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OVERVIEW OF THE ONCOPHARMACOLOGICAL STUDIES OF PLANT- DERIVED NATURAL PRODUCTS CONDUCTED AT THE FACULTY OF PHARMACY (MU-SOFIA)

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Abstract. The present review gives an outline of some oncopharmacological projects focused on the characterization of plant-derived natural products, conducted at the Faculty of Pharmacy at the Medical University of Sofia. These include collaborative phytochemical/pharmacological studies of a) alkaloids, b) saponines with immunomodulatory and anti-cancer effects; c) cytotoxic lignans; iii) phenolic compounds (acetylphloroglucinols, benzophenones, xanthones) from Hypericum spp. iv) various volatiles and crude extracts.

Keywords: antineoplastic, alkaloids, apoptosis, benzophenones, cytotoxicity, IL-2, lignans, phloroglucinols, podophyllotoxin, saponins, Astragalus, Crinum zeylanicum, Kigelia pinnata, Linum, Hypericum, multi-drug resistance

Introduction

Despite their essential therapeutic role, most of the existing antineoplastic agents share the distinction of having the narrowest therapeutic indices among the clinically useful drug classes and hence their clinical use is frequently accompanied by significant dose-limiting toxicities [1-3]. On these grounds there has been a profound interest towards elaboration of novel more selectively acting anticancer drugs with optimized efficacy and safety profiles [1, 3-6].

The most essential alternative to combinatorial synthesis and drug design as a source for lead identification is the screening of natural products and especially of plant-derived biologically active compounds as they comprise a vast, chemically diverse and generally unexplored source of biologically active agents [7-12]. Although the bioactivity guided fractionation and isolation of plant-derived agents are very time-consuming and expensive these natural products pose great interest as a source of potential drug candidates. On one hand the unique enzymatic machinery of plants allows them to biosynthesize a huge diversity of chemical substances including complex structures such as fused heterocyclic structures, complex compounds with many chiral centers and other highly varied structures which are virtually unobtainable via conventional synthetic routes [7, 8], while on the other hand the plant biodiversity especially in remote, unexplored regions of the planet is still a vast source of tumor-inhibiting lead compounds [7-9, 12].

On this ground it is not surprising that in the last several decades a number of potent natural products of plant origin have been commercialized as anticancer drugs, occupying significant shares of the oncological drug markets, e.g. the taxanes, the etoposide analogues, the camptothecins and the Catharanthus (Vinca) alkaloids, among others [1, 7-14]. In addition to the clinically applied analogues a huge number of plant-derived agents are currently in accelerated clinical phases of development [7-12, 15].

The phytochemical and pharmacognostic explorations have long history in the Faculty of Pharmacy (MU-Sofia) – the oldest and the most authoritative and internationally esteemed academic institution for higher pharmaceutical education in Bulgaria, as it began with the very establishment of the Department of Pharmacognosy, more than five decades ago. This long-standing interest towards natural products allowed the engagement of the Department in many
industry- and academia-coordinated projects, which through the years resulted in the development and commercialization of some clinically applied medicinal products and food supplements. The oncopharmacological studies of such natural products, however became possible after the establishment of the Lab of Experimental Chemotherapy and Molecular Pharmacology in the Department of Pharmacology and Toxicology, during the 1990’s. The collaborations between the groups from the Dept. of Pharmacognosy and the Lab of Experimental Chemotherapy, allowed the isolation and pharmacological characterization of a number of active principles with cytotoxic/antineoplastic activity within the frame of successful joint projects, which received financial support from either the National Science Fund (Ministry of Education, Youth and Science) or the Medical Science Council (MU-Sofia) [16-32].

This paper gives a concise overview of the oncopharmacological studies of the plant-derived natural products, carried out in the Faculty of Pharmacy (MU-Sofia), with an emphasis on recent collaborative projects.

Oncopharmacological evaluation of cytotoxic alkaloids

The emergence of multi-drug resistance (MDR) is one of the most important hurdles limiting the clinical success of antineoplastic chemotherapy [1, 33]. This type of resistance is mediated by the overexpression of ATP-binding cassette proteins (e.g. Pgp, MRP-1 etc.) which are capable of pumping out chemically diverse anticancer drugs from tumor cells, leading to subeffective intracellular levels [1, 33]. Thus much attention has been paid to elaboration of multi-drug resistance modifying agents in order to optimize the cancer treatment in case of proven or suspected MDR-phenotype [34-40].

Thaliblastin comprises an alkaloid mixture derived from Thalictrum aquilegifolium (Fam. Ranunculaceae). It is an original Bulgarian medicinal product with hybrid anticancer and multi-drug resistance–modulating properties [41, 42]. Within a joint project between the Lab of Oncopharmacology (National Centre of Oncology) and the Lab of Exp. Chemotherapy a series of thorough in vitro studies of thaliblastine were carried out. Its antiproliferative effects were tested against a panel of human tumor cell lines, namely HL-60 (acute promyelocyte leukemia), HL-60/DOX (a multidrug resistant variant of the former line, characterized by the expression of the MRP-1 transporter), REH (lymphoid leukemia) and HD-MY-Z (Hodgkin lymphoma). The alkaloid was found to exert dose-dependent inhibitory effects, and moreover collateral sensitivity was encountered with HL-60/DOX. Additionally, typical for apoptosis oligonucleosomal DNA fragmentation was detected in leukemia cells after with thaliblastine. Co-treatment of HL-60/DOX and anthracyclines was consistent with a reversal of the MDR-phenotype characteristic for this cell line [37].

A more recent project was focused on amaryllidaceaeous alkaloids derived from Crinum zeylanicum. This plant has been extensively used in folk medicine as a rubefacient in rheumatism, as antimalarial remedy and as a poison [43]. Complex alkaloid profiles in C. zeylanicum plant organs were revealed by GC-MS analysis, including several bioactive compounds. Crinine, 11-O-acetoxambelline, ambelline, 6-hydroxybuphanidrine and 6-ethoxybuphanidrine (an artifact of the isolation procedure) were isolated. Crinine, 6-hydroxybuphanidrine and 6-ethoxybuphanidrine exerted cytotoxicity in a panel of human tumor cell lines, crinine being the most active. The latter compound induced apoptosis in a dose-dependent manner in HL-60 and MDA-MB-231 cell lines. Structure-activity relationships in the studied molecules indicated that the hydrogenation of the double bond at C1-C2 leads to a loss of activity, whereas substitutions at C6, C8 and C11 modulate the cytotoxic properties [17].

Oncopharmacological assessment of biotechnologically produced arylnaphthalene lignans

Lignans comprise a large class of phenolic compounds defined as ββ’-dimers of phenylpropane (C10C6) units [10, 44, 45]. This important and ubiquitous group of natural products has drawn much attention due to the prominent cytotoxic effects of the 2,7’-cyclophanidodophyllotoxin (PTOX) – a potent mitotic spindle poison [10, 44, 46-49]. Although the extreme gastrointestinal toxicity and the low water solubility of PTOX precluded its use as potential antineoplastic agent, this compound is widely used as a starting material for production of the semi-synthetic anticancer drugs etoposide and teniposide, which despite of the great structural resemblance to the prototype act as topoisomerase II inhibitors [50], and are among the most important classes of clinically applied anticancer agents [51-53].

For large scale pharmaceutical preparations PTOX is obtained from the rhizomes of Podophyllum peltatum and Podophyllum hexandrum (Berberidaceae)
Unfortunately, due to extensive over-collection, and insufficient counter-cultivation, both species are becoming increasingly limited [44, 45]. *P. hexahendrum* is listed in the appendix II of CITES (Convention for International Trade in Endangered Species) [54]. Due to this serious supply issue, much effort has been focused on alternative sources for production of podophyllotoxin, including utilization of different plant species, chemical synthesis and biotechnological production [45, 55].

The research team from the Lab of Biotechnology at the Dept. of Pharmacognosy lead by Professor Iliana Ionkova has focused much attention upon the utility of diverse *Linum* spp. (incl. Bulgarian or Balkan endemic species) as sources for production of PTOX-related lignans [10, 25, 31, 32, 45, 56-62]. To meet this objective extensive phytochemical studies have been carried out in order to identify the major lignan constituents in different *Linum* species and also to elaborate and optimize approaches for establishing in vitro plant cultures for biotechnological production of lignans with pharmaceutical importance [19, 25, 31, 32, 45, 57-59, 61, 62].

A podophyllotoxin-analogue 4'-demethyl-6-methoxypodophyllotoxin (4'-DM-6-MPTOX), has been identified as a major lignan constituent of *Linum tauricum* Willd. Its cytotoxicity was assessed in three human leukemic cell lines (HL-60, BV-173 and

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**Fig. 1.** Chemical structures of plant-derived biologically active compounds tested for antineoplastic activity.
Overview of the oncopharmacological studies of plant-derived... PHARMACIA, vol. 60, No. 1/2013

LAMA-84) whereby 4’-DM-6-MPTOX demonstrated prominent efficacy, with IC₅₀ values being several fold lower than those of the referent antineoplastic agent etoposide. Moreover, it exhibited apoptogenic effects in BV-173 and HL-60 cells and was found to induce NF-kB inhibition in HeLa cells as assessed by the IL-6 luciferase gene reporter assay [32].

Another thoroughly explored cytotoxic lignan was justicidin B (Fig. 1), derived from genetically transformed cultures of Linum leonii. The oncopharmacological assessment of justicidin B demonstrated its strong cytotoxicity against human tumor cell lines: the chronic myeloid leukemia derived LAMA-8 and K-562 cell lines and the T-cell leukemia-derived SKW-3 cell line with IC₅₀ values of 1.11, 6.08, and 1.62 μM, respectively. A DNA-fragmentation analysis using agarose electrophoresis, ethidium bromide staining and UV-transillumination demonstrated its pro-apoptotic properties [31].

In a more recent detailed study justicidin B was tested for cytotoxic activity and induction of apoptosis in MDA-MB-231 and MCF-7 breast cancer derived cell lines. The tested lignan evoked strong, concentration dependent cytotoxicity in both cell lines, whereby MCF-7 proved to be far more sensitive as compared to MDA-MB-231. The 24 h treatment of both cell lines increased the level of apoptotic DNA fragmentation; however the proapoptotic activity is completely inhibited if the cells are co-incubated with the non-selective pan-caspase inhibitor Boc-Asp(OMe)-fluoromethyl ketone (PCI), which implies that justicidin B, activates programmed cell death via caspase -dependent mechanisms. Exposure of MDA-MB-231 cells with justicidin B leads to concentration dependent decrease in the expression of NFkB; whereas the treatment of MCF-7, is consistent with strong increase in the expression of this transcription factor [25].

Oncopharmacological evaluation of polyphenols isolated from Hypericum species

One of the main fields of research in the Dept. of Pharmacognosy is the phytochemical characterization of Hypericum (Guttiferae) – a large genus of herbs or shrubs, which grows widely at temperate regions, and is used as traditional medicinal plants in various parts of the world [63]. Much attention has been paid on the phytochemical investigation of Hypericum annulatum Moris subsp. annulatum also known as H. degenii Bornm – a herbaceous plant, endemic for the Balkan Peninsula and Sardinia. The exploration of this plant has demonstrated the presence of flavonoids, catechins, hypericins, xanthones, and benzophenones [64, 65].

The benzophenones annulatophenone, annulatophenonoside, acetylanulatophenonoside, neoannulatophenonoside, hypericophenonoside and the structurally related xanthone gentisine (1,3,7-trihydroxyxanthone) (Fig. 1) were tested in vitro in a panel of human tumor cell lines. The pharmacological screening was carried out in a panel of human tumor cells lines: HL-60 (acute promyelocyte leukemia), HL-60/Dox (selected in doxorubicin-containing medium; characterized by over-expression of MRP-1 and mdr phenotype), K-562 (chronic myeloid leukemia). The tested polyphenols exhibited concentration-dependent cytotoxicity, whereby gentisine proved to exert the highest relative potency. A pharmacodynamic study for genomic DNA fragmentation was conducted, which has shown that the effects of the polyphenols are mediated by induction of programmed cell death. When applied at sub-cytotoxic concentrations the investigated benzophenones and gentisine increased the chemosensitivity of HL-60/Dox cells to the anthracyclines doxorubicin, epirubicin, idarubicin and daunorubicin. These findings indicate that the tested compounds could be considered perspective for further, more detailed investigations as multidrug resistance modulators [18].

In another study the protective effects of neoannulatophenonoside, annulatophenonoside and acetylanulatophenonoside against epirubicin-induced anticolonogenic effects on murine bone marrow stem cells cultured in semi-solid medium. The anthracycline alone (at 1.25 μM) significantly inhibited the clonogenicity of bone marrow cells following 24 h exposure. All of the benzophenones were absolutely devoid of anticolonogenic activity themselves. The concomitant administration of neoannulatophenonoside decreased the detrimental effects of the anthracycline epirubicin towards bone marrow colony forming units, following 24 h exposure, whereas neither annulatophenonoside nor acetylanulatophenonoside exerted significant cytoprotective effects on this model system [27].

Another ongoing collaborative project between the Department of Pharmacognosy and the Lab of exp. Chemotherapy is focused at the pharmacological assessment of a recently identified acylphloroglucinol hyperatomarin (Fig. 1) from H. annulatum. The cytotoxic effects of this compound were investigated in a large panel of tumor cell lines, whereby it was found to exert potent, concentration-dependent antiproliferative effects with...
IC$_{50}$ values identical or even lower than those of the referent antineoplastic drug daunorubicin [24]. Moreover hyperatomarin proved to induce apoptosis in different tumor cell lines as evidenced by the characteristic oligonucleosomal fragmentation of genomic DNA following 24 h exposure. Flow cytometric analysis of the effects of hyperatomarin in KG-1 cells confirmed the proapoptotic potential of the compound and its ability to induce G1 cell cycle arrest (Fig 2). Preliminary investigation of the antiangiogenic potential of hyperatomarin revealed that it inhibited the VEGF-induced proliferation of human umbilical vein endothelial cells and induced apoptosis in these cells which firmly indicates the need for further examination of the angiostatic effects of this compound [23]. These data well correlate with the recently established potent cytotoxic, pro-apoptotic, antiangiogenic and antimetastatic effects of hyperforin, a structurally related to hyperatomarin acylphloroglucinol from Hypericum perforatum [66-68].

In a recent bioactivity guided phytochemical evaluation of Hypericum elegans Stepan ex Willd two cytotoxic phloroglucinols, namely elegaphe-none and 7-epi-clusianone, were isolated from the aerial parts of the plant. Both compounds showed prominent cytotoxicity on HD-MY-Z, K-562 and KE-37 tumor cell lines, causing 50% reduction of cellular viability at low micromolar concentrations, showing comparable potency. The established apoptotic fragmentation of genomic DNA following short-term (6 h) or long-term (24 h) exposure to the tested compounds clearly indicates that the induction of programmed cell death is an implicated in their cytotoxic mode of action [30].

**Cytotoxic/immunomodulatory saponins**

Saponins have been long known as antineoplastic and immunostimulant agents. The discovery of the potent broad-spectrum anticancer properties of betulinic acid however has further focused research interest on this class of plant secondary metabolites as possible anticancer lead compounds [69-77]. The identification and exploration of Bulgarian plants including endemic species as sources of biologically active saponins is one of the most important fields of research, carried out at the Department of Pharmacognosy, FF, MU-Sofia [20-22, 26, 78-81].

*Astragalus* sp. is the largest genus in the Leguminosae family and practically one of the largest genera of vascular plants, comprising approximately 2500 species of herbs and shrubs. The phytochemical exploration of *Astragalus* sp. demonstrated the occurrence of diverse secondary metabolites such as saponins, flavonoids, and polysaccharides, the former being the most important classes of biologically active compounds, conditioning the pharmacological activity (immuno-modulators, antineoplastic, antimicrobial, anti-inflammatory etc.) of these plants [82-85].

A purified saponin mixture (PSM) from *Astragalus corniculatus* Bieb. was used in an in vivo tumor model to demonstrate its protective effect against myeloid Graffi tumor in hamsters. The intraperitoneal administration of PMS decreased the tumor growth.
and transplantability during the early stages of tumor progression. The PMS application also led to an increase in the mean survival time and reduction of the percentage mortality [80].

The pharmacology of the aforementioned PSM from Astragalus corniculatus Bieber. was further evaluated for its immunostimulating properties on phagocytic cells in Graffi-tumor bearing hamsters (G-TBH). The cellularity, migration and phagocytic indexes of peritoneal macrophages (pMos) and of blood polymorphonuclear leukocytes (PMNs) were evaluated in a comparative fashion in healthy and Graffi-tumor bearing hamsters (G-TBH) treated with PSM. The number, migration and phagocytic activities of pMos, as well as the phagocytic ability of PMNs increased significantly in both healthy and G-TBH after i.p. application of the 50 mg/kg body weight PSM [81]. A successive phytochemical study of this saponin mixture has led to the identification of three new oleanane-type triterpene saponins [78, 79].

Another research project was focused on the pharmacological evaluation of a purified saponin mixture from A. hamosus. It was tested for cytotoxicity against a panel of human tumor cell lines. The saponin mixture demonstrated significant antiproliferative effects against a multi-drug resistant cell line HL-60/Dox, with a collateral sensitivity phenomenon, i.e. the IC_{50} value was lower in the resistant sub-line vs. the chemosensitive parent cell line HL-60 [21].

In a recent paper the cytotoxicity of different natural compounds, isolated from hairy roots of Astragalus membranaceus was reported for the first time. Root cultures of the plant, cultivated in bioreactor gave 18.5 g/l dry wt roots with the highest astragaloside production in vitro reported up to now - 1.64% (astragaloside I), 1.12% (astragaloside II) and 1.08% (astragaloside III). In this manner the production in airlift bioreactor can be used as means of reliable supply of cycloartane saponins to extend the research to human clinical studies. The compounds exerted concentration-dependent cytotoxicity and apoptogenic activity against cultured human tumor cell lines [20].

In another report two new glycosides of 30-normedicagenic acid were isolated from the aerial parts of Chenopodium foliosum Asch. The structures of the compounds were determined by means of spectroscopic methods (1D and 2D NMR, UV, IR) and HRMS-ESI. The compounds were tested for cytotoxicity on BV-173, SKW-3, and HL-60 human leukemic cell lines. In addition, the saponins showed stimulatory effects on interleukin-2 production in phytohemagglutinin/phorbol myristate acetate-stimulated Jurkat E6.1 human T-cells [29].

Other oncopharmacological studies of plant-derived natural products

In a collaborative project we have tested the antineoplastic potential of Kigelia pinnata DC (or K. africana, Bignoniaceae) stem bark extract. This plant also known as Sausage Tree, or Worsboom has a variety of traditional medicinal uses throughout Africa [86, 87]. Ethnopharmacological studies regarding the antineoplastic potential of the plant were fuelled by anecdotal reports for the traditional use of fruit and stem bark extracts for the treatment of melanoma and other skin neoplasms [86], as well as for the management of endometrial cancer [87], and hence much of the experimental chemotherapy research was focused on solid tumor-derived cell lines [88-91]. Considering the scarce data about the efficacy of Kigelia pinnata against hematological malignancies and the lack of evidence for in vivo antineoplastic activity in the available literature a study was conducted to assess the anticancer potential of a stem bark methanolic extract of the plant. A MTT-bioassay was carried in seven human tumor cell lines, namely SKW-3, REH, HL-60, K-562, DOHH-2, HD-MY-Z and MCF-7. TME displayed prominent cytotoxicity, being most active against the breast cancer derived MCF-7 and the acute T-cell leukemia SKW-3. A bioactivity-guided fractionation of the parent extract using SKW-3 cells as tumor test system was undertaken in order to identify of sub-fractions possessing biological activity. Of the 10 tested fractions however, only CF3 and CF7 chloroform fractions retained biologic activity, but both were significantly less active as compared to the TME (Fig 3). Treatment with TME induced DNA laddering in SKW-3 cells following 24 h exposure, which shows that its activity is mediated via induction of apoptosis (Fig 2). TME exerted strong antineoplastic activity against Lewis lung carcinoma in BDF-1 mice. The extract was applied at 37.5, 75 or 105 mg/kg, i.p. on study days 1,5,9. Prominent increase of the life span of treated animals was encountered at doses 75 mg/kg (66.67% vs. the untreated control) and 100 mg/kg (65.2 versus the untreated control). At all doses of TME hampered the enlargement of the tumor volume as compared to the untreated control, with TGI values ranging between ca. 80 and 90% (Fig. 4) [28].
In another project the cytotoxic potential of volatile fractions from four Astragalus species were tested for cytotoxic activity against human tumor cell lines, and were found to inhibit their proliferation and viability [26]. Volatiles from A. corniculatus were investigated in more detail using a spectrum of human tumor cell lines of different origin – leukemias and solid tumors. These volatiles proved to be the active antineoplastic agents, and were also found to induce a necrotic-type pattern of DNA-fragmentation in SKW-3 cells. These data indicate that the antineoplastic effects of the fractions are at least partly mediated via induction of cell death through necrosis/necroptosis [22].

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