Studies on Coumarins and Coumarin-Related Compounds to Determine their Therapeutic Role in the Treatment of Cancer

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Abstract: The Benzopyrones are a group of compounds whose members include coumarins and flavonoids. Dietary exposure to benzopyrones is quite significant, as these compounds are found in vegetables, fruit, seeds, nuts, coffee, tea and wine. It is estimated that the average western diet contains approximately 1g/day of mixed benzopyrones. It is, therefore, not difficult to see why extensive research into their pharmacological and therapeutic properties is underway over many years. Coumarin is a natural substance that has shown anti-tumour activity in vivo, with the effect believed to be due to its metabolites (e.g. 7-hydroxycoumarin). This review is based on recent studies of coumarins and coumarin related compounds. Therefore, the focus will be on these relevant compounds and their therapeutic importance.

A recent study has shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is overexpressed in many types of cancer. This knowledge may lead to its use in cancer therapy. Esculetin inhibits growth and cell cycle progression by inducing arrest of the G1 phase in HL-60 leukaemia cells, resulting from the inhibition of retinoblastoma protein phosphorylation. Recent studies investigating the potential of flavonoids as therapeutic agents have suggested they may have use in various therapeutic settings ranging from leukaemia treatment to the treatment of patients with HIV. Genistein is a well-known isoflavone and is a tyrosine kinase inhibitor. Studies have indicated that genistein elicits inhibitory effects on cell growth of various carcinoma cell-lines and may be a potential candidate for cancer therapy.

In our research, we have investigated the effects of coumarins and coumarin-related compounds on a panel of cell-lines. The most recent work involves two cell-lines, MCF-7 a breast carcinoma and A549 a lung carcinoma. Microtitre assays were performed along with real-time analysis of cell viability using a biosensor called the Cytosensor microphysiometer. These studies suggest that both genistein and esculetin exerted the most potent inhibitory effect on cell growth in comparison to the other two compounds.

Key Words: Coumarin, 7-hydroxycoumarin, esculetin, warfarin, genistein, benzopyrones, flavonoids, coumarin derivatives, furanocoumarins, pyanocoumarins, pyrone-substituted coumarins, pharmacokinetics, cytochrome P450 microtitre assay, cell proliferation, MTT, LDH, BrdU, Acid Phosphatase, Cytosensor Microphysiometer, MCF-7, A549, toxicity, signal transduction, cancer treatment, anti-proliferation, cytostatic.

INTRODUCTION

Coumarins owe their class name to ‘Coumarou’, the vernacular name of the tonka bean (Dipteryx odorata Willd., Fabaceae), from which coumarin itself was isolated in 1820 [1]. Coumarin is classified as a member of the benzopyrone family of compounds, all of which consist of a benzene ring joined to a pyrone ring [2]. The benzopyrones can be subdivided into the benzo-α-pyrones to which the coumarins belong and the benzo-γ-pyrones, of which the flavonoids are principal members (Fig. 1.1).

OCCURRENCE

There are four main coumarin sub-types: the simple coumarins, furanocoumarins, pyranocoumarins and the pyrone-substituted coumarins (Table 1.1). The simple coumarins (e.g. coumarin, 7-hydroxycoumarin and 6,7-dihydroxycoumarin), are the hydroxylated, alkoxylated and alkylated derivatives of the parent compound, coumarin, along with their glycosides. Furanocoumarins consist of a five-membered furan ring attached to the coumarin nucleus, divided into linear or angular types with substituents at one or both of the remaining benzoid positions. Pyranocoumarin members are analogous to the furanocoumarins, but contain a six-membered ring. Coumarins substituted in the pyrone ring include 4-hydroxycoumarin [3]. The synthetic compound, warfarin, belongs to this coumarin subtype. By virtue of its structural simplicity coumarin has been assigned as head of the benzo-α-pyrones, although it is generally...
accepted that 7-hydroxycoumarin be regarded as the parent compound of the more complex coumarins (Table 1.1) [4]. Genistein is an isoflavone and belongs to the benzo-γ-pyrones. It is a natural component of soy and has been intensively investigated as a chemopreventive agent, mainly against hormonally regulated breast and prostate cancers in animal models [5].

Coumarins comprise a very large class of compounds found throughout the plant kingdom [6-8]. They are found at high levels in some essential oils, particularly cinnamon bark oil (7,000 ppm), cassia leaf oil (up to 87,300 ppm) and lavender oil. Coumarin is also found in fruits (e.g. bilberry, cloudberry), green tea and other foods such as chicory [9]. Most coumarins occur in higher plants, with the richest sources being the Rutaceae and Umbelliferae. Although distributed throughout all parts of the plant, the coumarins occur at the highest levels in the fruits, followed by the roots, stems and leaves. Environmental conditions and seasonal changes can influence the occurrence in diverse parts of the plant [3]. Recently six new minor coumarins have been isolated from the fruits and the stem bark of Calophyllum dispar (Clusiaceae). The genus Calophyllum, which comprises 200 species, is widely distributed in the tropical rain forest where several species are used in folk medicine [10].

Although most of the natural coumarins in existence have been isolated from the higher plants, some members have been discovered in microorganisms (Fig. 1.2). Some important coumarin members have been isolated from microbial sources e.g. novobiocin and coumermycin from Streptomyces, and aflatoxins from Aspergillus species [11, 12]. The aflatoxins are a group of highly toxic fungal metabolites and the most commonly occurring member of the group is aflatoxin B1 [3]. Coumarin group antibiotics, such as novobiocin, coumermycin A1 and clorobiocin, are potent inhibitors of DNA gyrase. These antibiotics have been isolated from various Streptomyces species and all possess a 3-amino-4-hydroxy-coumarin moiety and a substituted deoxysugar; noviose, as their structural core that is essential for their biological activity. Chlorobiocin differs from novobiocin in that the methyl group at the C-8 of the coumarin ring is replaced by a chlorine atom, and the carbamoyl at the 3' of the noviose is substituted by a 5-methyl-2-pyrrolcarboxyl group. Coumermycin A1 contains two of the coumarin-noviose core joined by a 3-methyl-2,4-dicarboxyl pyrole

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Table 1.1. The Four Main Coumarin Subtypes. The Main Structural Features and Examples of Each Coumarin Subtype are Illustrated in this Table

<table>
<thead>
<tr>
<th>Classification</th>
<th>Features</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIMPLE COUMARINS</td>
<td>Hydroxylated, alkoxylated or alkylated on benzene ring</td>
<td><img src="image" alt="7-hydroxycoumarin" /></td>
</tr>
<tr>
<td>FURANOCOUMARINS</td>
<td>5-membered furan ring attached to benzene ring. 5-membered furan ring attached to benzene ring.</td>
<td>Psoralen <img src="image" alt="Psoralen" /></td>
</tr>
<tr>
<td></td>
<td>Linear or Angular</td>
<td>Angelicin <img src="image" alt="Angelicin" /></td>
</tr>
<tr>
<td>PYRANOCOUMARINS</td>
<td>6-membered pyran ring attached to benzene ring. 6-membered pyran ring attached to benzene ring.</td>
<td>Seselin <img src="image" alt="Seselin" /></td>
</tr>
<tr>
<td></td>
<td>Linear or Angular</td>
<td>Xanthyletin <img src="image" alt="Xanthyletin" /></td>
</tr>
<tr>
<td>PYRONE-SUBSTITUTED COUMARINS</td>
<td>Substitution on pyrone ring, often at 3-C or 4-C positions</td>
<td><img src="image" alt="Warfarin" /></td>
</tr>
</tbody>
</table>
linker and has the same substituted noviose as in chlorobiocin [13].

PHARMACOKINETICS

The pharmacokinetics of coumarin, including the excretion of various metabolites, were elucidated over many years. Coumarin is rapidly and almost completely metabolised with little unchanged compound excreted [14].

Absorption and Distribution

Following oral administration, coumarin is rapidly absorbed from the gastrointestinal tract and is distributed throughout the body [9]. Coumarin and 7-hydroxycoumarin are both poorly soluble in water (0.22 and 0.031 %, respectively). These percentages indicate coumarin may have reduced bioavailability in vivo, as 0.3% solubility in water is considered the critical value at which the distribution of a compound limits its rate of absorption. However, both compounds have high partition coefficients (21.5% for coumarin and 10.4% for 7-hydroxycoumarin), which is considered favourable for the rapid absorption of compounds once they are in aqueous solution. This coupled with the fact that coumarin is non-polar, suggests that in theory coumarin should cross lipid bilayers easily by passive diffusion [12].

Pharmacokinetic studies in humans have demonstrated that coumarin is completely absorbed from the GIT after oral administration and extensively metabolised by the liver in the 1st pass with only between 2 and 6% reaching the systemic circulation intact [9]. The low bioavailability of coumarin, in addition to its short half-life (1.02 hrs peroral v 0.8 hrs intravenous) has brought into question its importance in vivo and it is now accepted that coumarin is a pro-drug, with 7-hydroxycoumarin being the compound of main

Fig. (1.2). Important coumarin members isolated from microbial sources: [A] Novobiocin, [B] Coumermycin A₁, [C] Clorobiocin.
therapeutic relevance. At normal therapeutic plasma concentrations many drugs exist in the plasma mainly in the bound form. Ritschel [15] and colleagues have shown that 35% of coumarin and 47% of 7-hydroxycoumarin bind plasma proteins. Availability of the compounds at their target tissues should not be problematic since the proportions that bound were well below the accepted critical value of 80% binding. The pharmacokinetics of coumarin have been studied in a number of species including the rat, dog, gerbil, rhesus monkey and in man [9]. Specific antibody recognition for its antigen is the basis for very selective and sensitive analytical methods. This was exploited in numerous formats for the pharmacokinetic determination of coumarin and its derivatives. Immunoanalytical approaches have included ELISA-based methods for the detection of coumarin and 7-hydroxycoumarin in urine [16]. Antibody-based biosensors have also been employed, with either electrochemistry, or surface plasmon resonance (BIAcore) facilitating detection of coumarin compounds in various matrices [17, 18].

Metabolism

Traditionally coumarin has been viewed by pharmacologists as the ideal model for studying the complex metabolism of a structurally simple organic molecule, and as such, its metabolic fate has been extensively researched [9, 19, 20]. Determining the metabolic fate of coumarin is important in order to utilise the fact that it is metabolised at several sites, and to access the possible dependence of coumarin-induced toxicity on metabolism [14].

The superfamily of cytochromes P450 (CYPs) consists of microsomal hemoproteins that catalyse the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds. The CYP superfamily is divided into families and subfamilies according to their nucleotide sequence homology. Most biotransformations of xenobiotics (such as drugs) are performed by enzymes from the families CYP1, CYP2 and CYP3. The CYP2 family have been examined using the rat, mouse and rabbit model systems. This family includes seven subfamilies in mammals. In humans, the most important CYPs regarding drug metabolism are CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 [21].

Coumarin is metabolised initially by specific cytochrome P-450-linked mono-oxygenase enzyme (CYP2A6) system in liver microsomes, resulting in hydroxylation to form 7-hydroxycoumarin. After 7-hydroxylation, coumarin undergoes a phase II conjugation reaction resulting in a glucuronide conjugation associated with 7-hydroxycoumarin. The 7-hydroxylase activity is exceptionally high in human liver microsomes compared with its activity in the livers of other animal species. The activity of coumarin 3-hydroxylase is very high in rodent microsomes but is absent in human microsomes. Although coumarin may be metabolised by hydroxylation at all six possible positions (i.e. carbon atoms 3,4,5,6,7, and 8), the most common routes of hydroxylation are at positions 7 and 3 to yield 7-hydroxycoumarin and 3-hydroxycoumarin, respectively (Fig. 1,3). 7-hydroxylation has received the most attention among the various metabolic steps, predominantly because it is the major metabolic route in humans and is easily analysed. Hydroxylation at carbon 3 results in further metabolism via ring opening, yielding two further products, o-hydroxyphenyllactic acid (o-HPLA) and o-hydroxyphenylacetic acid (o-HPAA) [9, 14]. The expression of CYP enzymes (e.g. CYP2A6) varies between individuals due to genetic and environmental factors. These factors produce inter-individual variation in the metabolism of drugs such as coumarin. The frequency of poor metabolisers varies between species, races and ethnic groups. It has been shown that there exists large inter-species and inter-individual variability in the activity of these enzymes [21].

Table 1.2. Extent of Coumarin Metabolism to 7-HC in Various Species. *Coumarin Administered Orally Unless Otherwise Stated

<table>
<thead>
<tr>
<th>Species</th>
<th>Dose (mg/kg)</th>
<th>Collection time (hr)</th>
<th>Urinary 7-HC (% of Dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>100</td>
<td>890 or 120</td>
<td>0.4</td>
</tr>
<tr>
<td>Mouse</td>
<td>21. i.p.</td>
<td>22</td>
<td>25</td>
</tr>
<tr>
<td>Syrian Hamster</td>
<td>200</td>
<td>24</td>
<td>5</td>
</tr>
<tr>
<td>Squirrel Monkey</td>
<td>200</td>
<td>24</td>
<td>1</td>
</tr>
<tr>
<td>Baboon</td>
<td>200</td>
<td>24</td>
<td>60</td>
</tr>
<tr>
<td>Human</td>
<td>200mg/subject</td>
<td>24</td>
<td>79 (range 68-92)</td>
</tr>
<tr>
<td></td>
<td>200mg/subject</td>
<td>24</td>
<td>63 (range 40-97)</td>
</tr>
</tbody>
</table>

Table 1.2. Extent of Coumarin Metabolism to 7-HC in Various Species. *Coumarin Administered Orally Unless Otherwise Stated

aShilling et al. (1963). bEgan et al. (1990)
an oral dose of coumarin has been employed as a biomarker of human hepatic CYP2A6, the cytochrome P-450 (CYP) isoform which is responsible for coumarin 7 hydroxylation in human liver [14]. Some individuals can metabolise a considerable proportion of coumarin through pathways other that 7-hydroxylation such as the 3,4-epoxidation pathway to \( o \)-HPAA.
In humans, there are three genes in the CYP2A sub-family, however, CYP2A6 is mainly of greater importance, as the other two gene products (CYP2A7 and CYP2A13) are either inactive or are not expressed in the liver. CYP2A6 codes the enzyme catalysing coumarin 7-hydroxylation (about 10% of total P450) [26]. Recently, CYP2A6 has been reported to be polymorphically expressed in the human liver. It has been shown that CYP2A6 participates in metabolism of nicotine and its metabolite cotinine. Some drugs and chemicals, including coumarin, which is widely used as a probe substance for CYP2A6 both in vitro and in vivo, are also metabolised by this enzyme [27]. Substrates and inhibitors currently known to be metabolised by or interfere with CYP2A6 in vitro and in vivo have been summarised by Pelkonen [26]. Although 7-hydroxycoumarin is the main human metabolite, other hydroxylation pathways are important in humans and, as such, the therapeutic relevance of non-7-hydroxymetabolites should be examined rather than disregarded.

TOXICOLOGY

Since 1954, coumarin has been classified as a toxic substance by the FDA, following reports of its possible liver tumour-producing properties in rats [28]. The FDA banned its use, labelling as adulterated all foods containing coumarin [12]. Due to tests performed on rodents coumarin was referred to as a chemical carcinoenby by NIOSH [National Institute for Occupational Safety and Health]. However, caution needs to be taken in extrapolating this information to human situations. Various tests (Ames, micronucleus) have shown that coumarin and its metabolites are non-mutagenic [6]. Preliminary results from early studies indicated that coumarin was a toxin, but it has been shown since, that the rat is a poor model to compare with the human for this particular metabolism [29]. A number of studies have examined the acute, chronic and carcinogenic effects of coumarin in the rat and mouse. In studies involving the rat, hepatic biochemical and morphological changes have been examined for various periods of coumarin administration (1 week to 2 years). Depending on dose administered, coumarin treatment results in an increase in relative weight and changes in various hepatic biochemical parameters. Single oral doses of coumarin have been shown to produce liver necrosis and increase plasma transaminase activities in DBA/2 strain mice [9].

In contrast, studies involving baboons, syrian hamsters and certain mice strains seem to be resistant to acute coumarin-induced hepatotoxicity. Species differences in coumarin-induced toxicity in vitro have been investigated in cultured hepatocytes. These studies provide evidence for species differences in coumarin-induced toxicity in vitro. The relative resistance of human and cynomolagus monkey liver slices and/or hepatocytes to coumarin toxicity correlates with coumarin 7-hydroxylation, the major pathway of coumarin metabolism in these species, being a detoxification pathway of coumarin metabolism. However, while coumarin-7-hydroxylation pathway is a detoxification pathway this does not appear to be the only explanation for resistance of a species to coumarin-induced toxicity. In the rat coumarin-induced hepatotoxicity appears to be partially attributable to the excretion of coumarin metabolites in the bile. This may result in enterohepatic circulation enhancing the exposure of liver cells to toxic coumarin metabolites. Species such as syrian hamster, baboon, and humans excrete coumarin metabolites primarily in urine. Low level exposure to coumarin from diet and from fragrances used in cosmetic products would not be expected to produce any hepatotoxicity even in individuals with deficient 7-hydroxylase activity [9, 14].

APPLICATIONS OF COUMARIN AND COUMARIN DERIVATIVES

The coumarins are of great interest due to their biological properties. In particular, their physiological, bacteriostatic and anti-tumour activity makes these compounds attractive for further backbone derivatisation and screening as novel therapeutic agents. Weber [30] and co-workers have shown that coumarin and its metabolite 7-hydroxycoumarin have antitumour activity against several human tumour cell lines. Both coumarin and coumarin derivatives have shown promise as potential inhibitors of cellular proliferation in various carcinoma cell lines [12, 31, 32]. In addition it has been shown that 4-hydroxycoumarin and 7-hydroxycoumarin inhibited cell proliferation in a gastric carcinoma cell line [33].

SIMPLE COUMARINS

Coumarins and the benzopyrones are representative of a very diverse and potentially useful groups of drugs.

Clinical Uses

Due to its biochemical properties coumarin was proposed for use in clinical medicine. It had been evaluated for the treatment of various clinical conditions, resulting in the use of a variety of dosing regimes. Recommended doses range from 8mg for the treatment of venous constriction to 7000mg/day in anti-neoplastic therapies.

High Protein Oedema (HPO)

The lymph system is responsible for drainage of interstitial fluid within human tissues. Oedema interferes with the metabolism of the tissue cells and reduces oxygen transport, resulting in problematic wound healing [34]. In the case of high protein oedemas (HPO), there is an accumulation of protein in the tissue following trauma or inflammation, with resulting permeability of the capillaries causing water leakage in the tissue spaces. Many disease states are associated with high protein oedemas, ranging from extremely severe and chronic (e.g. lymphoedema and elephantiasis) through more common and acute forms (e.g. burns, accidental and surgical traumas). All forms have been shown to benefit from benzopyrone treatment [35]. Coumarin and numerous other benzopyrones have been tested in high protein oedema, and all have been shown to successfully reduce the swelling. However, according to Loprinzi [36] and colleagues, coumarin treatment alone is not effective therapy for women who have lymphoedema of the arm after treatment for breast cancer. It may be possible to increase the beneficial therapeutic effect of coumarin by using it with other compounds in combination treatments. The objective of a recent study was to evaluate the oedema-protective
effect of a combination vasoactive drug, coumarin/troxerutin (SB-LOT) plus compression stockings in patients suffering from chronic venous insufficiency after decongestion of the legs as recommended by the new guidelines. The study confirms the oedema-protective effect of SB-LOT in chronic venous insufficiency and provides a treatment option for patients who discontinue compression after a short time [37].

### Chronic Infections

In addition to its stimulatory effect on macrophages, coumarin has been shown to activate other cells of the immune system. In chronic brucellosis *Brucella abortis* infects macrophages, thus evading the immune response [38]. When immunostimulatory drugs such as coumarin are administered, the symptoms of chronic brucellosis disappear. These results have encouraged the use of coumarin in other chronic infections such as mononucleosis, mycoplasmosis, toxoplasmosis and Q fever. A new antiplasmodial coumarin has been isolated from the roots of *Toddalia asiatica*. This finding supports the traditional use of this plant for the treatment of malaria [39].

### Application of Simple Coumarins in Cancer Treatment

Anti-cancer drugs have traditionally been targeted to damage the aberrantly dividing cell by interrupting the cell division process [40]. Reagents used include DNA intercalating agents (e.g. adriamycin), DNA cross-linking agents (e.g. cis-platin), topoisomerase inhibitors (e.g. camptothecins), cytoskeleton-disrupting agents (e.g. vinblastin) and anti-metabolites (e.g. mercaptopurine). These drugs though effective, are cytotoxic, and thus exhibit severe side effects, particularly on normal proliferating tissues such as the haematopoietic system. Often combination therapies, whereby several cytotoxic agents are combined in the treatment regime, offer better results with fewer toxic side-effects, as they are carefully regulated to allow recovery of normal, but not malignant cells, from drug exposure [40].

Currently, chemotherapy, radiotherapy and surgery combined offer the best outcomes for cancer patients, and treatment combinations have been successfully applied to particular cancer types, for example, Hodgkin’s lymphoma, testicular cancer and various leukemias. Coumarins can be used not only to treat cancer but to treat the side effects caused by radiotherapy. A recent study investigated the efficacy of coumarin/troxerutine combination therapy for the protection of salivary glands and mucosa in patients undergoing head and neck radiotherapy. The results suggest that coumarin/troxerutine have a favourable effect in the treatment of radiogenic sialadenitis and mucositis [41]. The interest in coumarin and 7-hydroxycoumarin as anti-cancer agents, arose from reports that these agents had achieved objective responses in some patients with advanced malignancies.

### Coumarin in Malignant Melanoma

Early diagnosis of malignant melanoma facilitates surgical removal of the primary lesion and achieves a good prognosis. However, if the lesion progresses, the risk of recurrence becomes serious and represents a major challenge to the oncologist, as no satisfactory treatment for recurrent malignant melanoma currently exists. Studies have shown that five years after removal of the primary lesion, the recurrence of malignant melanoma is observed in 55-80% of high risk patients [42].

Original work with coumarin derivatives in the treatment of melanoma focused on the use of warfarin as a maintenance therapy. This compound was known to inhibit tumour spread, and to stimulate granulocytes, lymphocytes and macrophages [43]. Thones then began to assess the potential application of coumarin, the parent compound of warfarin, as an adjuvant therapy in melanoma. Like warfarin, the *in vivo* actions of coumarin were known to be macrophage-derived. Coumarin was non-toxic and conveniently administered, it had no anti-coagulant activity, and a previous administration resulted in subjective improvement in cancer patients [44, 45]. A more recent study by Velasco-Velazquez [46] and colleagues determined the *in vitro* effects of 4-hydroxycoumarin (4-HC) employing the murine melanoma cell line B16-F10 and the non-malignant fibroblastic cell line B82. 4-HC disorganized the actin cytoskeleton in B16-F10 cells, but not in B82 fibroblasts. Adhesion of tumour cells to extracellular matrix is required during the metastatic process, therefore, 4-HC might be useful as an adjuvant therapy for melanoma.

### Coumarin in Renal Cell Carcinoma

The clinical course of renal cell carcinoma (RCC) has been well documented, with long-lasting stable periods and rapid tumour growth its principal features. Surgery remains the standard care for patients whose tumour is confined to the kidney. However, many of these patients develop recurrent or metastatic disease within months, where the lungs, liver and bones are the common sites of secondary occurrence [47]. The outlook for patients with metastatic RCC is poor, with a 5-year survival rate of less than 10% [48].

Interest in the coumarin family of compounds stemmed from reports by Thones, of the immunomodulatory activity of coumarin and its utility in malignant melanoma [49]. The clinical activity of coumarin in renal cell carcinoma patients has been investigated by applying a treatment regime described by Thones (coumarin at 100mg/day oral dosage, with the addition of cimetidine 4 X 300mgs/day from day 15). This preliminary study yielded some interesting results, with 14 objective results among 45 patients with metastatic RCC, and almost no toxic side effects. Validation of this anti-tumour activity was further demonstrated by other investigators [50, 51].

Following this success, it became clear that additional information regarding doses and toxicities was required, and Marshall and colleagues implemented a phase I trial to define the maximally tolerated dose, and dose-limiting toxicities of coumarin and cimetidine. All coumarin doses were well tolerated, with the most common side effect of nausea attributable to the intense aroma of coumarin. Objective responses were observed in 7 patients, all with renal cell carcinoma, these responses being observed across a range of coumarin doses (600-5000 mgs) [52]. *In vitro* cytotoxic potential and mechanism of action of selected coumarins using renal cell lines have been investigated.
recently [8]. The results obtained suggest that the coumarins examined may have a potential therapeutic role to play in the treatment of renal cell carcinoma.

**Coumarin in Prostate Cancer**

Prostate cancer is the most common invasive malignancy in males and is characterised by a very slow growth rate and a wide biological variability, especially with regard to hormonal sensitivity [53, 54]. These two traits have curbed attempts at curative treatments for patients, as most effective chemotherapeutic drugs rely on fast growth kinetics in the tumour mass, and, due to differential hormonal dependencies, hormonal therapy (androgen deprivation) is not effective in all cases. At present, early detection, and removal of clinically significant tumours by surgery or radiation, have been the focus of clinical strategies. However, patient survival is dependent on metastases where principal metastatic sites are regional lymph nodes and bone. Eventually, almost every prostate carcinoma that initially regressed on androgen deprivation will relapse into a hormonal-insensitive state and grow in the absence of androgen. Evidently, better therapeutic approaches to control both metastases and hormone-insensitive prostate carcinomas are required.

As coumarin had previously appeared to exert immunomodulating effects in other cancers, a small-scale study to test the efficacy of coumarin in prostate cancer was set up [55]. A phase I trial involving 40 patients with metastatic, hormone-naïve or hormone-refractory prostate cancer was conducted [56]. Participants were administered 3g of coumarin daily, and evaluated for toxicity and anti-tumour responses. 3 partial responses occurred, all in patients with low tumour loads. One responder remained with 3 responsive bone metastases and stable prostate specific antigen (PSA) levels for 7 years following the trial. Myers [57] and co-workers examined the effects of various concentrations (0-500 μg/ml) of coumarin on the proliferation of two renal cell carcinoma cell lines (786-O and A-498) and two malignant prostatic cell lines (DU145 and LNCaP). After 5 days of treatment, coumarin inhibited the growth of the four cell lines. The LNCaP prostatic cell line was most sensitive to the inhibitory effects of coumarin.

**Coumarin in Leukaemia**

The effect of esculetin, coumarin and 7-hydroxycoumarin on the cell cycle and its regulatory molecules has been investigated. The cytostatic and cytotoxic nature of 8-nitro-7-hydroxycoumarin (8-NO2-7-OHC) was determined using both human and animal cell lines grown in vitro. This compound displayed cytotoxic properties in two human cell lines tested (HL-60 and K562), inducing cell death by apoptosis. This compound imposed a cytostatic effect on the three other cell lines tested, exerted through a perturbation in their cell cycle [31]. The effect of a number of coumarin compounds on the growth, metabolism and cell signalling of human tumour cell lines was examined by Cooke [12]. Overall esculetin exhibited the strongest antiproliferative effect on the carcinoma cell lines tested [32]. Given the importance of signalling anomalies in cancer cells, tests were performed to determine if the cellular target of 7-hydroxycoumarin was a signalling pathway component. Both 7-hydroxycoumarin and esculetin were found to inhibit tyrosine phosphorylation in EGF-stimulated tumour cells in a time- and dose-dependent manner. It appears that this effect may be achieved by reduction of the tyrosine kinase activity of the EGF-Receptor [12].

A recent study has presented evidence that esculetin affected phosphorylation of pRB thus inducing G1 arrest of human leukaemia HL-60 cells. The results demonstrated that the treatment with esculetin resulted in an accumulation of hypophosphorylated pRB in HL-60 cells along with reductions of both cyclin D1 and E. This induced the arrest of the cell cycle at the G1 phase. Among the released proteins, the E2F family of transcription factor has central position. Not only does E2F induce gene expression necessary for DNA synthesis, it also contributes to the regulation of the cyclin D1 and E genes. Esculetin treatment also induced enhanced expression of the CDK1 p27 and a reduced expression of CDK-4, thus inhibiting pRB phosphorylation [58].

In a separate study aiming to clarify mechanisms of action of 7-hydroxycoumarin and coumarin, the effect on cell cycle progression of the human adenocarcinoma cell line A427 was investigated. These cells are pRB positive and have homozygous deletions at the gene of p16INK4A. The results showed that 7-hydroxycoumarin had greater cytostatic activity than coumarin. The inhibition of the cell-cycle at transition G1/S is consistent with the cytostatic effect of 7-hydroxycoumarin. Furthermore, the decrease in the percentage of cells expressing cyclin D1 indicates that the action of 7-hydroxycoumarin involves early events in phase G1. Absence of changes in the level of cyclin D1 mRNA suggests a post-transcriptional effect of 7-hydroxycoumarin. A pathway which regulates post-transcriptionally the levels of cyclin D1 is the PI-3K/AKT pathway. If this pathway is inhibited it cannot inhibit phosphorylation of GSK-3 which leads to cyclin degradation [59].

**COUMARIN DERIVATIVES AND CANCER**

**Furanocoumarins**

The furanocoumarins are a therapeutically important subtype as they have various clinical applications. The furanocoumarins consist of a 5-membered furan ring attached to the coumarin nucleus. Two of the most important and well known furanocoumarins are psoralen (Linear) and angelicin (Angular). The terms linear and angular refer to the orientation of the furan ring with respect to the coumarin nucleus [3].

Psoralens are naturally occurring plant biosynthetic metabolites that have been used since ancient times in photochemotherapy to treat a number of skin disorders including mycosis fungoides, psoriasis and vitiligo [60]. Psoralens have recently found application in the regulation of human cervical carcinoma cell proliferation in conjunction with anti-sense technology [61]. Oligonucleotides and their analogs have been used to inhibit protein biosynthesis by suppressing the gene expression in a sequence specific manner. The method is called antisense strategy and has been applied to gene therapy for incurable diseases such as cancers and viral infections. Among various reports regarding antisense technology, many have presented clear
evidence that the antisense mechanism participated in the regulation of cell growth. Some of the antiproliferative effects of oligo nucleoside phosphorothioates S-oligo may be attributed to the interaction of S-oligo with certain proteins such as growth factors.

Upon UVA irradiation psoralen derivatives have the ability to crosslink covalently with pyrimidine bases (e.g. thymine and uracil). As psoralen derivatives can inactivate gene expression via cross-linking, they have been conjugated with oligonucleotides to reinforce antisense effects. During in vitro experiments psoralen-conjugated S-oligos have shown resistance to nuclease and, therefore, have exhibited significant inhibitory effects upon UVA irradiation. Psoralen-conjugated S-oligos (Ps-S-oligo) were prepared and used to inhibit the proliferation of human cervical carcinoma cells. Upon UVA irradiation of Ps-S-oligo-treated cells, Ps-S-oligo complementary to the initiation codon region (Ps-P-As) of human papillomavirus (HPV)18-E6*-mRNA of human cervical carcinoma cells significantly inhibited proliferation. The E6* protein is tightly correlated with the transformation of human cervical cells and therefore, its suppression may regulate cellular proliferation. The psoralen-conjugated antisense DNA has significant potential to regulate gene expression, which may provide useful information to explore novel gene regulating agents [61].

Pyranocoumarins

Plant materials have a long history of being successfully used in the treatment of cancer, both as chemotherapeutic agents and as complementary treatments. The pyranocoumarin compound (+)-3-angeloyl-4-acetoxy-cis-khel-lactone was isolated from Radix peucedani, a herb well-known for the treatment of respiratory diseases and pulmonary hypertension. Resistance of cancer cells to chemotherapeutic agents remains one of the major obstacles in achieving an effective treatment for cancer. The molecular mechanism of multidrug resistance (MDR) in cancer cells may involve over-expression of membrane drug efflux pumps, p53 mutations, and up-regulation of bcl-2, DNA repair or cellular detoxification enzymes. P-glycoprotein is over-expressed in various MDR cell lines and functions as an ATP-dependent drug efflux pump that rapidly extrudes anti-tumour drugs from target cancer cells which prevents the drugs from exerting their cytotoxic effects. (+)-3-angeloyl-4-acetoxy-cis-khel-lactone is a P-glycoprotein inhibitor and studies were recently performed to determine its effect on MDR cell lines. Work by Wu [62] and co-workers demonstrated that this pyranocoumarin causes apoptotic cell death for drug sensitive KB-3-1 and multidrug resistant KB-V1 cancer cell lines. Strong synergistic interactions were demonstrated when the pyranocoumarin was combined with common anti-tumour drugs such as vincristine, doxorubicin and paclitaxel. Hence, this pyranocoumarin may have beneficial therapeutic application as a MDR reversing agent. However, further research is necessary to confirm these current findings.

Warfarin

Metastasis involves several distinct steps, including one in which the tumour cell, after entry into the bloodstream, comes to rest in a capillary located at a distant site where a metastatic tumour will ultimately form. Components of the blood-clotting pathway may contribute to metastasis by trapping cells in capillaries or by facilitating adherence of cells to capillary walls. Conceivably, anticoagulants could interfere with this step in the metastatic process [63]. Warfarin has shown particularly promising results in the treatment of SCCL (Small Cell Carcinoma Lung) a tumour cell type that is characterised by a coagulation-associated pathway [64-66]. Studies by Mousa [67] have shown that anticoagulation with commonly used agents such as unfractionated heparin and warfarin (Coumadin) prevent tumour formation by limiting the ability of tumour cells to be retained in the pulmonary microvasculature.

Recent studies suggest that anticoagulant drugs and cimetidine therapy in malignancy may improve cancer survival and inhibit the metastatic process. A study investigated and compared the effects of anticoagulant drugs (e.g. warfarin and heparin), cimetidine and a combination of cimetidine with anticoagulants on adhesion of highly invasive breast cancer cell lines BT-549 and MDA-MB-231 in vitro. A high anti-adhesion effect was observed with cimetidine and warfarin. Anticoagulants such as warfarin can decrease adhesion and tumour angiogenesis. Application of cimetidine and anticoagulant drugs intensifies the anti-adhesion effect together with other anti-metastatic effects [68]. There is now considerable evidence that the blood coagulation system plays an important role in the biology of malignant tumours. This evidence has been derived from a combination of clinical, biochemical, histological, and pharmacological observations that point to the possibility of favourably affecting the course of malignant disease with agents that interfere with blood coagulation pathways [67].

SOIFLAVONES

For many years now, isoflavones have been investigated by clinicians, pharmacologists and plant physiologists. Isoflavones have exceptionally interesting, multidirectional therapeutic properties and the biological activity of these substances is conditioned by the location of the phenyl ring near the third carbon of the benzo-γ-pyrole. Hence, these compounds, in addition to antiinflammatory, antimycotic and radical scavenging properties, also exhibit both estrogenic and anti-estrogenic effects [69]. Genistein (4,5,7-trihydroxyisoflavone) is a natural isoflavone phytoestrogen present in soy (Fig. 1.4). In the gastrointestinal tract, the β-glucoside conjugates of soy are converted by the natural gut microflora into free genistein and other related isoflavones, which are present in circulating blood, accumulate in tissue and are

**Fig. (1.4).** The basic chemical structure of genistein. It belongs to the benzo-γ-pyrone subclass.
excreted in urine of people who consume high amounts of soy in their diet. Studies have revealed that individuals who consume a traditional diet high in soy products have a low incidence of certain types of cancer, such as breast, prostate and colon cancer [70]. Genistein has been shown to inhibit cancer cell proliferation in vitro and this effect may be attributed to the fact that it is a known tyrosine kinase inhibitor. Therefore, this compound was chosen to determine its effect on two cell lines in vitro. The following section gives background details on this therapeutically important compound.

GENISTEIN

Although genistein belongs to the benzopyrones is not classed as a coumarin (benzo-α-pyrone). It is a flavonoid and belongs to the benzo-γ-pyrones. Genistein is a phytoestrogen, which belongs to the 'isoflavone' class of compounds. These diphenolic compounds structurally resemble estradiol (E₂) and were shown to have some estrogenic activity [71].

Reports about environmental estrogens, or xenoestrogens, have been widespread in the last few years. There are important distinctions between estrogenic compounds of industrial origin and those that come from plants. Compounds such as insecticide DDT and industrial PCBs have been implicated by some researchers in causing estrogen-dependent cancers in exposed populations. Unlike some industrial xenoestrogens, which tend to bioaccumulate in adipose tissue and persist in the body for years, phytoestrogens are readily metabolised and spend relatively little time in the body. The timing of exposure, repeated exposures and levels of exposure to phytoestrogens are important. The two major classes of phytoestrogens that have captured the most scientific attention are isoflavones and lignans. Diets rich in natural anti-estrogenic substances such as isoflavones have been considered as one of the main reasons for significantly lower incidence of breast cancer in China, Japan, and South East Asia. Soybeans are a particularly abundant source of isoflavones such as genistein and daidzein [72].

Genistein in Cancer Research

Genistein has been shown to inhibit cancer cell proliferation in vitro. This effect is attributed to inhibition of several key enzymes, especially tyrosine kinase, which plays a critical role in cell proliferation and transformation. Tyrosine kinase is also associated with oncogene expression in breast cancer. Breast cancer is one of the most frequently diagnosed malignancies in women, and its incidence is increasing in industrialised nations. Tanos [71] and co-workers have investigated the effect of genistein on human dysplastic and malignant epithelial breast cell lines, expressing low and high metastatic potentials. They discovered that genistein has a significant in vitro inhibitory effect on the growth rate of dysplastic (fibrocystic cells) and cancerous breast cells. In vitro studies show that genistein also exhibited a synergistic additive effect when cancer cells were exposed to a genistein and tamoxifen treatment. These results indicate the potential of genistein administration alone or in combination with tamoxifen for the treatment of breast cancer [71].

A separate study has shown that genistein treatment has a role in triggering cell death and promotes cell cycle arrest on γ-irradiated K562 myeloid leukemia cells [73]. Genistein structurally resembles estradiol (E₂) and demonstrated weak estrogenic activity in several studies (70,71,74). In humans, this compound appears to have both estrogenic and anti-estrogenic effects, depending on the concentrations of circulating endogenous estrogens and estrogen receptor (ER). The estrogen agonistic activity in the absence of estrogen may account for the beneficial effects of genistein against atherosclerosis, coronary artery disease, osteoporosis and post-menopausal manifestations of hot flushes etc. The protective effect of genistein against certain forms of hormone-dependent cancers, such as prostate, may be due to similar mechanisms [74].

BENZOPYRONES AND BREAST CANCER

While a significant amount of research regarding the clinical applications of the benzopyrones has been reported it is important to emphasise that only the 'warfarin-type' anticoagulants belong to mainstream pharmacotherapy. Other types of coumarin compounds are either at the experimental or early clinical trial phase. Hence, additional studies on the elucidation of their mechanism of action is necessary to facilitate a greater understanding of these compounds so that they may find future clinical application. Recent work carried out in our laboratory involved the investigation of the effects of various benzopyrones on two carcinoma cell lines. The two cell lines were MCF-7, a breast carcinoma and A549, a lung carcinoma. However, the majority of research focused on the MCF-7 cell line especially with regard to signal transduction and cell cycle regulation studies. In our research the effect of benzopyrones (warfarin, 7-hydroxy-coumarin, esculetin, and genistein) on the growth and metabolism of human tumour cells was examined. Signal transduction studies were also performed in order to obtain information regarding the mechanism of action of warfarin, esculetin and genistein on MCF-7 cells.

Previous investigators have used cell proliferation-based assays in their chemosensitivity assessment of coumarin compounds [12, 57]. Recently an in vitro proliferation assay was utilised to determine the anti-tumour activities of SP500263, a novel next-generation selective estrogen receptor modulator (SERM), tamoxifen, and raloxifene in an in vitro and in vivo MCF-7 breast cancer model [75]. The insensitivity of many human tumour cell lines, previously tested to growth inhibition by coumarin, seems to confirm the generally held belief that coumarin is not responsible for the observed in vivo effects, but is a pro-drug for other active metabolites. Therefore, in our research we chose initially to examine three benzopyrone compounds, namely warfarin, esculetin, and genistein under similar assay procedures, with the MCF-7 cell lines and the A549 cell lines. The cell lines were exposed to each of the three compounds for 96 hours.

Proliferation assays yielded some interesting information on growth inhibition by benzopyrones:

1. The potency of growth inhibition by genistein was greater than esculetin which, in turn was greater than warfarin.
2. MCF-7 cells tested were quite sensitive to the effects of all three compounds.
3. A549 cells did not seem to be as sensitive to the effects of all three compounds in comparison to MCF-7 cells. However, both genistein and esculetin displayed significant inhibitory effects on A549 cells.

The most evident trend from the results was the sensitivity of both cell lines tested to growth inhibition by esculetin (6,7-dihydroxycoumarin). This reflects well previous observations by Kołodziej [76] and colleagues and Cooke & O’Kennedy [30], that a dihydroxy-function in either an ortho- or meta-format, was an extremely potent chemical structure for toxicity in human tumour cell lines. Since this potency was not evident in either of the single-hydroxycoumarin compounds, the added potency may be due to the existence of a double hydroxy-function on the coumarin ring.

Assessment of leakage of a wide variety of enzymes is often used for cytotoxicity testing endpoints. Lactate dehydrogenase (LDH) is a convenient marker because of the stability of the enzyme activity in the culture system [77]. In this study none of the four compounds tested (warfarin, 4-hydroxywarfarin, esculetin and 7-hydroxycoumarin) caused significant membrane damage in both cell lines (MCF-7 and A549) assessed by LDH assay. There are some drawbacks that should be noted when using the LDH assay. If serum contaminates the system with endogenous enzymes they can mask low levels of leached enzymes, which leads to underestimation of LDH present. The main drawback of this assay, however, is its reliance on the premise that membrane integrity and cellular viability are closely linked. This is not always the case, at least in the early stages of cell death; so these assays may not always be true / sensitive indicators of viability. Hence, other assays (MTT, AP; explained later in text) must be performed in order to obtain a complete picture of the effects of benzopyrones on cells under investigation.

The effects of warfarin, 7-hydroxycoumarin, esculetin, and genistein on cellular metabolism was examined using the MTT assay and the cytosensor (where only warfarin and esculetin have been tested on MCF-7 cells). The MTT Assay is a well-established colourimetric assay, which can be used to detect the effects of agents on cellular metabolism. The assay is based on the cleavage of the yellow tetrazolium salt, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), to purple formazan crystals by mitochondrial dehydrogenases. Therefore, cells must not only be alive, but also metabolically active for this reaction to occur [12]. The assay can be used in either a long- (96hr) or short-term (24hr) drug-exposure format, to assess the effects of a drug on cellular growth or metabolism, respectively. Previously, the MTT assay has been used to assess the anti-proliferative effects of coumarins [78].

An alternative method was used to evaluate the effect of coumarins on cellular metabolism. The cytosensor microphysiometer is a biosensing instrument that determines the extracellular acidification rate of cells, which is closely coupled to their basal metabolic rate. Monitoring of cellular metabolism is achieved using a pH-sensitive sensor, in ‘real-time’, which offers distinct advantages over the end-point nature of the MTT assay, when examining the effect of chemical agents on cellular metabolism [12]. The effect of 24hr exposure of MCF-7 cells to two coumarin compounds (warfarin and esculetin) was monitored using this technique. In these experiments the basal metabolic rate of the cells was determined prior to drug-exposure, and this value was normalised as 100%. This was achieved for each of the four separate sensor chambers in the cytosensor. Following drug exposure all subsequent metabolic rates were expressed as a percentage of the basal rate of the particular sensor chamber, ensuring each chamber with its encased cells acted as its own internal control.

An important result evident from the data collected was the fact that the cytosensor microphysiometer was more sensitive to the effects of the benzopyrones on cellular metabolism than the MTT assay. For example, the MTT assay detected a decrease in cellular metabolism of ~ 30% when MCF-7 cells were exposed to 20µg/ml esculetin for 24hrs. According to the cytosensor results, the cellular metabolism in cells exposed to 20µg/ml esculetin decreased by ~ 60% over this time period. In fact it would appear from comparing the MTT and cytosensor results for warfarin, esculetin and genistein, that the cytosensor microphysiometer is a much more sensitive predictor of metabolic inhibition. The reason for this may be due to the fact that the MTT assay relies on the activity of just one group of mitochondrial enzymes to predict adverse effects on metabolism, and in doing so may underestimate metabolic effects. The MTT assay has been shown to underestimate the growth inhibitory effects of interferons (IFNs) in the past [79]. Formazan production can also be induced by drugs that cause perturbations of the cell cycle [80]. Cells have also been observed to metabolise the tetrazolium dye when lethally damaged and have lost the ability to exclude vital dyes [81]. Additionally, the test compound may also react with other assay components, e.g. tetrazolium salts, causing interference, which again may lead to inaccurate results [32].

Apart from the increased sensitivity of prediction, the cytosensor has several other advantages over the MTT assay for the detection of metabolic suppression. The ‘real-time’ aspect of detection with the cytosensor yields large amounts of information on the nature of the metabolic suppression (e.g. time of detrimental effects, etc.), per experiment. To attain this information with an end-point system such as the MTT Assay would require multiple, kinetic end-point assays. In addition, the exposure set-up of the cytosensor (flow-through, perfusive) mimics the in vivo drug delivery/exposure more than the static exposure set-up of the MTT assay. Finally, the cytosensor microphysiometer allows for reversibility studies to be performed on drug-treated cells to assess their recovery, an experiment difficult, if not impossible to achieve with cells following the MTT assay. The tested benzopyrones (warfarin, esculetin and genistein on cytosensor) all displayed a dose- and time-dependent effect on cellular metabolism, with genistein followed by esculetin displaying the most potent effects. For warfarin, only the highest concentration (100µg/ml) irreversibly suppressed the metabolism of the MCF-7 cells tested.

The acid phosphatase (AP) assay is based on hydrolysis of pNPP by intracellular AP in viable cells to produce p-nitrophenol. The method shows higher sensitivity and reproducibility in comparison to cell proliferation assays based on reduction of tetrazolium salts (e.g. MTT assay) and
is especially suited to applications where a large number of samples are assayed [82, 83]. The AP assay was performed on both cell lines. Both esculetin and genistein were the most potent proliferative inhibitors. Genistein had a proliferative effect at low concentrations only in MCF-7 cells, which was expected and correlates well with previous studies [84]. Overall the AP assay was found to offer the best combination of sensitivity, linearity, flexibility, ease of performance, reproducibility and precision, of all the microtitre assays.

The investigation of how esculetin and warfarin affect the signal transduction cascade and cell cycle progression in MCF-7 cells was performed using three different assay formats. An ELISA for detection of tyrosine phosphorylation in whole cells was used in order to determine whether the agents tested inhibit tyrosine phosphorylation of growth factor stimulated EGF-R in MCF-7 cells. The cytosensor microphysiometer was utilised to determine if esculetin or warfarin inhibit tyrosine kinase activity in EGF-stimulated MCF-7 cells. The final essay again utilised the ELISA format, with the aim of determining to what extent the agents inhibited DNA synthesis in MCF-7 cells. Previous studies have investigated the effects of E2 on ER stimulation and signal transduction [85-87]. However, breast cancer cells can be stimulated to proliferate with growth factors in the absence of added estrogen or progesterone. MAP kinase activation increases in response to stimuli such as EGF and IGF-1. Urokinase type plasminogen activator, FGF-2 and insulin activate MAP kinase in MCF-7 cells [88-90]. Therefore, it was possible in this study to successfully stimulate the EGF-R in MCF-7 cells with EGF and, in turn, activate the MAP kinase pathway in the absence of E2.

The regulation of activated MAP kinase in MCF-7 cells involves inhibitory (TNF-α) as well as stimulatory (EGF) pathways. Research has shown that EGF stimulates activated MAP kinase within 1-2 minutes in MCF-7 cells. Maximal stimulation occurs at 6-8 minutes with a decrease in MAP kinase stimulation after this time period [91]. The cytosensor results reflect this stimulation pattern as the transient maximal stimulation of EGF-R (which will also transiently stimulate downstream MAP kinase activation), there is a decline in stimulation after a short time period of ~10 minutes. It was important to determine for this study that the EGF-R is tyrosine phosphorylated in MCF-7 cells. This has been demonstrated and tyrosine kinase activity was also detected in Western blots of whole-cell protein extracts from the MCF-7 cell line probed with an anti-phosphotyrosine antibody [92]. Therefore, when MCF-7 cells were pre-exposed to either esculetin or warfarin it was possible to determine if these agents inhibited tyrosine phosphorylation or not. The ELISA for detection of tyrosine phosphorylation in MCF-7 cells yielded results that indicated esculetin inhibited tyrosine phosphorylation in EGF stimulated cells by up to 20% more than control cells. Warfarin is a less potent inhibitor of cell proliferation and metabolic activity than esculetin and this may be partly due to the fact that it shows no significant tyrosine kinase phosphorylation inhibition in comparison to esculetin and the positive control, genistein. The observed results from the cytosensor microphysiometer correlated well with the ELISA results as both sets of results demonstrate that esculetin has the ability to inhibit both tyrosine kinase phosphorylation and its kinase activity. Therefore, from our research it is evident that esculetin alone inhibits cell proliferation, which can be partially attributed to its tyrosine kinase inhibition activity.

Esculetin is known to be a cytostatic drug and the inhibition of the cell-cycle at transition G1/S is consistent with the cytostatic effect of both 7-hydroxycoumarin and esculetin. It is also evident from previous studies that esculetin primarily effects/inhibits events in the G1 phase of the cell cycle [58, 59]. If cells are arrested in G1 phase before S phase progression there would be a significant decrease in DNA synthesis expected in these arrested cells. Therefore, the results obtained indicate there is a significant decrease in DNA synthesis in MCF-7 cells treated with either esculetin or genistein but not warfarin. It is possible that the decrease in DNA synthesis in esculetin-treated MCF-7 cells is due to the agent’s ability to induce G1 arrest with no progression to S phase where DNA synthesis occurs. Genistein seems to affect cell cycle progression differently to esculetin. Genisten results show that the decline in DNA synthesis occurs at a higher concentration than esculetin and this may be due to the fact that genistein does not arrest cells at G1 phase. Genistein has been shown to inhibit progression later at G2/M phase transition [5, 70, 73]. This is after S phase and therefore, some DNA synthesis may occur. However, this stop in G2/M phase usually is followed by apoptosis and not progression into mitosis as would happen in normal cycling cells. This leads to a decrease in DNA synthesis as cells die off due to apoptosis.

Taken together the signal transduction and cell cycle regulation results indicate what the possible target of esculetin in MCF-7 cells is and how it affects components of the activated MAP kinase pathway. This inhibition affects downstream molecules (e.g. Ras, ERK, Myc and Nuclear ER) with important roles in MAP kinase cascade [93]. Therefore, esculetin can inhibit the receptor tyrosine kinase activity of growth factor receptors such as EGF-R and ER. This in turn will prevent growth signals reaching other signalling intermediates such as Ras, or activation of transcription factors such as Myc. Another signal transduction pathway involves phosphatidylinositol-3-kinase (PI3-K) heterodimer, which is an important mediator of survival factors, protecting many cell types from multiple apoptosis-inducing stimuli. If PI3-K is inhibited (by esculetin inhibiting phosphorylation) the signalling pathway is interrupted and the cell is unable to progress into the S phase of the cell cycle.

CONCLUSIONS

Coumarin and coumarin-related compounds have proved for many years to have significant therapeutic potential. They come from a wide variety of natural sources and new coumarin derivatives are being discovered or synthesised on a regular basis. Coumarin is a simple molecule and many of its derivatives have been known for more than a century. However, their vital role in plant and animal biology has not been fully exploited. Coumarins have multiple biological activities including disease prevention, growth modulation and anti-oxidant properties. These compounds are known to exert anti-tumour effects and can cause significant changes
in the regulation of immune responses, cell growth and differentiation. Research involving coumarins and their antiproliferative effect on malignant melanoma, leukaemia, renal cell carcinoma, prostate and breast cancer are discussed in this review. Recent studies, including research performed in our laboratory have been presented, in order to give an overview of the various potential therapeutic applications of coumarins. It is evident from the research described that coumarin and coumarin-related compounds are a plentiful source of potential anti-cancer drugs deserving further study.

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REFERENCES

References 94-96 are related articles recently published in Current Pharmaceutical Design.


