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Flavonoids as a scaffold for development of novel anti-angiogenic agents: An experimental and computational enquiry

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ABSTRACT

Relationship between structural diversity and biological activities of flavonoids has remained an important discourse in the mainstream of flavonoid research. In the current study anti-angiogenic, cytotoxic, antioxidant and cyclooxygenase (COX) inhibitory activities of diverse class of flavonoids including hydroxyl and methoxy substituted flavones, flavonones and flavonols have been evaluated in the light of developing flavonoids as a potential scaffold for designing novel anti-antiangiogenic agents. We demonstrate anti-angiogenic potential of flavonoids using *in vivo* chorioallantoic membrane model (CAM) and further elaborate the possible structural reasoning behind observed anti-angiogenic effect using *in silico* methods. Additionally, we report antioxidant potential and kinetics of free radical scavenging activity using DPPH and SOR scavenging assays. Current study indicates that selected flavonoids possess considerable COX inhibition potential. Furthermore, we describe cytotoxicity of flavonoids against selected cancer cell lines using MTT cell viability assay. Structural analysis of *in silico* docking poses and predicted binding free energy values are not only in accordance with the experimental anti-angiogenic CAM values from this study but also are in agreement with the previously reported literature on crystallographic data concerning EGFR and VEGFR inhibition.

Introduction

Angiogenesis is a physiological process involved in the formation of new capillary blood vessels from pre-existing ones and is virtually required for maintaining normal as well as tumor vasculature. Being a normal physiological processes, angiogenesis takes place not only in developmental stages such as growth and development in embryos and adults but also, in wound healing, as well as in the female reproductive cycle [1]. Targeting tumor angiogenesis has remained a significant hope in the mainstream of anticancer research as it plays an important role in tumor growth, proliferation and metastasis [2]. The growing tumor cells have an absolute requirement for a persistent supply of new blood vessels to nourish their growth and to facilitate metastasis. Thus, tumor vascularization is a vital process for the progression of a neoplasm from a small localized mass of cells to an enlarging tumor with the ability to metastasize [1,3]. The most important manifestation of pathological angiogenesis is the presence of well developed network of vessels in the vicinity of solid tumors. Indeed, well-vascularized tumors expand both locally and by metastasis, whereas avascular tumors do not grow beyond a diameter of 1–2 mm [4]. The angiogenesis dependency of tumor growth has led to the development of anti-angiogenic therapies [5]. Despite of numerous complexities and challenges that are yet to be solved, still anti-angiogenic therapy is conceptually extremely appealing [6].

Angiogenesis is tightly regulated by large number of pro-angiogenic and anti-angiogenic factors. As the tumor enlarges, increasing need for oxygen causes hypoxia in the tissue, leading to increased production of angiogenic factors, which in turn, stimulate angiogenesis [7]. Commonly known proangiogenic factors are vascular endothelial growth factor (VEGF),[2] basic fibroblast

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[2] *Abbreviations used:* VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; COX, cyclooxygenase; ROS, reactive oxygen species; DPPH, (2,2-diphenyl-1-picryl hydrazine); SOR, superoxide anion radical; 4′-MF, 4′-methoxy flavone; 3H-7-MF, 3-hydroxy-7-methoxy flavone; 2′HFN, 2′-hydroxy flavonone; 4′HFN, 4′-hydroxy flavonone; 7HFN, 7-hydroxy flavonone; RMSD, Root Mean Square Deviation; CAM, chorioallantoic membrane.

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growth factor, epidermal growth factor (EGF), platelet-derived growth factor, placental growth factor, and matrix metalloproteinases. Endogenous anti-angiogenic factors include thrombospondin, angiostatin, tumstatin, and endostatin. Structural peculiarities of these proangiogenic factors and therapeutic opportunities these angiogenic factors hold are comprehensively reviewed [8]. The clinical importance of VEGF for tumor growth is supported by the fact that most tumors produce VEGF and that inhibition of VEGF-induced angiogenesis significantly inhibits tumor growth *in vivo* [9]. The major physiological stimuli for angiogenesis include tissue ischemia hypoxia, inflammation and shear stress. Tumor angiogenesis has been identified as a target site for therapeutic intervention because of its important role in tumor growth, metastasis, and inflammatory diseases [10]. It is known that VEGF receptor-ligand interactions, a common target of anti-angiogenic therapy [11], induce not only differentiation and proliferation of endothelial cells during vasculogenesis [12], but also plays a key role in establishment of tumors and wound healing.

Cyclooxygenase (COX) class of enzymes is believed to be key pacemaker between angiogenesis and neoplastic growth [13,14]. COX-II specifically mediates pro-angiogenic activity by modulating multiple facets of angiogenesis including VEGF production; initiating and maintaining germination of new vasculature, endothelial cell migration, and tube formation [15]. Moreover, recent studies have established that angiogenesis is driven by COX dependent mechanisms [16]. Simultaneous restriction of VEGF and COX pathways are recently reported to efficiently abolish tumor angiogenesis [17]. Considering these facts, we initiated a separate *in vitro* investigation targeting COX inhibition potential of flavonoids.

Free radicals are implicated in the initiation of variety of human ailments. Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress [18]. Body cells and tissues are continuously threatened by the damage caused by free radicals and ROS, which are either produced during normal oxygen metabolism or are induced by exogenous damage. Oxidative stress is now perceived as a prominent feature of many acute and chronic diseases including cancer and leukemias [19]. Recent studies validated that oxidative stress, along with influencing development and progression of breast cancer [20]; it eventually induces angiogenesis [21]. Therefore, exploring antioxidant properties of therapeutic agents can pave new avenues in understanding the role of oxidative stress in concern to cancer therapy. In this context, we have evaluated the free radical scavenging activity of selected flavonoids using DPPH (2,2-diphenyl-1-picryl hydrazine) and superoxide anion radical (SOR) scavenging assays.

Flavonoids are phenolic compounds, widely distributed as secondary metabolites in plant kingdom. The biological, pharmacological, and medicinal properties of flavonoids have been extensively reviewed [22]. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants [23]. They are powerful chain-breaking antioxidants. Their antioxidant effectiveness depends on the stability in different systems, as well as number and location of hydroxyl substitutions [24]. In many *in vitro* studies, phenolic compounds demonstrated higher antioxidant activity as compared to vitamin C and carotenoids [25]. The flavonoids display a remarkable array of biochemical and pharmacological actions [26], some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems. The flavones and catechins are described as the most powerful flavonoids for protecting the body against ROS [27] and thus potentially possess anti-neoplastic activities. The anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic attributes of flavonoids are well established [28].

Apart from currently used effective anti-angiogenic agents like Avastin (an antibody) that directly targets VEGF, various other strategies are in development phase that include development of anti-angiogenic small molecules like VEGF-AS (Veglin), ABT-510 and Dalteparin. Veglin is being developed at VasGene Therapeutics Inc. as an antisense oligonucleotide that binds with DNA and impairs VEGF expression. ABT-510 is a peptide mimetic of thrombospondin-1, designed for blocking angiogenesis [29,30]. Dalteparin targets heparin associated modulation of angiogenesis [31–33]. Previously we have described series of flavonoids as potential anti-angiogenic, anti-cancer and antioxidant agents. More specifically, the 3-hydroxy substitution was reported to be associated with effective anti-angiogenic activity in CAM model [34,35]. In continuation of our research interest in this area, we inspired to undertake the effect of structurally diverse flavonoids on CAM angiogenesis along with analyzing their cytotoxic effect on various cancer cell lines. The selected flavonoids were also tested for their COX inhibitory and antioxidant properties. *In silico* studies such as molecular docking was carried out to understand the possible underlying mechanism of anti-angiogenic activities of the selected flavonoids. Results from the present study focus the importance of formononetin, silibinin, silymarin, biochanin-A and myricetin as suitable molecules for the design of novel anti-angiogenic and anticancer agents.

Materials and methods

Chemicals and cell culture

The selected flavonoids (Fig. 1) such as 4′-methoxy flavone (4′-MF); 3-hydroxy-7-methoxy flavone (3H-7-MF); 7-hydroxy-4′-methoxy isoflavone (formononetin); 5,7-dihydroxy-4′-methoxy isoflavone (biochanin-A); diosmin; 3′,5,7-trihydroxy-4′-methoxy flavonone (hesperitin); 3′,5,7-trihydroxy-4′-methoxy flavonone-7-rhamnoglucoside (hesperidin), 2′-hydroxy flavonone (2′HFN), 4′-hydroxy flavonone (4′HFN), 7-hydroxy flavonone (7HFN), myricetin, taxifolin, silibinin, silymarin, naringenin, naringin, catechin and DPPH were obtained from Sigma–Aldrich Co. (St. Louis MO, USA). Nitroblue tetrazolium (NBT) and phenazine methosulfate (PMS) were purchased from Fine Chemicals Ltd. Mumbai. NADH and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was procured from Spectrochem Pvt. Ltd. Mumbai. The COX-I & II (human ovine) inhibitor screening assay kit [Catalog No. 760111] was obtained from Cayman, U.S.A., Fertilized chickens eggs were purchased from the local market at Nanded city MS (Maharashtra State). Cancer cell lines, HL-60 (leukemia), Hep-3B (liver cancer), HeLa-B75 (cervix cancer), MDA-MB-453 (mammary gland cancer) and Chang liver (normal liver) lines were purchased from National Centre for Cell Sciences (NCCS), Pune (MS), India; all other chemicals, solvents and reagents used were of analytical grade and were procured from the commercial sources.

Chorioallantoic membrane (CAM) assay

The CAM assay was carried out as per earlier reported method [36] with slight modifications [34,37,38]. In brief, the fertilized chicken eggs were kept in a humidified incubator at 37 °C. The eggs were positioned in a horizontal position and rotated several times. On 9th day of incubation the eggs were opened on the snub side and a 1 × 1 cm window was cut into the eggshell. The individual concentrations (10–100 μM) of selected flavonoids prepared in dimethyl sulfoxide (DMSO, 0.05%, v/v) were applied (20 μl/disc) to sterile glass discs (10 mm) separately and allowed to dry under laminar flow conditions. The discs loaded with samples were