydroxy-4-methylcoumarin	**V**	Extrasynthese (Genay, France)
7,8-Dihydroxy-4-methylcoumarin	**V**	Extrasynthese (Genay, France)
Esculetin	**I**	Fluka Chemie (Buchs, Switzerland)
Esculin sesquihydrate	**V**	Fluka Chemie (Buchs, Switzerland)
Fraxidin	**V**	Extrasynthese (Genay, France)
Herniarin	**V**	Sigma Chemical Co (St.Louis, MO, USA)
Imperatorin	**V**	Extrasynthese (Genay, France)
Isoscopoletin	**V**	Extrasynthese (Genay, France)
6-Methylcoumarin	**V**	Extrasynthese (Genay, France)
6-Methoxy-4 methylcoumarin	**V**	Extrasynthese (Genay, France)
7-Methoxy-4 methylcoumarin	**V**	Extrasynthese (Genay, France)
4-Methylumbelliferone	**V**	Extrasynthese (Genay, France)
Scoparone	**V**	Extrasynthese (Genay, France)
Scopoletin	**V**	Sigma Chemical Co (St.Louis, MO, USA)
Umbelliferone	**V**	Sigma Chemical Co (St.Louis, MO, USA)

4.3. SYNTHESIS OF 5-(HYDROXYMETHYL)ISOPHTHALIC ACID DERIVATIVES [I]

5-(hydroxymethyl)isophthalic acid derivatives (1a-j) for apoptosis studies were synthesized starting from diethyl-5-(hydroxymethyl)isophthalate as described in [**I**]. The chemicals and reagents used in the synthesis were commercially available from Aldrich Chemical Company (Schnelldorf, Germany) and Fluka Chemie (Buchs, Switzerland) and were used without purification. The structures of the 5-(hydroxymethyl)isophthalic acid derivatives are presented in Figure 2. The purity of the compounds was over 98%.



a	CH_2(*p*-*t*-Bu-Ph)	**f**	CH_2Ph
b	CH_2(*m*-CN-Ph)	**g**	CH_2(*m*-OMe-Ph)
c	CH_2(*m*-CF_3-Ph)	**h**	$CH_2(CH_2)_4CH_3$
d	CH_2(*m*-NO_2-Ph)	**i**	$CH_2(CH_2)_3CH_3$
e	CH_2(*m*-Cl-Ph)	**j**	CH_2(*c*-Hex)

Figure 2. The structures of the 5-(hydroxymethyl)isophthalic acid. R*indicates the places where substituents a-j are attached.

4.4. NATURAL PRODUCT EXTRACTION [II, VI]

Plants for the protein kinase C activity studies [**II**] were obtained from the herb collection of the Division of Pharmaceutical Biology, Faculty of Pharmacy, University of Helsinki. The plants were extracted with methanol (HPLC grade, Rathburn, Walkerburn, Scotland),

lyophilized and dissolved in dimethyl sulfoxide (DMSO) to yield a concentration of 40 mg/ml. The plants and their parts are presented in Table 5.

Table 5. Scientific names, parts studied (F= flower, L= leaf, S= stem, R= root) and herbarium numbers of plants used in PKC activity studies [**II**].

Scientific name	Studied parts	Herbarium numbers
Achillea millefolium L.	F, L, S, R	BH-01-F/L/S/R
Arctium tomentosum Mill.	F, L, S	BH-028-F/L/S
Berteroa incana (L.) DC.	F, L, S, R	BH-04-F/L/S/R
Calluna vulgaris (L.) Hull	F, L, S, R	BH-027-F/L/S/R
Campanula rotundifolia L.	F, L, S, R	BH-020-F/L/S/R
Epilobium parviflorum Schreb.	L, S, R	BH-06-L/S/R
Filipendula ulmaria (L.) Maxim.	L, S, R	BH-015-L/S/R
Hypericum maculatum Crants	F, L, S, R	BH-024-F/L/S/R
Lamium album L.	F, L, S, R	BH-014-F/L/S/R
Lysimachia vulgaris L.	F, L, S, R	BH-019-F/L/S/R
Melampyrum pratense L.	F, L, S, R	BH-021-F/L/S/R
Myosotis scorpioides L.	F, L, S, R	BH-017-F/L/S/R
Pteridium aquilinum (L.) Kuhn	L, S, R	BH-013-L/S/R
Ranunculus acris L.	F, L, S, R	BH-011-F/L/S/R
Sedum acre L.	F/L/S in same	BH-05-F/L/S
Silene dioica (L.) Clairv.	F, L, S, R	BH-03-F/L/S/R
Symphytum asperum Lepech.	L, S, R	BH-08-L/S/R
Symphytum officinale var. bohemicum L.	F, L, S, R	BH-026-F/L/S/R
Tanacetum vulgare L.	L, S, R	BH-023-L/S/R
Tripleurospermum inodorum (L.) Schultz Bip.	F, L, S, R	BH-018-F/L/S/R
Valeriana sambucifolia Mikan. f.	F, L, S, R	BH-016-F/L/S/R
Veronica longifolia L.	F, L, S, R	BH-025-F/L/S/R
Vicia cracca L.	L, S	BH-02-L/S

Ellagitannin and anthocyanin fractions and a concentrated extract of red raspberries [*Rubus idaeus* L. (v. Ottawa), Rosaceae] were used to evaluate effects on model drug permeability [**VI**]. The berries were extracted, ellagitannins and anthocyanins were isolated and phenolic

profiles were identified as described in Kähkönen et al. 2001 and 2003. The extract and fractions were dissolved in HBSS buffer solution pH 7.4 (Hank's balanced salt solution/Hepes buffer solution 1M, Biowhittaker, Verviers, Belgium) yelding the solutions of 0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml. The maintenance at pH 7.4 was checked after dilution of the samples.

4.5. APOPTOSIS ASSAYS [I]

HL-60 human promyelocytic leukemia cells and Swiss 3T3 fibroblasts for apoptosis studies were kindly provided by Prof. S.O. Døskeland (University of Bergen, Norway). The cells were maintained as described in **I**. 15,000 cells were plated on 96-well plates in HL-60 experiments and on 48-well plates in Swiss 3T3 experiments. The test compounds (Table 4 and Figure 2) were dissolved in DMSO and diluted so that the DMSO concentration in the assays did not exceed 0.1%. Natural compounds apigenin, esculetin, kaempherol, myricetin and quercetin were used to apoptosis assay development with morphological evaluation at concentrations of 20 μM, 60 μM and 100 μM (unpublished results). The variation between and within days was evaluated as well as the repeatability the sample preparation and assay performance. Novel derivatives of 5-(hydroxymethyl)isophthalic acid were first screened at concentrations of 20 μM, 60 μM and 100 μM with three replicates in the same plate and repeated at least two times in different days. Apoptosis inducing activity of 1c and 1h was studied further at concentrations of 10 μM, 20 μM, 60 μM, 100 μM and 10 μM, 20 μM, 40 μM respectively in leukemia cells.

DNA binding HOECHST 33342 (2'-[4-Ethoxyphenyl]-5'-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) stain was used to visualize degradation of the nucleus and to count the ratio of apoptotic cells by fluorescence microscopy. Cells were incubated with test compounds for 24 hours at 37ºC in a humidified atmosphere with 95% air and 5% CO_2. After incubation the cells were fixed with 2% formaldehyde in PBS solution (pH 7.4) with HOECHST 33342 indicator. 200 cells were evaluated by fluorescence microscopy (Nikon, type 108, Japan) from each well and the number of apoptotic cells was counted.

The most potent apoptosis inducers in HL-60 cells were studied further. The concentrations yielding 50% inhibition (IC_{50}) were determined by fitting the data into four parameter logistic

curves using SigmaPlot2002 for Windows Version 8.0 software (SPSS Inc., Chicago, IL, USA). In addition changes in mitochondrial membrane potential ($\Delta\psi m$) were detected by flow cytometry with JC-1 (5,6-dichloro-2-[3-(5,6-dichloro-1,3-diethyl-1,3-dihydro-2H-benzimidazol-2-ylidene)-1-propen-1-yl]-1,3-diethyl-iodide) staining and the activity of caspase-3 was detected using a commercial EnzChek Caspase-3 Assay Kit according to the manufacturer's instructions.

4.6. ASSAY FOR PROTEIN KINASE C ACTIVITY (II)

High-throughput PKC assay for natural product matrices was set up on a Biomek FX robotic workstation (Beckman Coulter Inc., CA, USA). The protocol was designed to handle four 96-well plates at a time. The assay included dilution of the compounds, addition of the reaction mixture, initiation of the enzymatic reaction with protein kinase C isolated from rat brain, and stopping the reaction with phosphoric acid. After a 12-minute enzymatic reaction samples from the 96-well plates were transferred to a 384-well filter plate and filtered with centrifugation before LC/MS analysis. The flavonoid compounds luteolin, myricetin and quercetin were used as reference substances.

The PKC modulating activities of 81 plant extracts of Finnish origin were investigated from altogether 23 plants at concentration of 40 mg/ml. The plants studied are listed in Table 5. One active extract was then investigated in more detail. The extract was acid hydrolyzed at 80°C for 30 minutes, fractionated by HPLC into 30-second fractions and analyzed by LC/MS. Identification of the active compound was based on retention time, UV spectra and MS studies.

4.7. PERMEATION STUDIES [III-VI]

Caco-2 cells (originating from American Type Culture Collection) for permeation studies were kindly donated by Professor Arto Urtti, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Finland. The cells were maintained as described in **III-V**.

For the permeation experiments, cells were seeded onto polycarbonate filter membranes with a pore size of 0.4 μm and growth areas of 1.0 cm^2 in clusters of 12 wells (Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.75×10^5 cells/cm^2 [**III-IV, VI**] or onto polycarbonate filter membranes with a pore size of 0.4 μm and growth areas of 0.11 cm^2 in clusters of 96 wells (MultiScreenTM Caco-2, Millipore, USA) at a density of 0.93-4.2x10^5 cells/cm^2 [**III**] and 2.1x10^5 cells/cm^2 [**V**]. Cells were grown on 12-well plates for 21-27 days [**III-IV, VI**] and on 96-well plates for 7 days [**III, V**]. The insert membrane on which cells were grown for the experiments is illustrated in Figure 3.

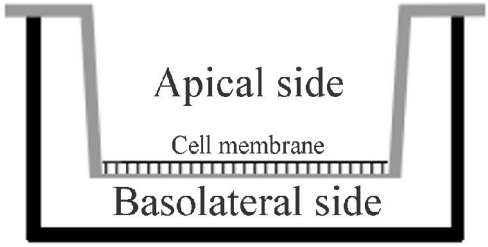


Figure 3. Insert membrane on which cells were grown for the permeation experiments.

The permeation test was performed at pH 7.4 in an incubator (Stuart, orbital incubator S150, Bibby Sterilin Limited, USA), in humidified atmosphere with shaking at 75 rpm at 37°C. Transepithelial electrical resistance (TEER) was measured before and after the experiments with an EVOMX voltohmmeter (EVOM™, World Precision Instruments Inc. USA) to evaluate the integrity of the monolayers. By the use of sink conditions, i.e. the quantity of transported compound in the acceptor chamber did not exceed 10% of the quantity in the donor compartment, the influence of the drug diffusing back from the receiving to the donor compartment could be minimized.

Samples from Caco-2 permeation experiments were analyzed using a reversed-phase HPLC system with a diode array detector. The apparent permeability coefficients P_{app} (cm/s) were calculated according to the equation (Artursson 1990):

$$P_{app} = (\Delta Q/\Delta t) / (A \times C_0)$$

where $\Delta Q/\Delta t$ is the flux of the compound across the monolayer, A is the surface area of the monolayer (cm^2), and C_0 is the initial drug concentration in the donor compartment (µg/ml).

4.7.1. Caco-2 permeability assay development and natural product screening [III, V]

To develop a new accelerated Caco-2 cell model, 7-, 10- and 21-day experiments were performed with several different cell densities and monolayer formation was evaluated daily by measuring transepithelial electrical resistance (TEER) [**III**]. The permeation of paracellular markers, fluorescent Lucifer Yellow (LY) and radioactive ^{14}[C]-mannitol was used after adequate cell growth for evaluation within experiments. The ^{14}[C]-activity was determined with Microbeta® Trilux liquid scintillation counter (PerkinElmer Life and Analytical Science/Wallac Oy, Turku, Finland) and the Lucifer Yellow permeation was measured with a Varioskan scanning spectrofluorometer and spectrophotometer (Thermo Electron Corporation, Vantaa, Finland) with exitation-λ 430 nm and emission-λ 535 nm. Permeability of 250 µM model compounds (FDA 2000) metoprolol, ketoprofen, verapamil, naproxen and hydrochlorthiazide across Caco-2 monolayer on 12-well plates and on 96-well plates apical to the basolateral (AP-BL) direction was compared. Samples were collected after 0, 20, 40, 60 and 120 minutes in both cases. The functionality of efflux protein P-glycoprotein (P-gp) was assessed using permeation studies with the known P-gp substrates rhodamine 123 alone and in the presence of verapamil (250 µM) AP-BL direction (50 µM) and basolateral to the apical (BL-AP) direction (5 µM). Samples were collected from AP-BL experiments after 0, 20, 40, 60 and 120 minutes and from BL-AP experiments after 0, 3.5, 7, 10 and 15 minutes to maintain sink conditions. The Varioskan scanning spectrofluorometer and spectrophotometer with exitation-λ 485 nm and emission-λ 535 nm was used to obtain the results. Monolayer formation and P-gp expression were confirmed by means of confocal laser microscopy using actin antibodies according to Maimone and Merlie (1993). Cells from passage numbers of 50 to 72 were used for the experiments.

Automated Caco-2 permeation experiments were set up on a Biomek FX workstation equipped with a 96-channel head. Pipetting parameters and robotic handling during the

process were optimized and the results of these experiments were compared to those obtained from manually performed experiments. The workstation was used to perform all pipetting steps as well as for sampling.

The permeability of a set of coumarins was evaluated using the 96-well Caco-2 permeation method [**V**]. Coumarins were first dissolved into DMSO, then diluted into HBSS (pH 7.4) to yield a final concentration of 250 μM. Coumarin 18 was prepared similar in manner with the exception that MeOH was used instead of DMSO. The final DMSO or MeOH concentration did not exceed 1%. Permeation tests were performed both apical to the basolateral (AP-BL) direction and basolateral to the apical (BL-AP) direction. Samples were collected after 0, 20 and 120 minutes and kept at -20°C until analyzed. The efflux ratio ((ER) = BL-AP/AP-BL)) was calculated according to Polli et al. (2001).

4.7.2. Evaluation of natural products in traditional Caco-2 permeability model [IV, VI] and in phospholipid vesicles [IV]

Caco-2 permeation experiments were conducted to study the apical to basolateral (AP-BL) transport of a set of flavonoids and alkyl gallates (**IV**) and to investigate the influence of natural product extracts and fractions on the permeation of model drug compounds (**VI**). Cells from passage numbers of 31 to 47 were used for the experiments [**IV**]. The flavonoids and alkyl gallates studied are presented in Table 4 and used at concentrations of 1000 μM (naringin, naringenin, methyl gallate, propyl gallate), 750 μM ((-)-epicatechin), 500 μM (morin, (+)-catechin, luteolin), 200 μM (octyl gallate), 100 μM (flavone) and 75 μM quercetin. The concentrations were selected based on toxicity studies. The studies were conducted AP-BL direction. Samples were collected from basolateral compartment after 0, 15, 30, 45, 60, and 90 minutes and from apical compartment after 60 and 90 minutes.

The permeation of 250 μM metoprolol, ketoprofen, verapamil and paracetamol was evaluated both alone and with raspberry samples at concentrations of 0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml. Samples were added to the apical compartment and the samples from basolateral side were collected after 0, 15, 30, 60, and 90 minutes. Cells at passage 31-42 were used for the experiments. Samples were kept at -20°C until analyzed.

Membrane affinity experiments [**IV**] were performed at the Department of Biochemistry and Pharmacy, Åbo Akademi University (Turku Finland). The association of model compounds with bilayer membranes comprising 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC) (Avanti PolarLipids, Birmingham, AL, USA) as bilayer forming phospholipid was studied using a fluorescence quenching method (Verkman 1980, van Dijk et al. 2000).

4.8. CYTOTOXICITY ASSAYS [I, IV-VI]

The LDH test was used to evaluate cytotoxicity by necrosis with the Swiss 3T3 and HL-60 cells in apoptosis studies [**I**]. Lactate dehydrogenase leakage was determined after 3 h incubation with the test compounds at concentrations of 20 µM, 60 µM and 100 µM using the commercial colorimetric cytotoxicity assay CytoTox 96® according to the manufacturer's instructions. The absorbance data was collected using a 96-well plate reader (Victor 1420 multilabel counter, PerkinElmer Life and Analytical Sciences/ Wallac Oy, Turku, Finland) at 490 nm.Assays were performed using 96-well plates with four replicates in the same plate and repeated at least twice on different days.

The MTT toxicity test was used with traditional 12-well Caco-2 cell experiments [**IV, VI**]. The assays were performed using 96-well plates and concentrations of 1000-100 µM flavonoids or 0.01 mg/ml, 0.1 mg/ml and 1.0 mg/ml raspberry samples with four replicates on the same plate. After 90 minutes test compound treatment absorbance data was collected using ELISA plate reader at 550 nm.

The WST-1 cell proliferation assay was used with 96-well Caco-2 cell experiments with 250 µM coumarins [**V**]. The assay was performed with a robotic workstation (Biomek FX) according to the manufacturer's instructions. Cells were incubated with test compounds for 2 hours. Absorbance data was collected using Varioskan scanning spectrofluorometer and spectrophotometer (Thermo Electron Corporation, Vantaa, Finland) at 440 nm. The assays were repeated at least twice on different days.

Cell viability in the presence of the test compounds was calculated as a percentage of the viability of untreated cells.

5. RESULTS

5.1. APOPTOSIS INDUCTION IN SWISS 3T3 FIBROBLASTS AND HL-60 PROMYELOCYTIC LEUKEMIA CELLS [I]

5.1.1. Method development with natural compounds [I]

Apoptosis is characterized by morphological features such as cell shrinkage, chromatin condensation and cellular fragmentation (Kerr et al. 1972). The process is rapid and causes no cellular injury to adjacent cells since cell fragments are rapidly phagocytosed (Steller 1995, Reed 2000). Fluorescence microscopy images of normal cells and an apoptotic cell are presented in Figure 4. In the apoptotic cell (B) the nucleus is fragmented and is clearly distinguishable from the nucleus of the healthy cells (A).

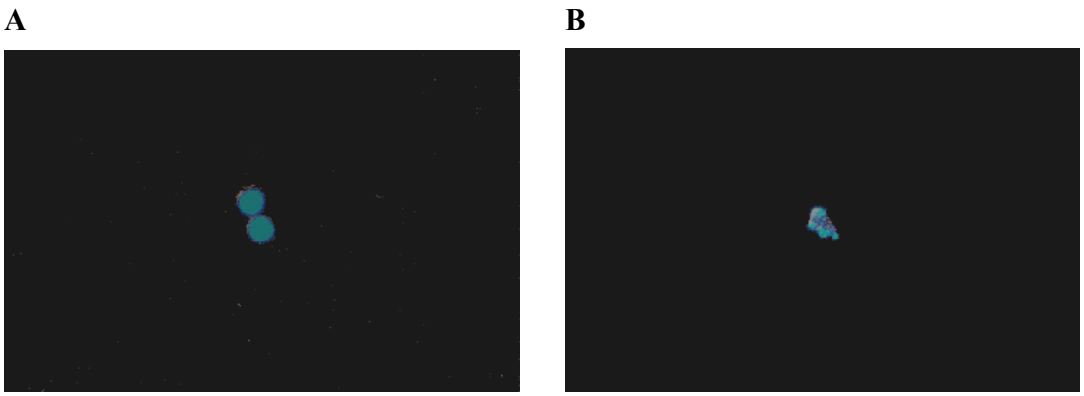


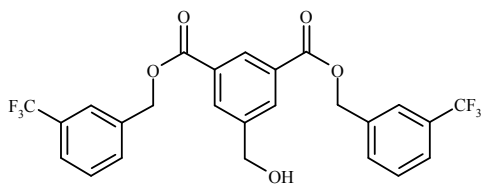
Figure 4. Microscopy image of HL-60 cells stained with the fluorescent dye HOECHST 33342. A normal cells, B apoptotic cell induced by 3% DMSO (Photograph taken by Anu Surakka 2002).

Cell growth density had no effect on spontaneous apoptosis in HL-60 cells between 200 000-600 000 cells/ml. In our experiments the starting cell density was 150 000 cells/ml, which was expected to double during incubation. Dimethyl sulfoxide (DMSO) was concluded to be the best solvent because of its ability to dilute compounds even from complex matrices and because of its low toxicity to these cells at concentrations below 1% (unpublished results).

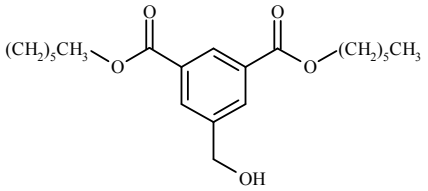
The apoptosis assay performance was first assessed with the known apoptosis inducers esculetin, quercetin, kaempherol, myricetin and apigenin at concentrations of 20 µM, 60 µM and 100 µM. All the compounds studied induced apoptosis in HL-60 cells but not markedly in fibroblasts. Experiments conducted on different days it was found that the deviation averaged 17% between days and 6% within days. This was seen even if the same dilutions of the compounds were used on separate days and different dilutions were used on the same day. The dilutions were stable at lest for ten days. It can be concluded that the assay performance is good and that cell growth is the major deviating factor in these experiments (unpublished results).

5.1.2. Apoptosis-inducing activity of derivatives of 5-(hydroxymethyl)-isophthalic acid [I]

The optimized method for measuring apoptosis was tested for a set of synthetic derivatives. After HL-60 and 3T3 Swiss cells were incubated with derivatives of 5-(hydroxymethyl)isophthalic acid for 24 h their morphology was evaluated. The most potent apoptosis inducers on leukemia cells were compounds 1c (Bis[3-trifluoromethylbenzyl]5-[(hydroxymethyl)isophthalate) and 1h (Dihexyl5-(hydroxymethyl)isophthalate), which had IC_{50} values of 41 µM and 23 µM, respectively, while their effects on fibroblasts were insignificant. The structures of 1c and 1h are presented in Figure 5. Other compounds were weaker apoptosis inducers in leukemia cells or they were non-selective. Changes in mitochondrial membrane potential ($\Delta\psi m$) and an increase in caspase-3 activity confirmed the apoptotic effects of 1c and 1h in HL-60 cells. Clear loss of $\Delta\psi m$ was observed in HL-60 cells after two hours' incubation with 40 µM of 1c and 1h and activation of caspase-3, one of the major effector caspases, was clearly increased after only one hour's incubation.



1c



1 h

Figure 5. Structures of the most potent apoptosis inducers of derivatives of 5-(hydroxymethyl)isophthalic acid 1c (Bis[3-trifluoromethylbenzyl]5-(hydroxymethyl)-isophthalate) and 1h (Dihexyl 5-(hydroxymethyl)isophthalate).

5.2. PKC ACTIVITY OF NATURAL COMPOUNDS AND PLANTS GROWING IN FINLAND [II]

The PKC-modulating activity of natural compounds and extracts from plants growing in Finland was investigated using a Biomek FX automated workstation followed by LC/MS analysis. The assay for 384 samples took about 1.5 hours and the samples could be analyzed by LC/MS in 27.2 hours. The values obtained from PKC inhibition studies with known PKC active compounds showed that our results correlated with values from the literature (Table 6).

Table 6. Results from analysis of known PKC-active compounds in relation to the literature values (Manuscript, Jokela et al.). IC50 is the concentration yielding 50% inhibition, activity %shows to which level (%) the PKC activity declined from full activation (100%) and inhibition % shows how much (%) the activity of PKC was inhibited.

Sample	Our study	Ref. value	Reference
	IC50 (µM)		
Staurosporine	1.3^{-3}	$0.9\text{-}2.7^{-3}$	Tamaoki et al. 1986, Vegesna et al. 1988, Combadière et al. 1993
Luteolin	44.6	15.5	Tammela et al. 2004
Quercetin	20	13.5	Tammela et al. 2004
Myricetin	18.8	7.8	Tammela et al. 2004
	Activity % at 60 µM		
Quercetin	28	22	Agullo et al. 1997
Myricetin	20	24	Agullo et al. 1997
	Inhibition % at 50 µM		
Quercetin	68	64	Ferriola et al. 1989
Luteolin	52	70	Ferriola et al. 1989

The developed method was used to evaluate plant samples. Twenty-one plant extracts from 7 plants inhibited protein kinase C significantly at concentration of 40 mg/ml (Figure 6). All extracts of *Calluna vulgaris, Epilobium parviflorum, Filipendula ulmaria, Hypericum maculatum* and *Lysimachia vulgaris* reduced the PKC activity to <5%. In addition the root extract of *Myosotis scorpioides* and leaf and root extracts of *Pteridium aquilinum* greatly inhibited PKC. Other plant extracts showed little or no PKC activity. No effects typical of a particular plant family could be seen with this set of compounds. On average, flower and leaf extracts were more potent PKC inhibitors than stem and root extracts. The activity was usually detected in all those parts of the plant studied.

The PKC inhibiting flavonoid compound quercetin could be identified from the active fractionated leaf extract of *Filipendula ulmaria*. The concentration of the quercetin was 30 μM. The results indicate that the method is suitable for finding active components from natural product matrices and provides a quick and low-volume non-radioactive alternative for PKC experiments.

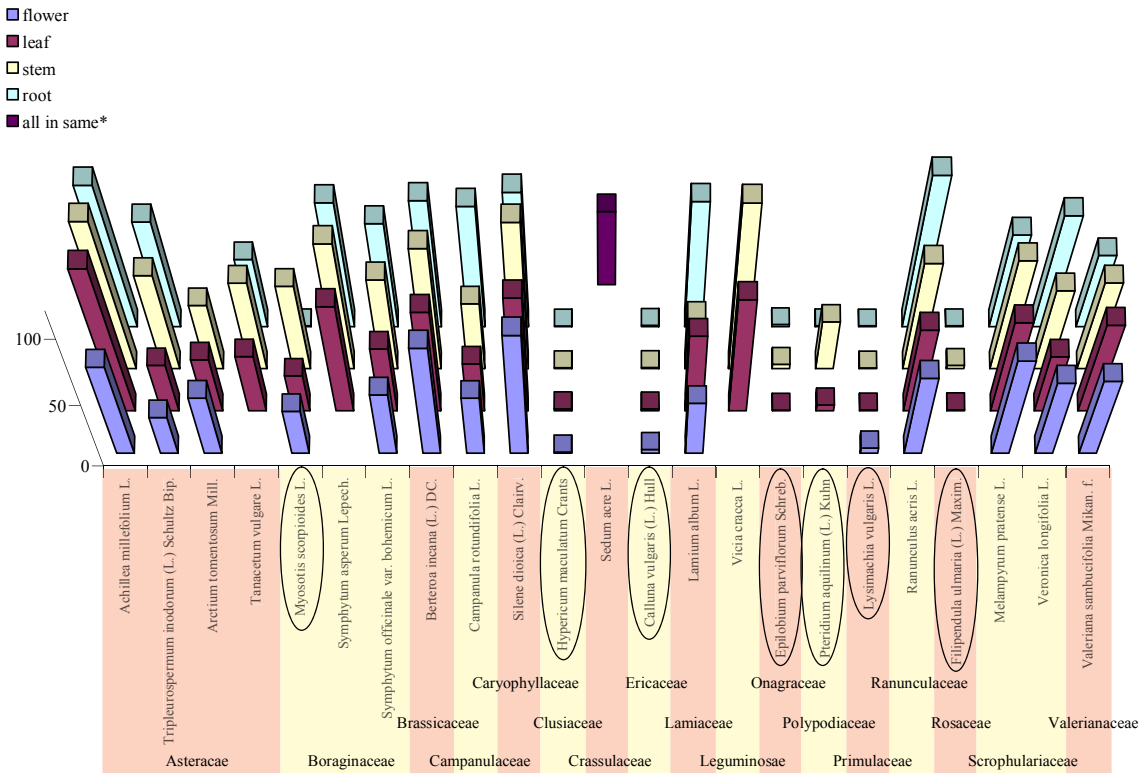


Figure 6. The PKC inhibition of Finnish plant extracts. The figure shows to which level (%) the PKC activity declined from full activation (100%). The most actively inhibiting plants are circled. Empty spots indicate that no such plant parts were available.
*used as whole plant

5.3. PERMEABILITY STUDIES WITH THE CACO-2 CELL METHOD [III-VI]

5.3.1. Development of the accelerated 96-well Caco-2 cell method [III]

Transepithelial electrical resistance measurement (TEER), which measures the resistance to passive ion transport and reflects the tightness and confluence of monolayers, was used to evaluate the formation of monolayers during cell growth with cell densities from 0.93×10^5 to 4.2×10^5 cells/cm^2 and growth times from 7 to 21 daysn at pH 7.4. With the lowest cell density the TEER values were low and a plateau was not reached until after 9 days' cell growth. With a density of 2.1×10^5 cells/cm^2 the TEER values obtained were most reproducible and stable at 600±70 Ωcm^2 (n=16). When the TEER data was combined with results from ^{14}[C]-mannitol

and Lucifer Yellow permeation experiments the results clearly indicated the advantage of this density. The ^{14}[C]-mannitol permeation rate stayed under 0.5% per 60 min and Lucifer Yellow rejection was 99.3±1.7% (n=160). The confocal imaging data confirmed that Caco-2 cells form monolayers when cultured for 7 days on MultiScreen^{TM} Caco-2 plates.

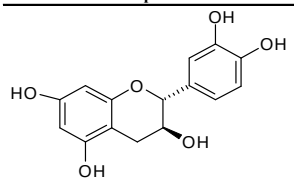
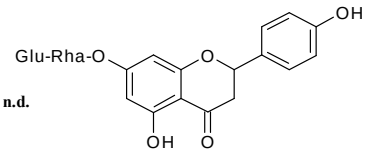
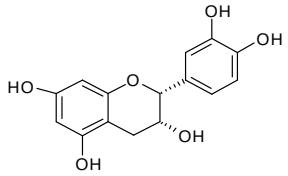
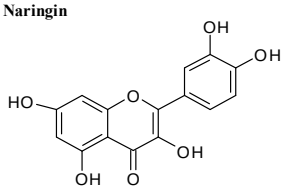
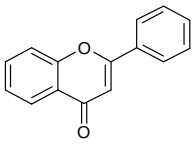
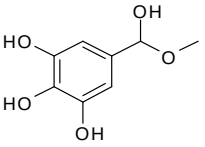
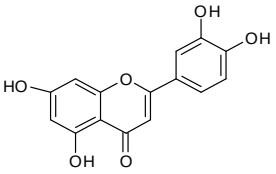
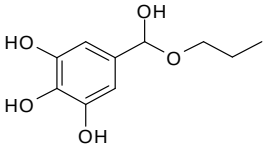
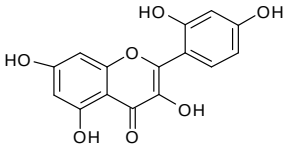
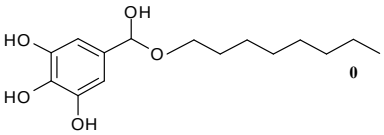
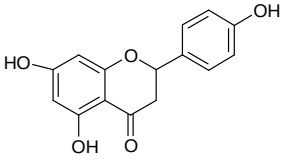
The P_{app} values obtained from transport experiments for 250 µM metoprolol, ketoprofen, verapamil, naproxen, and hydrochlorthiazide were 29.0±3.9 x10^{-6}cm/s, 24.5±3.5 x10^{-6}cm/s, 25.6±3.0 x10^{-6}cm/s, 32.5±0.9 x10^{-6}cm/s and 7.2±2.7 x10^{-6}cm/s on 12-well plates and 27.3±4.8 x10^{-6}cm/s, 33.4±1.6 x10^{-6}cm/s, 27.6±6.4 x10^{-6}cm/s, 36.9±1.6 x10^{-6}cm/s and 7.7±3.6 x10^{-6}cm/s on 96-well plates, respectively. The permeabilities on 96-well plates were comparable to those obtained from traditionally used 12-well plate experiments; the exception was ketoprofen. The permeation of ketoprofen in the 96-well plates was significantly higher than in the 12 well plates according to T-test ($p<0.01$).

Permeation of the P-gp substrate rhodamine 123 was higher in the BL-AP direction than the AP-BL direction. This difference was reduced in the presence of the P-gp inhibitor verapamil. These findings, together with those from the confocal laser microscopic studies, confirmed that P-gp was active in our cells.

5.3.2. Caco-2 permeability and membrane affinity of flavonoids and alkyl gallates [IV]

Caco-2 cells and phospholipid vesicles were used to study the transport and membrane interactions of flavonoids and alkyl gallates at pH 7.4. Rapidly permeating ketoprofen and paracetamol, with P_{app} values of 24.6x10^{-6}±4.8x10^{-6}cm/s and 22.0x10^{-6}±5.0x10^{-6}cm/s in Caco-2 cells and K_d values of 77.1±1.0 µM and 233.5±2.5 µM in phospholipid vesicles, respectively, were used as reference compounds. The P_{app} and K_d values obtained are presented in Table 7. Permeability coefficients from Caco-2 cell studies were obtained for flavone, naringenin, methyl gallate and propyl gallate, while polyhydroxylated flavonoids and long-chain alkyl gallates were retained on the cell membrane.

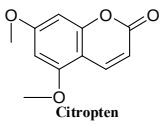
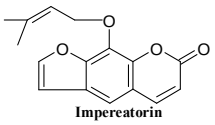
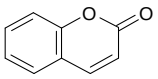
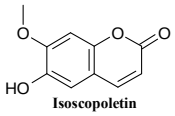
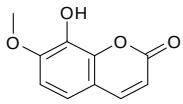
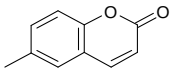
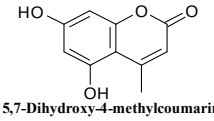
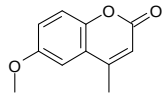
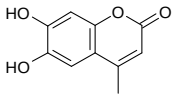
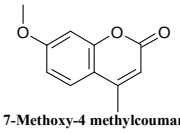
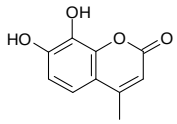
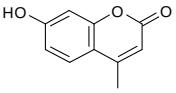
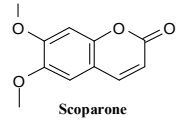
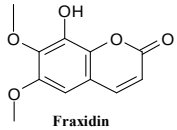
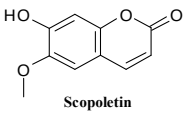
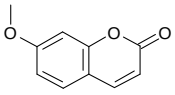
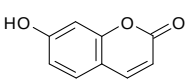
Table 7. Apparent permeability coefficients (P_{app}) from Caco-2 permeability studies and apparent partition coefficients (K_d) into phospholipid vesicles and structures for flavonoids and alkyl gallates. Values are means ±SD (n=3-4).
0= no transport to basolateral compartment detected.
n.d.= not determinable with the method employed.
Rha= rhamnoside; Glu= glucose

Compound	P_{app} (10^{-6}) cm/s	K_d µM	Compound	P_{app} (10^{-6}) cm/s	K_d µM
 (+)-Catechin	0	n.d.	 Naringin	0	n.d.
 (-)-Epicatechin	0	n.d.	 Quercetin	0	7.5±0.1
 Flavone	380.0±32.0	115±1.0	 Methyl gallate	2.9±0.2	n.d.
 Luteolin	0	7.1±0.2	 Propyl gallate	7.3±0.5	n.d.
 Morin	0	28.7±0.5	 Octyl gallate	0	n.d.
 Naringenin	29.4±3.1	11.2±0.3			

5.3.3. Permeability of coumarins across the Caco-2 cell monolayer [V]

The modified Caco-2 permeability model was used to evaluate the permeability of 18 coumarins with different numbers of OCH_3, OH and CH_3 groups at concentration of 250 μM at pH 7.4. The structures of these coumarins are presented in Table 8. The permeation test was performed in both AP-BL and BL-AP directions at pH 7.4. All coumarins permeated the Caco-2 cell monolayer rapidly. P_{app} values varied from 41×10^{-6} cm/s to 210×10^{-6} cm/s in the AP-BL studies and from 18×10^{-6} cm/s to 70×10^{-6} cm/s in the BL-AP studies. The compounds were more permeable in the AP-BL direction than in the BL-AP direction, which means the efflux ratio (ER) was less than 1 in all cases. The type and position of substituents affected permeability more than the number of substituents. No difference in the permeability of any compound was observed between days ($p>0.01$) according to the T-test.

Table 8. Structures and apparent permeability coefficients (P_{app}) ±SD (n=4) of coumarins from permeation studies with the accelerated Caco-2 cell model.

Coumarin structure	P_{app} (10^{-6}) cm/s AP-BL	P_{app} (10^{-6}) cm/s BL-AP	Coumarin structure	P_{app} (10^{-6}) cm/s AP-BL	P_{app} (10^{-6}) cm/s BL-AP
 Citropten	40.5±0.34	17.7±4.4	 Impereatorin	143.4±10.3	48.3±3.2
 Coumarin	147.2±0.50	64±1.6	 Isoscopoletin	102.9±11.8	45.5±0.68
 Daphnetin-7-methylether	119±2.9	51.8±4.4	 6-Methylcoumarin	71.1±0.86	47.1±4.7
 5,7-Dihydroxy-4-methylcoumarin	106.4±1.8	32.9±1.1	 6-Methoxy-4 methylcoumarin	137.6±2.1	70.4±1.4
 6,7-Dihydroxy-4-methylcoumarin	106.4±1.8	-	 7-Methoxy-4 methylcoumarin	68.4±1.4	37.2±3.2
 7,8-Dihydroxy-4-methylcoumarin	209.7±1.8	69±1.2	 4-Methylumbelliferone	160±18.4	44.7±4.6
 Esculin sesquihydrate	85.6±0.5	22.6±1.3	 Scoparone	101.4±2.24	53.4±1.55
 Fraxidin	118.1±13.5	52±0.11	 Scopoletin	122.9±9.9	52.4±1.18
 Herniarin	105.8±6.4	48.9±3.7	 Umbelliferone	105.6±10.1	57.3±2.32

5.4. EFFECTS OF RASPBERRY EXTRACT AND FRACTIONS ON THE PERMEABILITY OF MODEL DRUG COMPOUNDS [VI]

In order to evaluate the effect of concentrated natural products on pharmacotherapy, the effect of raspberry extract and fractions at concentrations of 0.01 mg/ml. 0.1 mg/ml, 1.0 mg/ml on the permeability of commonly used drugs at concentrationof 250 μM was studied using the Caco-2 cell model at pH 7.4. The P_{app} values obtained from transport experiments with metoprolol, ketoprofen, verapamil and paracetamol without raspberry samples were 32.5±2.4 $x10^{-6}$cm/s, 27.4±5.9 $x10^{-6}$cm/s, 27.8±1.7 $x10^{-6}$cm/s and 22.0±1.9 $x10^{-6}$cm/s, respectively. The effects of the raspberry samples on model drug permeability are presented in Figure 7.

5.5. CYTOTOXICITY STUDIES [I, IV-VI]

Cytotoxicity evaluations were performed during different stages of the study. The natural compounds used in the apoptosis studies were not cytotoxic for either Swiss 3T3 fibroblasts or HL-60 promyelocytic leukemia cells, and neither were the derivatives of 5-(hydroxymethyl)isophthalic acid 1c and 1h according to LDH cytotoxicity test [**I**]. Permeability studies with Caco-2 cells and flavonoids and alkyl gallates were not disturbed by toxic effects on mitochondria according to the MTT test [**IV**]. Neither did the raspberry samples in the Caco-2 permeation studies show any toxicity in the used concentrations [**VI**]. Of the coumarins studied [**V**], 4-methyl umbelliferone reduced the viability of the Caco-2 cells below 80% at a concentration of 250 μM. Daphnetin-7-methylether, herniarin, citropten and imperatorin increased viability by over 20% at a concentration of 250 μM.

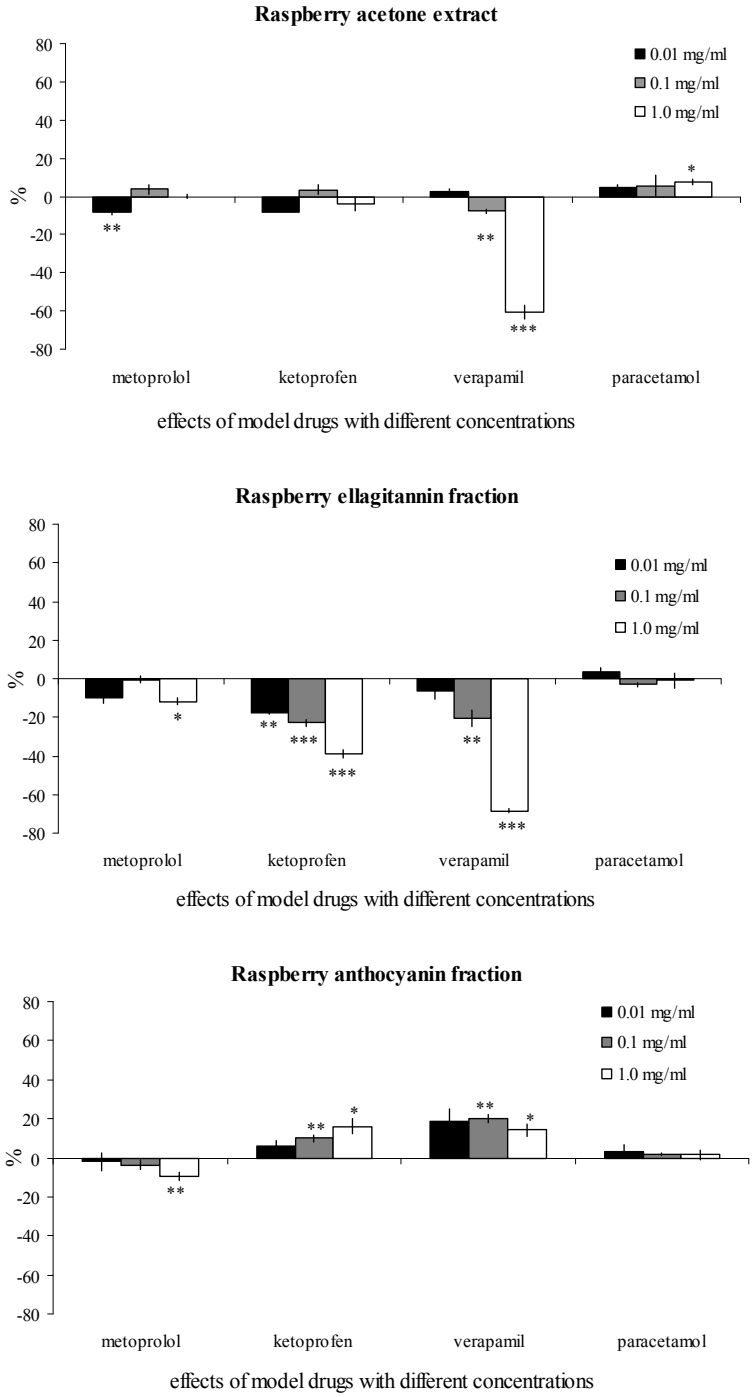


Figure 7. Changes (%) in permeabilities of metoprolol, ketoprofen, verapamil and paracetamol in the presence of raspberry acetone extract, raspberry ellagitannin fraction and raspberry anthocyanin fraction at concentrations of 0.01 mg/ml (black), 0.1 mg/ml (grey), or 1.0 mg/ml (white) across Caco-2 monolayers. Values are mean percentage differences (n=3) ±SD from the control values (model drugs without raspberry samples). *p < 0.1, **p < 0.05, ***p < 0.01.

6. DISCUSSION

6.1. APOPTOSIS INDUCTION AND PKC-INHIBITION IN RELATION TO CANCER PREVENTION [I-II]

Apigenin, esculetin, kaempherol, myricetin and quercetin were used in the development of apoptosis assay by means of morphological evaluation [**I**]. All these compounds are known to induce apoptosis in cancerous HL-60 cells (Xiao et al. 1998, Wang et al. 1999, Yamashita and Kawanishi 2000, Chu et al. 2001, Ramos 2007). They reduce the activity of anti-apoptotic Bcl-2 protein, stimulate release of cytochrome C from mitochondria and activate caspase 9 and caspase 3 and thereby induce apoptosis (Wang et al. 1999, Chu et al. 2001, Ramos 2007). It can be concluded from the results of this thesis that these natural compounds do not harm fibroblasts but are moderate to good apoptosis inducers in HL-60 cells [**I**]. In line with the results, flavonoids are shown to be more cytotoxic to malignant cells than to non-cancerous cells and more cytotoxic to human cells than to mouse cells (Fukai et al. 2000). These differences are assumed to be caused by metabolic differences between the mammalian species (Breinholt and Dragsted 1998).

Most of the natural compounds used in the studies of this thesis are regarded as having the ability to prevent cancer and to induce apoptosis (Table 9). The antiproliferative activity of flavonoids is said to be related to the existence of the C2-C3 double bond and the lack of a C6-hydroxyl group (Kawaii et al. 1999, Rusak et al. 2005). All flavones and flavonols used in the studies of this thesis meet these criteria [**IV**]. On the other hand, Kanno et al. (2006) and McMillan et al. (2007) have shown that naringenin, (+)-catechin and (-)-epicatechin, which have no C2-C3 double bond, have inhibitory effects on tumor growth. Studies with simple hydroxylated coumarins have indicated that the most potent and cancer cell selective compounds were 6,7-dihydroxylated coumarins such as esculetin and 6,7-dihydroxy-4-methylcoumarin (Kawase et al. 2003). Concentrations up to 200 µg/ml caused only minimal cytotoxicity in normal cells, while a concentration-dependent cytotoxicity was detected in tumor cells. These findings agree with those reported by Kawaii et al. (2000 and 2001). Alkyl substituents in positions 4 and 3 increased the activity even more (Kawase et al. 2003). However another study indicated that the 7,8-dihydroxy moiety is the most beneficial for anticancer activity (Riveiro et al. 2008).

The signaling pathways leading eventually to cancer prevention are very complicated and it is evident that phytochemicals act through multiple targeted pathways (Yang et al. 2006a, Heber

2008). It is difficult to fully compare the results from different studies and to decide the best cancer preventive structure, since the studies have been conducted with different cell lines, different sets of compounds and different concentrations. Nevertheless some structures seem to be more beneficial than others and the studies give ideas and templates for drug discovery purposes.

Table 9. Effects of the natural compounds studied on the topics discussed. The apoptosis induction and cancer prevention results are based on studies within the thesis or the literature cited. The PKC inhibition, Caco-2 permeation and Caco-2 cytotoxicity results are based on studies within this thesis or previous studies in our laboratory (Tammela et al. 2004). .* Indicates to the studies of this thesis and "indicates unpublished results in our laboratory. The + mark indicates action detected based on the literature cited and the – mark indicates that no action was observed. No mark indicates that no results were found for this kind of study.

	Apoptosis induction	PKC inhibition	Cancer prevention	Caco-2 permeation	Caco-2 Cytotoxicity	Ref.
Flavonoids						
Apigenin	+*	-	+*		-*	
(+)-Catechin	+	-	+	-*	-*	McMillan et al. 2007
(-)-Epicatechin	+	-	+	-*	-*	McMillan et al. 2007
Myricetin	+*	+*	+*		-*	
Naringenin	+	-	+	+*	-*	Kanno et al. 2006
Naringin	-	-	-	-*	-*	Chen et al. 2003
Flavone		-		+*	-*	
Kaempherol	+*	+	+*		-*	
Luteolin	+	+*	+	-*	-*	Yang et al. 2008
Morin	+	-	+	-*	-*	Kuo et al. 2007
Quercetin	+*	+*	+*	-*	-*	
Alkyl gallates						
Methyl gallate		-		+	-*	
Octyl gallate		-		-	-*	
Propyl gallate		-		+	-*	
Coumarins						
Citropten		-"		+*	**>120***	
Coumarin	+	-"	+	+*	-*	Chuang et al. 2007
Daphnetin-7-methylether		-"		+*	**>120***	
5,7-Dihydroxy-4-methylcoumarin		-"		+*	-*	
6,7-Dihydroxy-4-methylcoumarin	+	-"		+*	-*	Kawase et al. 2003
7,8-Dihydroxy-4-methylcoumarin	+	-"	+	+*	-*	Riveiro et al. 2008
Esculetin	+	-"	+	+*	-*	Riveiro et al. 2008
Esculin sesquihydrate		-"	+	+*	-*	Yang et al. 2006b
Fraxidin		-"		+*	-*	
Herniarin		-"		+*	**>120***	
Impereatorin	+	-"	+	+*	**>120***	Pae et al. 2002
Isoscopoletin		-"		+*	-*	
6-Methylcoumarin		-"		+*	-*	
6-Methoxy-4 methylcoumarin		-"		+*	-*	
7-Methoxy-4 methylcoumarin		-"		+*	-*	
4-Methylumbelliferone		-"	+	+*	**<80***	Yoshihara et al. 2005
Scoparone		-"	+	+*	-*	Yang et al. 2007
Scopoletin	+	-"	+	+*	-*	Manuele et al. 2006
Umbelliferone	+	-"	+	+*	-*	Elinos-Baez et al. 2005

The flavonoid compounds kaempherol, quercetin, flavone, naringenin and apigenin have been shown to inhibit inflammatory nitric oxide production and the activation of nuclear factor κB (NF-κB) (Hämäläinen et al. 2007). NF-κB is a central coordinator of immune responses and plays also a critical role in cancer development and progression (Karin 2006, Okamoto et al. 2007, Johnson 2007). Also PKC inhibition has been shown to down-regulate nitric oxide production but in an NF-κB-independent manner (Salonen et al. 2006). Based on Table 9, apoptosis induction and cancer prevention are not related to protein kinase C inhibition. The protein kinase C experiments referred to were conducted with rat brain homogenate, which is assumed to contain at least α, βI, βII, γ, δ and ζ PKC isoforms (Kishimoto et al. 1989, Tammela et al. 2004). As in this case, the different isoforms may possess opposite effects related to apoptosis; the activation of some isoform may be repealed with another and thus overall PKC inhibitory effect can not be seen.

Derivatives of 5-(hydroxymethyl)isophthalic acid were designed *in silico* to bind PKC [**I**]. They showed high affinity for the diacylglycerol binding site of PKC and were thought to modify the function of PKC. Unfortunately *in vitro* studies did not confirm this presumption. Some of the compounds were able to modify phorbol ester binding indirectly via a conformational change of the phorbol ester binding site (manuscript, Boije af Gennäs et al.). It is possible that these compounds also modify the activity of protein kinases other than PKC. Nevertheless, derivatives of 5-(hydroxymethyl)isophthalic acid 1h and 1c showed to be potent and selective apoptosis inducers based on the morphological evaluation, loss of mitochondrial membrane potential (Δψm) and increase in caspase-3 activity. Dissipation of Δψm and release of cytochrome C from mitochondria have been shown to be key events during apoptosis (Green and Kroemer 2004) and were thus used to verify the preliminary results from the morphological evaluation. The results show that these compounds trigger the mitochondrial pathway of apoptosis, which is thought to be important in response to cancer treatment (Sjöström and Bergh 2001). The results indicate that compounds with this core structure can be useful as anticancer agents and should be studied further.

Besides apoptosis induction, luteolin, myricetin and qurcetin have been shown to inhibit PKC (Ferriola et al. 1989, Agullo et al. 1997, Tammela et al. 2004) and thus these compounds were used as reference compounds for evaluation of new PKC assay [**II**]. The new PKC assay makes possible to enhance the sample throughput and accuracy of the method. The old

method suffered the radioactive [γ^{32}P]ATP detection not suitable for automation as well as low repeatability due to filtration step. The detection in the new method with LC/MS enables structure determination of the active compound in line with the analysis. The results from analysis of the reference compounds showed that the new method gives reliable results. All of the studies compared to, were done with radioactive method using rat or bovine brain homogenate. Although some differences are seen, especially compared to the results of Tammela et al. (2004) the ranking order of the results is still the same. The results from studies of Ferriola et al. (1989) and Agullo et al. (1997) correlated more to our results even if Agullo et al. (1997) used bovine brain homogenate instead of rat brain homogenate.

The new PKC assay was the used to study Finnish plant eftracts [**II**]. Seven of the 21 extracts showed significant PKC inhibition when rat brain homogenate with α, βI, βII, γ, δ and ζ PKC isoforms (Kishimoto et al. 1989, Tammela et al. 2004) was used [**II**]. At least to our knowledge these plants have not previously been reported to possess PKC activity although they have been reported to contain PKC-active flavonoids (Rzadkowska-Bodalska and Olechnowicz-Stepien 1975, Hiermann 1982, Allais et al. 1991, Lamaison et al. 1992, Martonfi et al. 2006). In our earlier studies *Filipendula ulmaria* was shown to possess antimicrobial activity, and *Filipendula ulmaria* and *Calluna vulgaris* were shown to possess antioxidant activity (Kähkönen at al. 1999, Rauha et al. 2000). *Filipendula ulmaria* has been used in Russia as a folk medicine (Bespalov et al. 1992, Spiridonov et al. 2005). It has been found to inhibit carcinogenesis and suppress cell growth (Bespalov et al. 1992, Spiridonov et al. 2005) and also to promote healing of chronic ulcers (Barnaulov and Denisenko 1980). These studies were made with an aqueous or ethanol extract of *Filipendula ulmaria* flowers, whereas we demonstrated PKC inhibition with methanol extracts of leaf, root, and stem samples. Quercetin, which was shown to be one of the compounds responsible for the PKC-activity in this plant, can be extracted in both ethanol and methanol, and to some extent even in water. Quercetin is also usually distributed throughout the plant.

6.2. ABSORPTION STUDIES [III-V]

6.2.1. Permeation method development with Caco-2 cells [III]

In order to make absorption studies more effective, a rapid permeability screening method was developed for the drug discovery process [**III**]. The method can be used to provide first

information about the permeability of pharmacologically active compounds. Traditional Caco-2 cell cultures are grown on 12- or 24-well plates for 21 to 28 days, which is time consuming, laborious, expensive and low throughput to gain firsthand information about the permeability of compounds. To make these experiments more efficient we aimed to reduce the cell growth time, to automate the permeation study and to miniaturize the format.

The results obtained from this study are in good correlation with studies of Alsenz and Haenel (2003) on 96-well plates. Although their plate and feeding system was different, they used seeding densities comparable to ours. The ratio of the cell count between 21-day growth on 12-well plates and 7-day growth on 96-well plates was the same (Alsenz et al. 1998, Alsenz and Haenel 2003). Balimane et al. (2004) and Marino et al. (2005) used lower cell densities on 96-well plates, but they grew the cells for three weeks.

Cell behavior was stable during the passages 50-72 used in this study. The optimum passage range has been suggested to be 28-65 (Briske-Anderson et al. 1997) but our study indicates the usefulness of higher passages as well. In some cases high passages are reported to promote rapid cell growth and monolayer formation (Briske-Anderson et al. 1997, Yu et al. 1997). Nevertheless inter-laboratory differences are high emphasizing the use of standardized growth protocols and cell behavior monitoring in each case during cultivation.

The efficacy of many drugs depends on their ability to cross cellular barriers. P-gp and other efflux proteins act as a permeability barrier. P-gp is present on the apical surface of enterocytes and the efflux ratio P_{app}BL-AP/P_{app}AP-BL is extensively used to evaluate how P-gp-mediated efflux activity affects the transport of P-gp substrates (Polli et al. 2001, Troutman and Thakker 2003, Balimane et al. 2006). In our study, secretory transport of P-gp substrate rhodamine 123 was affected in the presence of P-gp inhibitor verapamil and no effect was seen on the absorptive flux. These findings, together with those from confocal laser microscopic studies, confirmed that P-gp was active in the cells. It has been reported that the functionality of MDR1 decreases in passages 45+ (Siissalo et al. 2007), but this was not the case with our cell line. Monitoring P-gp functionality during passages 50-72 showed that expression was steady during these passages. The results emphasize the importance of defining activity when using cells of different origins and different passages.

Studies with model compounds showed that, as expected, metoprolol permeation was high and hydrochlorthiazide permeation was low. Verapamil permeability was high and the efflux

was probably saturated (Garrigos et al. 1993, Balimane et al. 2006) and the effect of P-gp could not be seen. Data analysis with the unpaired t-test showed a small difference in the permeability of naproxen and a significant difference in the permeability of ketoprofen. These acidic compounds are transported not only via the transcellular route but also by active transport (Tamai et al. 1995, Choi et al. 2005). In Caco-2 cells carrier-mediated transport can be variable and is generally lower than seen *in vivo* (Hu and Borchardt 1990, Lennernäs et al. 1996). Carrier expression might differ under different growth conditions, which could explain the differences between these two formats. On the other hand, the deviation was very small, especially in the case of naproxen, indicating that permeation is stable. Despite the small difference in naproxen permeation, the magnitude of the permeation data from the 96-well plate and 12-well plate experiments and permeation characteristics are similar. This indicates that acidic compounds carried by active transporters can also be studied accurately with our 96-well permeation model.

The permeability assay was set up on a Biomek FX workstation. The suitability of the assay for automation was evaluated by conducting several experiments. During aspiration, the height of the pipet tips was studied carefully to obtain as much liquid as possible while at the same time trying to avoid harming the cells. Automation of the protocol made it more accurate and allowed a large number of samples to be handled. Also, when the 96-well plate method is used, 18 times less medium is needed during cultivation and the growth time is 1/3 of that needed in the old 12-well plate method. These modifications significantly reduce labor and material costs and allow more experiments to be carried out in less time. The permeation studies with coumarins indicated that the repeatability of the new permeation model is good and that the results are accurate. Based on the obtained results we managed to build up a fast miniaturized and automated protocol to make a first permeation evaluation of new compounds in the drug discovery process.

6.2.2. Absorption characteristics of natural compounds [IV-V]

To have beneficial effects in the human body, compounds have to be absorbed. Even small modifications in molecular structure may change a compound's biological and biochemical properties (Rice-Evans 2004). The structural differences between flavonoids relate to the chemistry of the C ring, variations in the number and distribution of phenolic hydroxyl groups

and the extent and nature of their substitution. The planar molecular configuration of flavonols favors membrane interactions in contrast to the tilted configuration of flavanones (van Dijk et al. 2000). Flavone, which has no hydroxyl groups, was transported very rapidly across the Caco-2 cell membrane and had poor affinity for the phospholipid membrane, whereas strong membrane affinity was associated with poor transport across the Caco-2 monolayer [**IV**]. As the number of OH-groups in the flavonoids increased, permeability decreased and membrane affinity increased. Naringenin, which has three hydroxyl groups, had a stronger affinity for the membrane and a lower permeation rate than the analog with no hydroxyl groups. Naringin – the rhamnoglucoside conjugate of naringenin – was not able to permeate the Caco-2 monolayer. In nature, flavonoids are present mainly as glycosides. Flavonoid glycosides are in general poorly absorbed (Ameer et al. 1996, Fuhr 1998, Murota 2000, Serra et al. 2008). Removal of the sugar moiety enhances absorption (Murota et al. 2002, Serra 2008), as was seen in the present study [**IV**]. A slight lengthening of the alkyl chain had no significant effect on the behavior of alkyl gallates. Both methyl and propyl gallates were transported across the Caco-2 cell monolayer. When the alkyl chain was long, as in the case of octyl gallate, no transport across the Caco-2 monolayer was observed. This finding also indicates poor absorption of such compounds from the gut lumen.

Coumarins are part of the human diet. Common herbs like dill (*Anethum graveolens*) and parsley (*Petroselinum crispum*) contain many coumarins including for example scopoletin and esculetin, and coumarin and xanthotoxin, respectively (Murray et al. 1982). Grapefruit juice has been shown to inhibit CYP3A4 activity, a molecule involved in the metabolism of about 50% of all drugs (Edwards et al. 1996, Kakar 2004). A furanocoumarin compound 6',7'-dihydroxy-bergamottin is said to be the main inhibitor, but the parent compound bergamottin and many furanocoumarin monomers and dimers have also been shown to be CYP3A4 inhibitors as well (Paine et al. 2005, Row et al. 2006 a, b, Oda et al. 2007). The furan ring has proved to be essential for CYP inhibition. Grapefruit juice components are also able to inhibit P-glycoprotein (P-gp) and thus modify the disposition of drugs that are P-gp substrates (Ohnishi et al. 2000, De Castro et al. 2007). In the small intestine CYP3A4 and P-gp and other efflux proteins act synergistically and serious unwanted effects might occur if substrates for them are administrated simultaneously. On the other hand, premeditated administration of certain furanocoumarin derivatives might enable doses of drugs to be reduced, which would in turn reduce side effects (Oda et al. 2007). These issues evoked an idea to study the permeation of coumarins [**V**]. The compounds were selected according to their structure, the aim being to discover how the type, position and number of substituents

can affect permeability. The results suggest that all the coumarins studied are highly permeable in the gut lumen and that P-gp efflux does not limit absorption. The sink conditions (the amount of the receiver compartment <10% of the doneor compartment) were not maintained, but this does not change the conclusions made by the results since the permeation was so high. Moreover, it can also be concluded that the type and position of substituents affect permeability more than the number of substituents on the structure.

6.3. ACTIVITY ON CONTEMPORARY PHARMACOTHERAPY [VI]

Interest in preventive health care is growing and the food industry is producing not only added value products containing minerals and vitamins, but also functional foods containing a range of different ingredients (Tulp et al. 2006). Besides benefiting health, natural products may influence the absorption of other compounds simultaneously existing in the intestine. Even common foods may cause serious undesirable effects, especially if consumed solely and/or excessively. In the case of medication, the risk for adverse effects cumulates**.** People often do not even know that they are exposed to certain compounds that may affect their wellbeing. Some drugs appear to be safe when taken with food, but less safe if taken with fortified foods (Rates 2001, Wallace and Amsden 2002). Numerous *in vitro* and *in vivo* studies have shown that herb products and dietary supplements interact with many drugs (Hu et al. 2005, Chavez et al. 2006, Pal and Mitra 2006, Venkataramanan et al. 2006). The interactions often involve transporter systems and drug metabolizing enzymes, especially CYP3A4 (Fuhr 1998, Fugh-Bergman 2000, Plant 2007).

Raspberry extract and ellagitannin fraction showed very similar effects on the permeation of the model drugs studied, whereas the effect of the anthocyanin fraction was smaller and opposite [**VI**]. All the model drugs are transported mainly via the transcellular route. Moreover ketoprofen is thought to be transported by the monocarboxylic acid transporter MCT1 (Tamai et al. 1995, Choi et al. 2005) and verapamil is a known substrate of P-glycoprotein (Tsuruo et al. 1981, Orlowski et al. 1996, Collet et al. 2004). All the raspberry samples studied lowered metoprolol permeability slightly. However the effect was so small that severe adverse effects are unlikely with this medicine and these kinds of raspberry samples. The permeability of verapamil decreased considerably during co-administration with the raspberry extract, with an ellagitannin content of 50% of total phenolics (554 mg/g of

freeze dried extract), and the ellagitannin fraction with an ellagitannin content of 80% (Vuorela et al. 2005). Ketoprofen permeability was markedly decreased by the ellagitannin fraction in a dose-dependent manner. On the contrary, the anthocyanin fraction increased the permeabilities of ketoprofen and verapamil, although the effect was not that clear as with the ellagitannin fraction. The permeability of paracetamol was not affected by any of the raspberry samples. High TEER values after the experiments (500-600 Ωcm^2) indicated that paracellular spaces were not affected and that the monolayers were intact. This shows that the permeability differences seen are due to raspberry components and not to rupture of the cell membrane.

Ellagitannnis are not absorbed in human body as such but are metabolized by the intestinal flora to yield urolithins (Cerda et al. 2005, Espin et al. 2007). Caco-2 cell studies with ellagic acid, complexed in the form of ellagitannins, have showed that apical to basolateral (AP-BL) transport across cell membrane was minimal (Whitley et al. 2003). Ellagitannins and ellegic acid have been indicated to accumulate to the cells (Simon et al. 2003, Whitley et al. 2003). The interaction with the cell membranes could explain also the reduced permeation of ketoprofen and verapamil. Absorption study of anthocyanins across Caco-2 cell monolayers indicate that anthocyanins can be transported cross the cell membrane in intact glycone form although the transportation efficiency was relative low (Yi et al. 2006, McGhie and Walton 2007). Moreover metabolism of anthocyanins by CYP3A4 isoform, methylation and conjugation with glucuronide has been described (McGhie and Walton 2007, Dreiseitel et al. 2008,). Although the CYP activity in Caco-2 cells is generally low, the metabolism of anthocyanins might have a contribution to the permeability changes of model drugs. The regional change in the membrane due to slowly permeating anthocyanins could enhance the permeability of verapamil and ketoprofen as well. Nevertheless, more mechanistic studies must be conducted to fully characterize the mechanisms of actions.

The results from studies with raspberry extracts and fractions clearly showed that when components are used in more concentrated form, adverse effects may occur even though a less concentrated mixture of the same components lacks the effects. In some cases the effects might even disappear (Houghton 2000). For example, a mixture with synergistically acting substances can have an activity even though no single compound in the mixture has the effect. That is why different products have to be examined case by case and generalizations should not be made. For example, in our earlier studies (Laitinen et al. 2004) both the glycosidic and aglyconic raspberry extracts exhibited only very minor effects on drug permeation. Numerous

factors, including soil and climate, ripeness at the time of harvest, extraction, processing and storage affect the polyphenol content of plants. Different batches of the same product can thus have different effects, highlighting the importance of chemically specified samples (Kyle and Duthie 2006).

6.4. CYTOTOXICITY [I, IV-VI]

Cytotoxicity studies were included to eliminate the possibility of false positives caused by compound cytotoxicity. The LDH toxicity assay, used with the apoptosis studies [**I**], measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis into the supernatants (Korzeniewski and Callewaert 1983). LDH oxidizes lactate to pyruvate which then reacts with tetrazolium salt to form formazan. The amount of red formazan formed is proportional to lysed cells. The MTT-assay, used with traditional Caco-2 cell experiments [**IV, VI**], measures mitochondrial function (Mosman 1983). MTT is a yellow colored tetrazolium salt (3-[4,5-dimethylthiezol-2yl]-2,5-dipenthyltetrazolium bromide) which is cleaved by mitochondrial succinate dehydrogenases of living cells into dark blue formazan. In dead or damaged cells this reaction is not happening since the cells posses reduced or no succinate activity at all. The extent of the formazan formed is used for a colorimetric assay to determine viability. The WST-assay, used automated environment with 96-well plate Caco-2 experiments [**V**], is more developed version of MTT-test (Ishiyama et al. 1995). The assay is also based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells. The assay is simpler due to the soluble end product. In MTT assay the formed formazan is poorly dissolved which complicate the measurement.

The low cytotoxicity of the compounds used in the apoptosis studies confirmed that the cell death caused by these compounds at concentrations of <100 μM was due to apoptosis and not necrosis [**I**]. In some other studies quercetin has been shown to be cytotoxic to HL-60 cells over 100 μM concentrations (Dičkancaitė et al. 1998) and myricetin has been shown to be safe for HL-60 cells at concentrations up to 100 μM (Dimas et al. 2000).

Of the tested coumarin derivatives, 4-methyl umbelliferone reduced the mitochondrial enzyme activity of the Caco-2 cells, while daphnetin-7-methylether, herniarin, citropten and

imperatorin increased the enzyme activity by over 20% [**V**]. Compounds similar in structure to 4-methyl umbelliferone in addition of one OH-group did not decrease the mitochondrial enzyme activity. In some other studies 4-methyl umbelliferone has been shown to inhibit cell proliferation in human keratinocytes (Rilla et al. 2004) and imperatorin to affect energy transfer in rabbit mitochondria at a concentration of 300 μM (Kramar and Kaiser 1968). However, the toxic effects of certain compounds in our studies do not seem to affect the permeability of compounds since the cell monolayers remained intact. Based on the results, these compounds did not harm the Caco-2 cells at the concentration of 250 μM.

7. SUMMARY AND CONCLUSIONS

To create new methods for drug discovery, especially for natural product research, apoptosis and protein kinase C (PKC) inhibition assays were built up. The apoptosis study was optimized using natural compounds that are not harmful to Swiss 3T3 fibroblasts but are moderate to good inducers of apoptosis in human promyelocytic leukemia HL-60 cells**.** The assay for apoptosis was used for a group of synthesized derivatives of 5-(hydroxymethyl)isophthalic acid which were designed to inhibit PKC. Two of these compounds seemed to be potent and selective apoptosis inducers in HL-60 cells. The compounds trigger the mitochondrial pathway of apoptosis, which is thought to be important in response to cancer treatment. Thus these compounds should be studied further for their possible use in cancer therapy. The study of the PKC-inhibiting activity of plant extracts indicated that plants in Finland are a rich source of PKC bioactive compounds. The method seems suitable for PKC screening purposes from complex matrices and provides a quick and low-volume non-radioactive alternative to traditional PKC experiments.

The Caco-2 permeation model is widely used mehod for evaluation of the absorption of different compounds. To adapt the model to the needs of screening, we made the permeation experiments more efficient by reducing cell growth time, automating the permeation study and miniaturizing the protocol. The method cuts labor and material costs and increases the throughput of the experiments.

The absorption characteristics of flavonoids, alkyl gallates and coumarins were evaluated using Caco-2 cells. The degree of hydroxylation, molecular configuration and the length of the side chain governed ability of flavonoids and alkyl gallates to cross the Caco-2 cell monolayer and to be retained on phospholipid membranes. The studies with coumarins indicated that the type and position of substituents affected permeation of these compounds more than the number of substituents in the structure. The results indicated that all the coumarin compounds are higly permeable and P-gp efflux is not limiting the absorption.

The absorption studies with concentrated raspberry samples with concomitant medicines suggest that when components are used in more concentrated form adverse effects might occur even though a more dilute mixture of the same components had no such effects. Raspberry extract and ellagitannin fraction showed very similar effects on the permeation of the model drugs studied, whereas the effect of the anthocyanin fraction was smaller and

opposite. Caution must always be kept in mind when using natural products, especially in concentrated forms and together with medication. Problems might occur when a natural product inhibits or activates crucial enzymes with wide substrate specificity. This may lead to an accumulation or a reduction of drug compounds and cause serious unwanted effects, especially within drugs with narrow therapeutic window.

With efficient methods the screening of natural products is fast and easy despite the complex matrices. When various methods are combined, extensive knowledge of the behavior of natural compounds can be gained for the benefit of health.

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