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Gentisic Acid, a Quinonoid Aspirin Metabolite in Cancer Prevention and Treatment. New Horizons in Management of Brain Tumors and Systemic Cancers

Meric A Altinoz[1*]
İlhan Elmaci[2]
Aysel Ozpinar[3]

[1]Neuroacademy Group, Istanbul, Turkey
[2]Department of Neurosurgery, Memorial Hospital Istanbul, Turkey
[3]Department of Medical Biochemistry, Acibadem University, Istanbul, Turkey

Abstract

Gentisic Acid (GA) is an endogenously synthesized quinonoid phenolic acid in plants, where it functions as an immune molecule against viral plant pathogens via modifying microRNAs. In mammalians, GA is both consumed from exogenous sources (from fruits and vegetables) and produced endogenously as an endogenous siderophore and as a byproduct of tyrosine catabolism. Besides the edible plants, aspirin is also an important source of GA, since its catabolism produces GA. Noteworthy, humans with variant alleles of CYP2C9 which are incapable to produce GA during aspirin catabolism do not benefit from aspirin in reduction of adenomas in the large bowel. In past, GA was succesfully used in the treatment of rheumatological diseases in humans with high biosafety. GA has an affinity to connective tissue proteins and a higher retention of exogenous GA was demonstrated in humans with cancer. GA has both direct and indirect strong antioxidant effects as a free radical scavenger molecule and as an agonist of NRF2 (Nuclear factor erythroid-derived 2-like 2), an important transcription factor which regulates synthesis of antioxidant molecules. GA blocks cancer promotion in animal models in association with reduction of free radical products and stimulating antioxidant molecules. GA also exerts prominent anti-inflammatory effects while stimulating elements of acquired immunity, lymphocytes; which likely occur due to its strong efficacies to block cyclooxygenases, 12-lipoxygenase and acting as a ligand of GPR35/CXCR8. GA also specifically inhibit protumorigenic signaling of Fibroblast Growth Factor (FGF) and cyclin dependent kinase-1 (CDK1). Sulfonated derivative of GA (2,5-dihydroxyphenylsulfonate, dobesilic acid) blocks subcutaneous growth of C6 glioma; and GA also acts as an agonist of Vasoactive Intestinal Polypeptide (VIP) pathway, which suppresses invasion of glioma cells *in vitro*. GA also inhibits OAT3/SLC22A8, which involves efflux of chemotherapeutics from the brain which may help to achieve therapeutic concentrations of anticancer agents in brain tumors. In future, combined aspirin-GA preparates may be tested for potential activity in cancer prevention and treatment.

Introduction

Immense efforts are performed to discover novel molecules to treat cancer, which is undoubtedly very essential to find better treatment strategies. On the other hand, repurposal of old drugs or metabolites of cheap drugs may also constitute an additional and logical approach to fight against this deadly disease. Soluble drugs with simple molecular structure and with high cost effectiveness may provide novel resources in this continuing fight. We believe that aspirin metabolite gentisic acid (GA) (Figure 1) highly merits to be studied in diverse cancer models as an anticancer adjuvant. To provide rationale for our proposal, we conducted a comprehensive librarial analysis and classified our findings as subheadings described below.

Blood levels of GA during aspirin intake

Aspirin treatment could lead formation of significant amounts of GA in human body. Aspirin metabolism in liver via mixed function oxidase system yields the formation of GA as a secondary quinonoid metabolite. The term 'quinonoid' describes resemblance to the quinone by having a structure characterized by a benzene nucleus containing two instead of three double bonds within the nucleus, which contains two external double bonds attached to the nucleus either at ortho or para positions. Aspirin is first metabolized to salicylic acid, which has a much longer life and progressively accumulates; salicylic acid is eliminated from the body by a combination of processes including the formation of GA, as a first-order process. Hence, in all cases when aspirin is employed at high doses, these kinetic characteristics are responsible for an accumulation of GA [1]. A dose-dependent increase of GA in man was reported after aspirin administration, which accounted up to 3.3% of aspirin doses during a schedule of 4 g daily [1]. In human liver microsomes fortified with NADPH, salicylic acid reproducibly generates 2,3- and 2,5-DHBA (GA) but acetylsalicylic acid

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*Corresponding author:

Meric A Altinoz, MD
Neuroacademy Group
Department of Biochemistry
Acibadem University, Istanbul, Turkey
Tel:90 536 201 8327
E-mail: maltinoz@gmail.com

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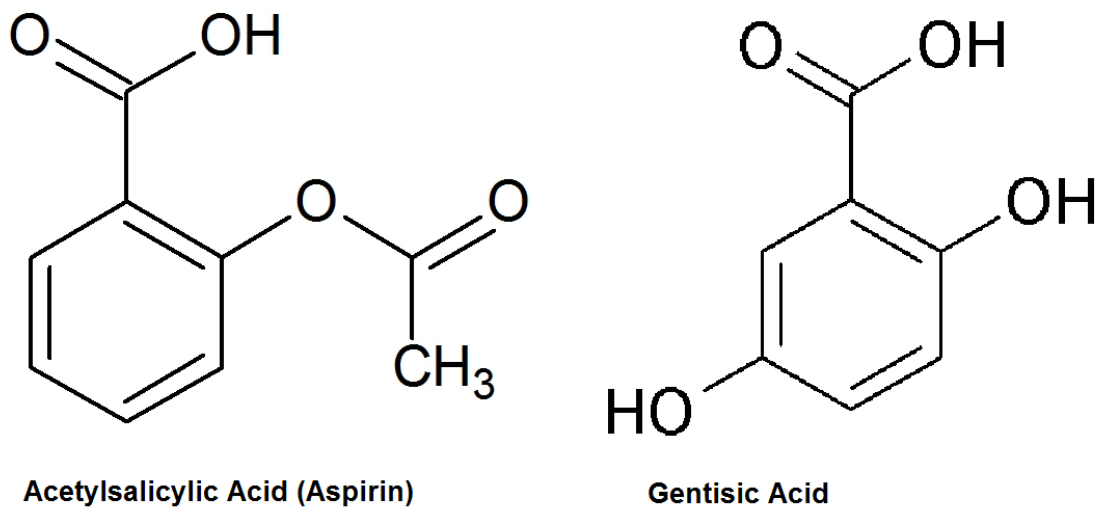

Figure 1: Molecular Structure of AcetylsalicylicAcid (Aspirin) and GentisicAcid

generates only GA [2]. Free radical attack of ·OH upon salicylate also produces GA, 2,3-dihydroxybenzoate and, to a much smaller extent, catechol; and formation of GA following salicylate administration is employed as a method to determine in vivo formation of·OH radicals [3]. In 16 patients with knee effusions receiving six to eight 650 mg aspirin tablets daily (3.9 to 5.2 gram), plasma levels between 5-25μM GAwere measured [4]. In human plasma, levels of 2 mM salicylate, which can be reached under aspirin therapy, lead to levels of about 20 μM GA [5].

Blood levels of GA during its direct employment as a medicine in rheumatismal disease treatment and its high biosafety

In rats, the minimum lethal dose of GA is 8.5 to 9 g/kg, which approximately correspond to 600 to 800 g/kg for a 70 kg person, which are huge dosages and revealing high safety [6]. No ill effects on growing rats were shown from feeding sodium gentisate in amounts upto 20 g daily for a normal person and the prothrombin time remained normal by feeding sodium gentisate for 18 weeks at the 30 and 40 g levels [6]. A two to four fold increase in the urine ascorbic acid content was revealed, which further enhanced as the feeding level of sodium gentisate is elevated. Experiments with ^{14}-C GA injection in rats showed wide dissemination in the organism, yet prolonged feeding of rats with 2% GA produced negligible storages in liver, omental fat, and brain, suggesting that continuous exposure to GA would be necessary to test various therapeutic potentials of GA [7]. In mongrel dogs, which were fed 50 mg/kg sodium gentisate three times a day for the first five days, and twice on the sixth day of each week for seven weeks, no histopathological changes were seen in biopsies from aorta, heart, lung, liver, kidney, spleen, stomach, small and large intestine, bladder, pancreas, adrenal and thyroid tissues [8].

GA is eight-to-ten times less toxic than salicylates in animal studies and preserves the plasma alkali reserve [9]. Salicylate blocks hyaluronidase at very high dosages *in vitro*, whereas GA exerts the same effect at a few μg/ml [7,10]. Since hyaluronidase involves in damage to the cartilage, GA has been introduced as an antirheumatic agent in 1948 [10]. Surprisingly, GA at a comparable dose to salicylate, reduced pain, edema, and heat in the joints with accompanying decrease in body temperature and sedimentation. Moreover, patients who can not tolerate aspirin due to duodenal ulcer, did not complain of gastric irritation while taking GA [7,10].

In the past, very high levels of GA were given to patients and the obtained levels in blood varied according to the applied doses. Some authors have proposed that GA is as activetherapeutically as sodium salicylate and it produces much fewer toxic effects even in doses up to 18 g per day [11]. Consden and Stanier [12] reported that GA blood levels between 200 to 300 μg/ml could be obtained at high doses; for instance, 300 μg/ml of GA was measured in the blood of a patient 24 hours after treatment with 22.5 g of GA. When GA was given as a single per oral application to 5 adults at a dosage of 37 mg/kg (2.6 g for a 70 kg subject), blood levels between 110 to 130 μg/ml were obtained, which declined following 4 hours after treatment [13]. Daily blood levels of GA between 40 to 80 μg/ml were measured in subjects given GA at a dose of 20 g per day in divided doses [11].

Some authors proposed that analgesic and antipyretic actions of GA is superior to salicylates and declared that GA controlled rheumatic fever as promptly as salicylates and did not produce any toxicity including prolongation of prothrombin time, changes in the blood count, liver or kidney function [15]. Clarke et al. [16] administered GA to patients who had a primary attack of rheumatic fever. The drug was given for from 60 days to 6 months, with a starting schedule of 1.0 or 1.2 g of sodium gentisate every three or four hours day and night (total 6 to 9.6 g/day). As improvement occurred, the amount of drug was gradually reduced to a minimum of 1.2 g every four hours four times daily (total 7.2 g/day). Some of these patients obtained symptomatic relief within 24 hours after the GA therapy was started, a few in four weeks, but most of these patients were symptom free within the first two weeks of treatment [16].

The temperature and heart rate became normal within two to three weeks and the sedimentation rate was normal in one to six weeks or in an average of four weeks. The only toxic reaction observed was nausea in two children, but when the sodium gentisate was given in a liquid vehicle the nausea promptly disappeared [16]. Moreover, GA was also efficient in reducing other cardiac symptoms (murmur, enlargement) of acute rheumatic fever. They found sodium gentisate to be more satisfactory than the salicylates in the treatment of the acute, recurrent and persistent forms of rheumatic fever, but they also underlined that gentisate is rapidly eliminated from the body, which requires that it be administered at frequent intervals throughout the entire day [16]. They also concluded that GA blood levels between 35 to 50 μg/ml (227.3 to 324.7 μM) can be achieved with these treatment regimes, which are lower concentrations than salicylates [16].

Sandler [17] had observed that GA treatment (5 times 2 g per day) provided faster relief of symptoms, faster reduction in erythrocyte sedimentation rates with lesser signs of toxicity (lack of tinnitus, nausea, gastric complaints and extension of prothrombin time) than aspirin in acute rheumatic fever [17]. Batterman [18] reported that GA exerts analgesic action comperable to aspirin yet

with significantly lesser side effects (11.1% versus 40% for GA and aspirin, respectively). On the other hand, Rosenberg et al. [8] reported lesser clinical efficacy of sodium gentisate in rheumatic diseases, while one patient with erythema nodosum reported rapid relief of pain and disappearance of lesions of the lower extremities.

Another feature of GA is that it does not influence platelet activity even at 500 µM, suggesting lack of hemorrhagic risk with GA consumption [19]. Kleinsorge and Pohl [9] also underlined that GA treatment does not cause any hepatic, liver and hematological side effects in their patients series. GA treatment at high dosages does not also confusion/delirium like side effects which may be observed with treatments including high dose salicylates [20]. Considering embryotoxicity, while GA doses upto 1.9 mM did not embrytoxicity in cultured rat embryos, salicylate induced prominent lethality in embryos suggesting again high biosafety of GA [21].

While salicylate induces dose dependent loose coupling, uncoupling and swelling of isolated mitochondria, GA does not induce mitochondrial uncoupling or swelling in healthy cells [22]. Moreover, when GA is added to a combinatory treatment including salicylate, it did not hinder its clinical efficacy and did not enhance toxicity [23]. Aspirin poisoning occurs when the total salicylate concentration exceed 3 mM with symptoms including increased oxygen consumption and hyperthermia (which also resemble to symptoms of Reye Syndrome), suggesting oxidative phosphorylation is a primary factor in salicylate toxicity [22].

GA upto concentrations reaching 20 mM do not inhibit proton transfer through phospholipid bilayers in opposite to aspirin and salicylate; hence it is safer in terms of general metabolic actions [22]. Nonetheless, as will be suggested below, very high levels of GA (5 mM, about 770 µg/ml) could block certain mitochondrial enzymes; such levels can not be reached even at high dose GA treatment; but retential of GA in cancerous tissues may selectively hamper metabolic activities in cancer cells.In dog studies, it was revealed that GA pathway of catabolism is exclusively by conjugation at the 5-hydroxyl group [7]. Following single oral doses of GA (190 to 310 mg/kg), about 60% of the drug is excreted unchanged in the urine, increased *O*-sulphate output accounts about 20% to 28%, *O*-glucuronide output accounts between 7% to 15%. 5-O-methylgentisic acid formation was detected at trace amounts. In fevered humans, dehydroxylation to salicylate is also reported [7].

GA as an immune defence molecule from bacteria to plants

Aspirin or acetylsalicylic acid is first synthesized by treatment of salicylic acid with acetic acid. In fact, salicylic acid is found as a sugar conjugate in salicin, after the latin name for the white willow (Salix alba). Very interestingly, salicylic acid derivatives act like "plant interferons" in various plants and suppress propagation of certain viral pathogens. As living organisms from plants to humans have some common mechanisms of immunity, it is very tempting to speculate whether aspirin and aspirin derivatives could also act like chemical interferones to suppress viral and malignant growth.

As early as in 1951, it was shown that GA could block T2r+ coliphage growth in *E. Coli* [24]. The lowest phage inhibiting concentration of GA was 150 µM but at least 100-fold higher concentration was required to inhibit the bacterial growth suggesting a specific inhibitory effect of GA against viral growth [24]. Later on it was suggested that salicylic acid (a precursor of GA) mediated Systemic Required Resistance (SAR) against pathogens in plants [25]. SAR is characterized by a hypersensitive response characterized by necrotic lesions limiting the site of infection in association with generation of reactive oxygen species, cell death, overproduction of phenolic compounds, deposition of lignins and expression of PR (pathogenesis related) proteins [25]. First in 1999, GA was shown to accumulate in tomato plants infected with RNA pathogens, Citrus exocortis viroid (CEVd) and tomato mosaic virus (ToMV) and induced accumulation of the CEVd-induced pathogenesis-related (PR) proteins P23, P32, and P34. These proteins were not induced by the GA-precursor salicylic acid, which is shown to be rapidly converted to GA in healthy leafs of tomato plants, suggesting that GA acts as a pathogen induced signal for activation of plant immune defense genes [26].

GA accumulation was also shown to occur in *Cucumis sativus* and *Gynura aurantiaca*, infected with either prunus necrotic ringspot virus (PNRSV) or the exocortis viroid (CEVd), respectively(Belles et al. 2006). Both pathogens produced systemic infections and induced production of large amounts of the GA, which was found mostly in a conjugated (β-glucoside).

GA also accumulated in nonnecrotic infections with low level inoculations with *Pseudomonas syringae* pv, but not in necrotic infections with higher Pseudomonas-inoculations suggesting systemic immunoactivating roles of this unique compound, which was able to induce peroxidase activity in both Gynura and cucumber plants and to induce polyphenol oxidase activity in cucumber in a stronger manner than salicylate [27]. Lastly,GA was also found to induce RNAsilencing-related genes in tomato, such as ToRDR1 and DCL2 against RNA viruses [28].

GA as an edible phenolic acid from fruits and vegetables

Phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids are secondary plant products, and are well known to possess antioxidant, antirheumatismal and antitumoral properties [16,29]. The structures of antirheumatismal phenolic acids exert common features which may be responsible of their antirheumatic efficacy [16]. They all possess an aromatic ring (with unique antiseptic effects without toxicity in man), they are unstable in the body and exert antipyretic and analgesic features [16]. All of these molecules contain one carboxyl group and have one or more hydroxyl groups, with a hydroxyl group adjacent to the carboxyl group, a relationship which is probably of biochemical and therapeutic importance [16]. The total adult intake of hydroxybenzoic acids amounts to 11 mg/day with 75% of salicylic acid intake coming from plant-derived foodstuff including fruits and juices [30].

GA belongs to the plant derived phenolic acids and is found in gentians (*Gentiana spp*.), olives (*Olea europaea*), in virgin olive oil, artichokes (*Helianthus tuberosus*), citric fruits (*Citrus spp*.), grapes (*Vitis vinifera*), sesame (*Sesamum indicum*), and red sandalwood (*Pterocarpus santalinus*) [31]. GA is also present in fruits including kiwi fruit, apple, bitter melon, blackberries, grapes, pears, and in aloe vera and mushrooms [32]. Therefore, it is not surprising that in healthy subjects, who do not consume aspirin, significantly detectable levels of salicylic acid, GA and 2,3-DHBA are measured in plasma [33]. GA and its sodium salt are used as antioxidants in the stabilization of edible fat, after ingestion around 300 mg/kg dosages in the rat and dogs, it is excreted via urine more than half of the dosage unchanged, and the remainder as conjugates (mainly with glucuronide) at the 5-hydroxyl group, which is usual for metabolism of phenols and phenolic acids [7]. GA also exists in wine, at levels of 2.25 mg/L in white wine and at slightly higher concentrations in red wine [34]. Lastly, GA may be intentionally added to oils, fats and other foodstuffs to minimize rancidification [35].

GA existence in medicinal plants with antiinflammatory and anticancer activities and GA inhibition of tumor promotion

Carcinogenesis exerts a multistep course involvinginitiation, promotion and progression. Of the three stages of cancer, initiation is the first and irreversible phase and progression is the last phase in carcinogenesis, so the most plausible way to block carcinogenesis shall target the initiation phase. Free radicals involve in all the three stages of carcinogenesis and to defend against free radicals, the body has versatile antioxidant compounds and enzymes (glutathione, catalase, superoxide dismutase, etc.). However, exposure of carcinogens generate free radicals and cause prominent declines in the body's antioxidant defense [30]. *Hibiscus rosasinensis*L. (Malvaceae)(Figure 2), commonly called China rose, isused in folkloric medicine in many countriesincluding India, China, Japan, parts of Africa and Americafor the treatment of fever, certain microbial diseases, as an abortifacient

and is also employed topicallyto cancerous tumors and wounds. Radical scavenging, antipyretic and antioxidant activities of this plant were also shown in experimental studies. *Hibiscus rosa sinensis* extract contains quercetin, carotene, niacin, riboflavin, malvalic acid, margaric acid and noteworthy, the GA [30].

Sharma *etal*. [30]analyzed the role GA in the chemopreventive activity of Hibiscusrosa sinensis extract on 7,12-dimethyl benz(a) anthracene(DMBA)/croton oil-stimulated carcinogenesis in mice skin through 12-O-tetradecanoyl phorbol-13-acetate (TPA)-inducedtumour promotion and oxidative stress. *H. rosa sinensis* extract significantly reduced the number of skin tumors induced by DMBA and croton oil and also delayed the latency period for tumor promotion. A single topical application of TPA significantly lowered reduced-glutathione, activities of its metabolizing and antioxidantenzymes (catalase, glucose-6-phosphate dehydrogenase, glutathione, glutathione peroxidase, glutathione reductase and glutathione-S-transferase), while malondialdehyde (MDA) formation, H_2O_2 content, ornithine decarboxylase (ODC) activity and DNAsynthesis were significantly enhanced [30]. Importantly, pretreatment with *H. rosa sinensis* extract (3.5mg and 7 mg/kgbody weight) and GA (2.0 µg and 4.0 µg/0.2 ml acetone per animal) restored the levels of GSH, and itsmetabolizing and antioxidant enzymes and reduced MDAformation and H_2O_2 content. GA also inhibited ODC activity which was not dose dependent; but it also blocked DNA synthesis in a dose-dependent manner. The same group also investigated the effects of GA on the carcinogenesis-associated biochemical pathways in murine skin induced by benzoyl peroxide (BPO) (20 mg/0.2 ml/animal) and ultravioletradiations (UVR) (0.420 J/m^2/s) [36]. BPO and UVR treatments induced tumor promotion and oxidative stress and significantlyreduced the antioxidant and detoxification enzymes in association with increases in MDA formation,H_2O_2 generation, ODC activity and unscheduled DNA synthesis. GA restored the levels of these parameters (such as via increasing catalase and glutathione peroxidase activity) in a dose-dependent manner except the ODC activity [36].

Solanum nigrum Linn (SN) belongs to the Solanaceae family, is a plant growing in South Asia, and has been used in folkloric medicine for antipyretic, diuretic,anticancer, and hepatoprotective effects [37]. *S. Nigrum* Linn has been reported to induce apoptosis in MCF-7 breast cancer and Hep-G2 liver cancer cells, necrosis in SC-M1 stomach cancer cells, and autophagy in Hep-G2 cells. HPLC analyses revealed that SN leaves containedthe highest concentration of GA, luteolin, apigenin, kaempferol, and m-coumaricacidand exerted highest tumoricidal activity in comparison to other parts of the plant (leaves, fruits, etc.). A significant tumoricidal effect of SN leaf extract was witnessed on AU565 breast cancer cells via autophagy at lower and apoptosis+autophagy at higher doses(<100 µg/ml) [37].

Ginseng (Panax ginseng Meyer) is a medicinal plant belonging tothe *Araliaceae* family being used for more than 2,000 years mostly as a 4 to 6-yr-old root [38]. Ginseng harbours versatile pharmacological features including immunostimulation, antitumoral activity, antiemetic and antioxidant actions. More than 10 phenolic compounds, including GA, caffeic acid, ferulic acid, vanillic acid, p-hydroxybenzoic acid and syringic acid were reported in fresh and/orprocessed ginseng. In 3 to 6-yr-old ginseng roots, GA and naringenin were defined as the major phenolic compounds. In the ginseng fruit, chlorogenic acid and gentisic acid were the most abundant phenolic compounds, followed by rutin, p-coumaric and salicylic acid [38].

Tremella fuciformis Berkis an edible mushroom and its extract (TFB) has long been used as a traditional medicine in Asia and exerts antioxidant, anti-inflammatory and anticancer features. Chronic inflammation prominently involves in metabolic diseases, inflammatory bowel disease and cancer. Upon exposure to antigens, innate immune cells are robustly stimulated and triggerinflammation by increasing the synthesis of inflammatory mediators including nitric oxide (NO) and prostaglandin E2 (PGE_2).Additionally, inflammatory

Figure 2: *Hibiscus Rosa* Plant Rich in Gentisic Acid

cytokines such as IL-1β, TNF-α, and IL-6 are increasingly being produced which significantly contribute to inflammation. The transcription factor NF-κB has a major role in inflammation. In the inactive form, NF-κB dimers localize in the cytoplasm bound to their suppressor, IκB.Following inflammatory stimuli, IκB is degraded upon phosphorylation by IκB kinase (IKK), liberating NF-κB to translocate from the cytoplasm into the nucleus where it binds to the NF-κB response elements to activate genes including iNOS, COX-2, IL-1β, TNF-α, and IL-6 [39].

Recent studies have demonstrated that mitogen activated kinases (MAPKs) and activator protein-1 are heavily involved in inflammation via the associated production of NO and PGE2.In RAW 264.7murine macrophages, TFB significantly blocked LPS-induced iNOS/NO and COX-2/PGE2 synthesis as well as IKK, IκB, and p65 phosphorylation andtranslocation of p65 from the cytosolinto the nucleus. Additionally, TFB inhibited LPS-induced phosphorylation of MAPKs. In an acute inflammation study, oral treatment with TFBreduced LPS-induced IL-1β, IL-6 and TNF-α production and iNOS and COX-2 expression. The major bioactive compounds from TFB extract were revealed as GA, protocatechuic acid, 4-hydroxybenzoic acid, and coumaric acid [39].

Leonurus sibiricus L. is a plant existing in southern Asia and Siberia which is widely used as a medicinal plant for anti-inflammatory, antibacterial, antioxidant, antiviral and anticancer effects [40]. Chromatographic analysis of TR extract demonstrated the presence of various phenolic compounds (GA, 4-hydroxybenzoic acid, vanilic acid, 1,3-dicaffeoylquinic acid, α-resorcylic acid). Transformed root extract (TR) of *Leonurus sibiricus* L. blocked proliferation of glioma cells after 24 h of treatment. TR root extract triggered apoptosis on various grades (I-III) of human glioma cells by the generation of reactive oxygen species (ROS) in association with loss of mitochondrial membrane potential. TR also changed mRNA levels of Bax, Bcl-2, p53, Caspase-3, Caspase-8 and Caspase-9 involved in apoptosis suggesting its potential to stimulate both the intrinsic and extrinsic pathways of programmed cell death [40].

GA as a byproduct of tyrosine catabolism

Both eukaryotes and prokaryotes catabolize phenylalanine and tyrosine to homogentisate, a central intermediate prior to gentisate [41]. In rat liver cytosol, homogentisate is oxidized to gentisaldehyde which is then oxidized to gentisate by aldehyde dehydrogenase [4]. In rats during the growth of the AH130 ascites tumor, the activity of the gentisic aldehyde forming enzyme first increased and then decreased, while the activity of gentisic aldehyde dehydrogenase decreased throughout the whole time [42]. This is a very interesting result which may suggest that tumoral tissue may try to avoid biosynthesis of GA, as GA may be an important inherent defense against tumor progression. Here, it shall be also noted that 4-HPP (4-hydroxyphenyl pyruvate)-dehydrogenase enzyme necessary to convert 4-HPP to homogentisic acid requires ascorbic acid (Vitamin C) [43], which is a powerful redox modifier and which intake is reversely linked to cancer incidence as shown by several studies [44].

GA as a constituent of mammalian iron chelating-siderophore

Iron delivery to cells is critical for growth and survival as cells need an unterrupted supply of iron, as functional components of mitochondrial heme and hemoproteins such as iron-sulfur (Fe-S) cluster-containing proteins [45]. Iron is delivered to almost all mammalian cells via receptor-mediated endocytosis of iron-loaded transferrin. After intracellular uptake, levels of iron shall be precisely regulated, as excess free iron catalyzes the production of toxic reactive oxygen species (ROS) in the cytoplasm. Iron levels in mitochondria shall also be tightly regulated; an insufficient delivery of iron can hinder the metabolic and respiratory functions of the organelle, whereas supraphysiological levels of mitochondrial iron could also trigger the formation of ROS, which are produced as a side reaction of mitochondrial electron transport [45]. Members of the solute carrier (SLC) family of proteins mediate iron import into mitochondria. SLC25A37, also called mitoferrin 1 (Mfrn1), and SLC25A28, also called mitoferrin 2 (Mfrn2), exert essential roles for mitochondrial iron import into erythroid and nonerythroid cells, respectively [46].

A high percentage of the cytoplasmic iron is tightly bound to proteins, but a certain percent of cytoplasmic iron is complexed with a low molecular weight carrier (siderophore) to form the labile or chelatable iron pool. One of these siderophores is the siderocalin/lipocalin 24p3 (also known as lipocalin-2 (Lcn2)) which binds iron and delivers it to or remove it from cells. Lipocalins are secreted proteins that can bind small molecular weight ligands. The mammalian antibacterial protein siderocalin/lipocalin 24p3, functions by sequestering iron as bacterial siderophore complexes to reduce iron levels necessary for bacterial growth. Indeed, lipocalin deficient mice are profoundly susceptible to bacterial infections [47]. Lipocalins also regulate versatile biological processes including programmed cell death and innate immunity [45]. Lipocalin/siderophore-free Scn (apo-Scn) is secreted upon cytokine withdrawal or during carcinogenesis and internalized by a receptor mediated pathway to sequester and export intracellular iron, driving apoptosis through autocrine, paracrine, or exocrine mechanisms such as during IL-3 withdrawal induced hematopoietic cell apoptosis as shown by certain groups but not others. Internalization of lipocalin/siderocalin is mediated by brain-type organic cation transporter (BOCT; also SLC22A17 or 24p3R), providing access of apo-Scn to crucial intracellular iron pools essential for metabolic and proliferative pathways [47].

Bacterial siderophores, which ablate binding of iron to mammalian lipocalin, are called 'stealth' siderophores and allow pathogens to evade the mammalian lipocalin defense, permitting bacterial acquisition of iron during infection [47]. 24p3 does not directly bind iron, rather via small molecular weight, iron-chelating compound, such as enterobactin, which is utilized by the bacteria in association with 2p43. The iron-binding component of bacterial enterobactin is 2,3-DHBA (2,3 dihyroxybenzoic acid) [45]. The human homologue of enterobactin A (EntA) was identified as short chain dehydrogenase/reductase family member (DHRS6) now reffered to as BDH2 (3-Hydroxybutyrate Dehydrogenase 2) (Figure 3 depicts the crystal structure of D-3-hydroxybutyrate dehydrogenase from *Pseudomonas fragi* complexed with NAD^+) and its labile iron binding component was defined as GA. Mammalian cells without this siderophore accumulate improperly intense levels of cytoplasmic iron, resulting in increased levels of ROS [45]. On the other hand, it would not be inappropriate to presume that excess GA would lead accumulation of abnormally high levels of iron within the

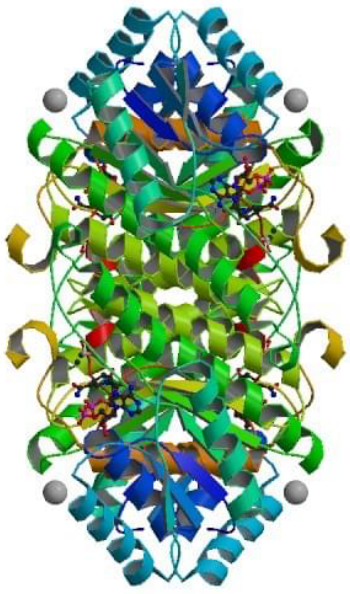

Figure 3: Molecular Structure of 3-Hydroxybutyrate Dehydrogenase

mitochondria causing mitochondrial ROS production and trigger mitochondrial pathways of apoptosis.

Some other research groups contradicted that GA can serve as an endogenous siderophore at neutral pH as binding studies lacked to demonstrate that GA could form high-affinity ternary complexes with lipocalin 2p43 and iron [47]. Nonetheless, more recent studies continued to show such a function of GA [46,48].

Decreasing of the mammalian siderophore component GA by blocking expression of bdh2 causes supraphysiological accumulation of intracellular iron and mitochondrial iron deficiency in cultured yeast and mammalian cells and in zebrafish embryos [46]. bdh2 null mice also suffered from microcytic anemia and tissue iron overload, especially in the spleen and, to a lesser extent, the liver, which is alleviated with exogenous GA supplementation [46].

The iron overload became more prominent when the mice are placed on an iron-rich diet, which lead to premature mortality. Additionally, bdh2 null mice exhibit reduced serum iron and in contrast to the phenotype of *bdh2* null mice on a high-iron diet, iron deficiency resulted in extreme anemia. Significantly, severe alopecia in *bdh2* null mice was witnessed that were received a low-iron diet which is similat to the anemia of chronic disease/inflammation in which iron stores are elevated in these tissues and are prevented from being exported. Thus, it is more likely that BDH2 may regulate iron export from cells and not intracellular import [46]. On the other hand, mice which could synthesize endogenous GA are more resistant to *E. Coli* infections, likely due to the fact that bacteria may "steal" endogenous GA for capturing iron [48]. Here, one may bring the concern that GA treatment may increase opportunistic infections; however, no such report exist for about a 20 years period when GA was used at high doses for treatment of rheumatological diseases.

Either, high doses of GA may reduce systemic levels of iron; and/or activate other pathways of immunity which would explain lack of enhanced infection risk with treatment of GA. For instance, GA constitutes 24% of the bound phenolics mango ginger (*Curcuma amada*), which phenolic extracts block the growth of *Helicobacter pylori* [49]. Very strikingly; GA was found to be superior to norepinephrine inpreventing cardiovascular collapse, mitochondrial dysfunction of the liver and lactic acidemia in *Pseudomonas aeruginosa* septic shock in dogs [50]. This effect was attributed to the GA induced reduction of lactate [50]; nonetheless if GA would be a significant promoter of bacterial growth, such a protective effect may have not been observed.

Protein affinity and fluorescence of GA and its accumulation in connective tissue and in cancer-bearing host

In ultraviolet light, GA exerts a prominent bluish-white fluorescence so robust that its trace amounts can be detected [51]. Following intravenous injection into mice, connective tissue flourescence could easily be witnessed highest in skin, tendons, bones, and cartilages (Figure 4). GA is excreted rapidly in the urine and the fluorescence of the skin and the tendons largely vanished within two hours. However, cartilages and bones retained the whitish fluorescence for 5 to 6 hours, perhaps because these tissues have a slower blood circulation, although the likelihood also exists that GA exerts a firmer attachment to the connective tissues [51]. *In vitro* binding of gentisate by tissues was analyzed by exposure of pieces of pigs' cartilage and mouse cartilage, muscle, liver, kidney, and tendon to 1 per cent solutions of GA and then washing them briefly in running tap water. Fluorescence was witnessed in each fragment and immersion in saline for several hours resulted in disappearance of fluorescence from all tissues except cartilage and tendon [51].

Significant binding of GA by both bovine and human serum albumin was also shown in molar ratios similar to salicylate. GA is the reductant in an oxidation reduction system [Eo3 0.796 m.v. at pH 0.028] of which the oxidant, gentisoquinone, is unstable and incapable to be isolated [51]. GA oxidation products can be separated electrophoretically into different components. Some of these appear to be highly bound not only by albumin but also by connective tissue proteins, e.g., collagen. Gentisate is easily oxidized in air at neutral pH to brown or reddish brown pigments, likely quinone which has partly polymerized [51]. Also, when pieces of collagen or skin powder were incubated and shaken with gentisate solutions at neutral pH, pink to reddish brown coloration of the proteins could be noted, which was discernible at GA concentrations as low as 0.08 mg. per cent (5×10^{-6} M). Connective tissue affinity of gentisate seemed to exceed that of salicylate. Moreover, GA (1.5 mM) was shown to induce formation of collagen fibrils from collagen solutions [52].

In the ochronosis of alkaptonuria, the black colour of the cartilages, sclerae, and tendons is due to oxidation products of homogentisic acid [51](Figure 5). The peculiar distribution of the ochre pigment, i.e., its affinity for the connective tissues mostly for cartilage and bone was explained with the fact that these tissues have low blood supply with lack of poising effect of hemoglobin allowing that the quinone from the homogentisic acid can be formed. A similar mechanism may also account for GA. In the alkaptonuria, the blood levels of homogentisic acid is no higher than 1 to 3 mg per cent, while after oral administration of GA serum levels as high as 15 to 30 mg per cent may be achieved [51]. Hence, it was commented that the antirheumatic efficacy of the phenols may involve their oxidation *in vivo* to quinones.

GA inhibition of mitochondrial respiration and fatty Acid β-oxidation may involve in its antineoplastic Potential

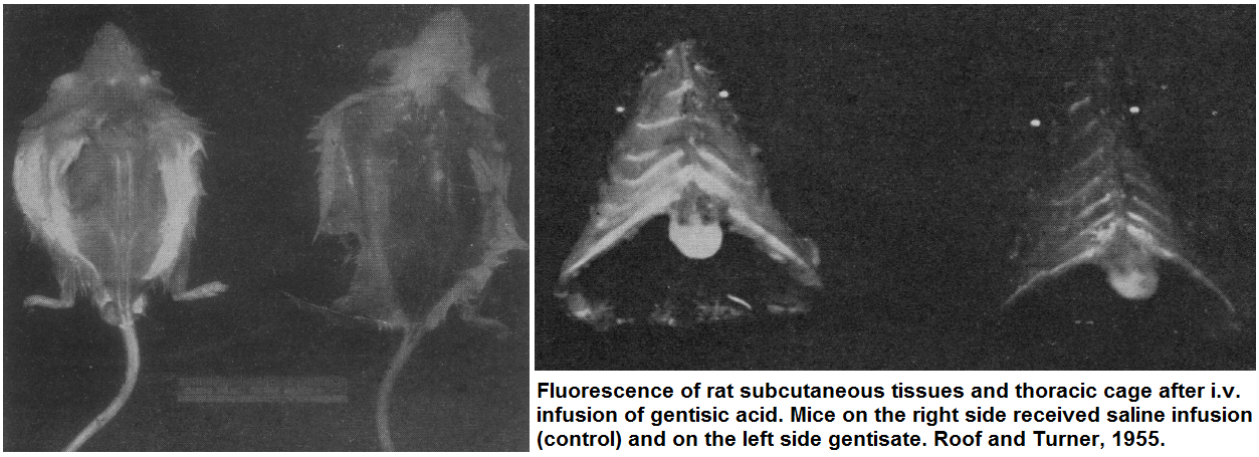

Figure 4: Fluorescence of Rat Connective Tissues Following i.v. Infusion of GentisicAcid [51]

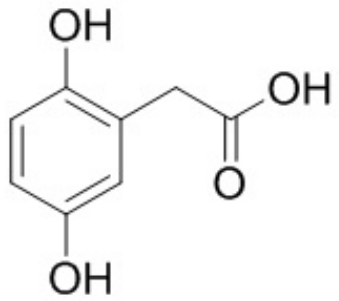

Figure 5: Molecular Structure of HomogentisicAcid, the Alkaptonuria Pigment

Eukaryotic cells produce their energy mainly through mitochondrial oxidative phosphorylation. Nonetheless, many cancer cells produce their energy principally from glycolysis even in the presence of sufficient oxygen, which is called aerobic glycolysis or Warburg effect (named in honour of Otto Warburg) [52]. Otto Warburg proposed that this shift in metabolism is the central route of carcinogenesis. However, the modern view mostly accepts that carcinogenesis is mostly driven by tumor suppressor gene mutations or activated oncogenes and the Warburg effect is a result of these mutations rather than a cause. Still, agents which inhibit glycolysis are actively being investigated as potential therapeutics. Whether glycolysis is the result or cause of carcinogenesis, one thing is certain. Cancer cells have a much higher demand of energy than normal benign cells due to their high proliferative rates. Thus, it is not surprising to discover that both glycolysis and tricarboxylic acid cycle+aerobic oxidative phosphorylation contribute to the energy production of malignant cells [54-56].

Cancer cells also increase mitochondrial β-oxidation of fatty acids [57]. Additionally, cancer cells shall neatly control certain mitochondrial pathways to overcome mitochondria associated production of reactive oxygen species and mitochondria-driven apoptosis [58,59]. For instance, NADH-ubiquinone oxidoreductase (Complex I) is the largest complex of the mitochondrial electron transport chain and contributes largely to the proton motive force essential for mitochondrial ATP synthesis and has a central role in redox control of cancer cell proliferation, apoptosis, and metastatic capability [60]. In parallel, metformin, a safe anti-diabetic agent, which blocks mitochondria oxidative phosphorylation complex I exerts prominent anticancer activity [61]. Shutting power of the both pathways, glycolysis and oxidative phosphorylation in cancer cells is even a more powerful strategy. For instance, blocking cytosolic NADH production by aldehyde dehydrogenase inhibition, combined with oxidative phosphorylation inhibition, resulted in up to 80% decrease of ATP production and significant regression of tumor growth in lung cancer models [62]. As will be discussed below, GA could block both aerobic metabolism and fatty acid oxidation induced energetic pathways in mitochondria.

Succinate dehydrogenase (SDH) or succinate-coenzyme Q oxidoreductase (SQR) or respiratory Complex II is a complex of enzymes residing in the inner mitochondrial membrane, which is the only enzyme complex participating both in the tricarboxylic acid cycle and the electron transport chain. Low concentrations of GA (100 μM) prominently inhibited the succinate oxidase and succinate-cytochrome C reductase activities in guinea pig liver mitochondria. GA exerted a specific effect a point on the electron chain between succinate dehydrogenase and cytochrome C [63]. Further experiments suggested that the formation of 2-carboxy 1:4-benzoquinone from GA may be responsible for this inhibitory efficacy, since quinones are powerful inhibitors of succinate oxidase activity and tissue preparations containing cytochrome C and cytochrome oxidase oxidise phenols [63]. Indeed, GA is readily oxidized to its corresponding quinone because the two hydroxyl groups are in the para positions of the benzene ring, whereas neither salicylate nor γ-resorcylate are capable of being converted to similar molecules and fail to inhibit succinate oxidase complex at low concentrations [63].

When the effects of salicylate derivatives on the incorporation of radiocarbon from [1:4-$^{14}C_2$] in the soluble intermediates of testis mitochondria were analyzed, it was revealed that GA at very high levels (5 mM) increased the radioactivity in fumarate, malate and decreased the radioactivity in aspartate which can be ascribed to competition with NAD and NADP and inhibition of aspartate transaminase [64]. Aspartate transaminase (AST) or aspartate aminotransferase is a pyridoxal phosphate -dependent transaminase which catalyzes the reversible transfer of an α-amino group between aspartate and glutamate and involves in aminoacid synthesis and catabolism. In guinea pig testis, GA at high dose (5 mM) blocks several dehydrogenases including malate, isocitrate, lactate (NAD→NADH), lactate (NADH→NAD), glyceraldehyde-3-phosphate, α-glycerophosphate and glucose-6-phosphate dehydrogenases at percentages of 46%, 31%, 16%, 13%, 17%, 13% and 21%, respectively [65].

β-oxidation is a catabolic biochemical process by which fatty acids are broken down to generate acetyl-CoA, which enters the citric acid cycle and NADH and $FADH_2$, essential coenzymes of the electron transport chain. This process is named as such because the β-carbon of the fatty acid is oxidized to a carbonyl group. Peroxisomal β-oxidation catalyzes the catabolism of very long chain fatty acids and mitochondrial β-oxidation catalyzes the catabolism of short, medium and long chain fatty acids. Recent studies demonstrated that β-oxidation of fatty acids may be as important as glycolysis in sustaining energy and growth of cancer cells [66]. β-oxidation may play even greater role than glycolysis in certain cancer types, such as prostate cancer. Increased glycolysis to cope with energy demand of rapid cell proliferation, is the basis for tumor imaging through glucose analog FDG (2-deoxy-2-fluoro-D-glucose) with positron emission tomography. One of the noteworthy features of prostate cancer is low glycolysis and low FDG avidity and it is shown that enhanced fatty acid β-oxidation is responsible to sustain energy in prostate cancer [67].

To illuminate the underlying mechanism behind the aspirin induction of Reye Syndrome (RS), Glasgow et al. [68] studied effects of aspirin metabolites on β-oxidation by fibroblasts obtained from RS patients and healthy controls. Aspirin did not inhibit at up to 15 mM, but salicylate, hydroxyhippurate and GA all exerted significant, dose dependent inhibition of palmitate oxidation in control and RS cells. RS patients cells were more vulnerable, where salicylate at 5 mM inhibited β-oxidation in controls by 11%, while RS cell rates were inhibited by 29%. Hydroxyhippurate and GA also inhibited β-oxidation beginning at a level of 1 mM, reaching 30% by 6 mM and 50% by 12 mM. GA similarly influenced β-oxidation in control and RS fibroblasts in different dose ranges, which was interpreted as a diminished risk with GA to trigger a RS like-effect [68]. But again, it shall be admitted that GA concentrations to block β-oxidation is almost impossible to be achieve in clinical application; but selective accumulation GA in tumors may lead concentrations capable to block β-oxidation in cancerous tissues.

As will be also mentioned below; GA, by virtue of its dihydroxy group in para position, also harbours oxidative features and inhibits glucose-6-phosphate dehydrogenase (G6PDH), but very high dosages above 5 mM (greater than 770 μg/ml) exerts this efficacy [70].

GA formation in large bowel and association of GA with aspirin's cancer preventive efficacy

Sulfasalazine is employed in treatment of inflammatory bowel diseases and its therapeutic activity is attributed to the formation of

5-aminosalicylate (5-ASA), which is a metabolite formed during the cleavage of sulfasalazine by intestinal microflora [70]. Dull et al. [70] demonstrated that activated mononuclear cells and granulocytes, and the products of the Fenton reaction, transformed [14C]5-ASA to a number of metabolites, including salicylate and GA. 5-ASA was oxidized by the oxidative burst of leukocytes and acted cytoprotective against reactive oxygen radicals as the lethal effects of superoxide radical or H_2O_2 on Chinese hamster ovary cells were blocked by 5-ASA [70]. Besides oxygen radicals, 5-ASA isa potential scavenger of other oxidants, such as hypochlorous acid (HOCI), released by neutrophils present in inflammatorybowel disease [72].

Liu et al. [71]studied the oxidation of 5-ASA by HOCI anddefined the reactive intermediates by mass spectral analysis, which revealed theformation of iminoquinone and quinone reactive intermediates. The major stable product formed was identified as GA.Regular consumption of nonsteroidal anti-inflammatory drugs (NSAIDs), and particularly aspirin has a preventive effect against colon cancer, but polymorphisms in NSAID-metabolizing enzymes may influence this efficacy. Aspirin is metabolized by cytochrome P450 CYP2C9 (Figure 6), which has a slow-metabolizing variant form. Bigler et al. evaluated whether the slow allele can modify aspirin chemoprevention against colon neoplasia in the Minnesota Cancer Prevention Research Unit (CPRU) adenomatous polyp case-control study by genotyping 474 adenoma cases and 563 controls. Aspirin use was inversely associated with adenoma risk, yet this protective efficacy was absent in aspirin users who carried the *CYP2C9*variant allele. It was underlined that the catabolic pathway of aspirin to GA is hampered in carriers of the *CYP2C9* variant alleles [72]; thus it would not be illogical to assume that GA itself exerts potential anticancer features.

Antioxidant activity of GA with emphasis to anticancer activity

Redox active biological compounds could exert both antioxidant and prooxidant effects dependent on the intracellular cytoplasmic trace metal content and block tumor growth via both mechanisms. While this seems paradoxical at the first glance, antitumor actions of these two opposite pathways were demonstrated in many studies. Intracytoplasmic free radicals and Protein Kinase-C mutually interact in malignant cell transformation and tumor progression and antioxidant molecules may hamper these cascades leading inihibiton of tumor growth [73-75]. On the other hand, robust levels of free radicals may also trigger proliferation inhibition and apoptosis of cancer cells [76,77]. As a third possibility, chronic oxidative stress elicited by redox active compounds may stimulate tumor growth at long term – at least theoretically -, if cancer cells could survive. We propose that such a mechanism would account for larger tumors in selenium-treated and long living animals with solid Ehrlich tumors.

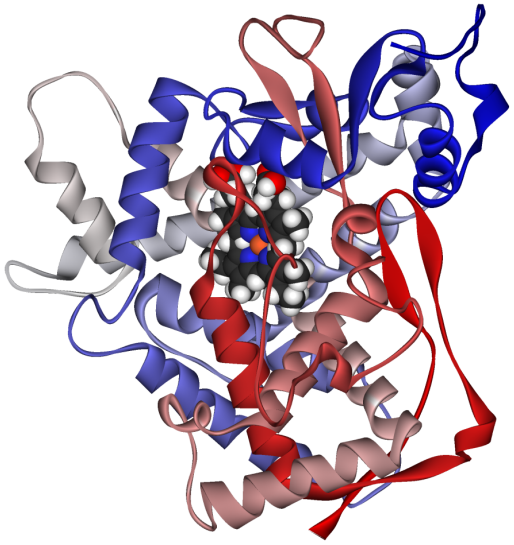

Figure 6: Molecular Structure of Cytochrome CYP2C9, Which Metabolizes Aspirin toGentisicAcid

GA has higher antioxidant capacity than trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble analog of vitamin E) [78] and forms stable chelates with biologically important cations Cu^{2+}, Fe^{3+}, Al^{3+} with the following orders of chelate stability: salicylic >gentisic >salicyluric acid, and Fe^{+3}>Al^{+3}>Cu^{+2} [79]. Broinizi et al. [80] assessed the antioxidant potentials of phenolic compounds that exist in the cashew seed and the cashew apple (*Anacardium occidentale*) and among these, GA showed the highest antioxidant potential when in relation to most of the other compounds that were found defined in this plant [80].

Tyrosyl radicals generated by myeloperoxidase (MPO) can promote low density lipoprotein(LDL) oxidation and the LDL oxidation by tyrosyl radicals involves in the onset of atherosclerosis [81]. GA at an easily achievable concentration (3 μM) inhibited LDL oxidation via myeloperoxidase driven tyrosyl radicals, in a process where even salicylate exerts prooxidant efficacy. Even when lipid oxidation was maximally induced by salicylate, the LDL oxidation was inhibited in presence of GA at salicylate/GA ratios that could be achieved in plasma of patients receiving aspirin medication [81]. When GA as well as aspirin, salicylate and o-anisic acid in the DPPH (diphenyl picryl hydrazyl) free radical (100 μM) scavenging assay is evaluated, only GA (50 μM) exerted scavenging features, all other salicylic acid derivatives (50 μM) were without effect [81]. GA forms a more stable phenoxyl radical than salicylate and thus may not be included into a radical chain with polyunsaturated fatty acids. Furthermore; phorbol-myristate-acetate (PMA)-activation of neutrophils resulted in an about 30-fold increase in diene formation; which was further increased by two fold when neutrophils were exposed to salicylate; while addition of GA (200 μM, 30.8μg/ml) to this salicylate system blocked this oxidation reaction and decreased diene formation to about 50% [81].

Glucose-derived radicals may oxidize LDL which is suggested to involve in the aetiology of atherosclerosis in diabetes [5]. The autoxidation of glucose causes formation of diverse reactive products like superoxide, hydroxyl, hydroxyalkyl and peroxylradicals and hydrogen peroxide. Exner et al. studied whether aspirin, salicylate and its metabolites GA and 2,3-dihydroxybenzoic acid (2,3-DHBA) could block LDL oxidation by glucose. Only GA and 2,3-DHBA inhibited LDL oxidation and the increase in endothelial tissue factor synthesis induced by glucose-oxidised LDL without effecting the glycation reaction of LDL. The antioxidative actions of DHBA were attributed to free radical scavenging and/or chelation of transition metal ions catalysing glucose autoxidation, but GA protection against LDL oxidation was not mediated via Fe^{+2} chelation like 2,3-DHBA. Glucose mediated oxidation of LDL was inhibited by achievable concentreations of GA (20 to 50 μM) where aspirin was ineffective [5].

Ashidate et al. also investigated whether GA inhibits LDL oxidation and the production of lipid hydroperoxides in human plasma [82]. The susceptibility of LDL oxidative modification was investigated by a method using 2,2V-azobis (4-methoxy-2,4-dimethylvaleronitrile, MeO-AMVN) or Cu^{2+}. MeO-AMVN is a compound which triggers free radical-mediated peroxidation of lipids in organic solution and in micelles, membranes, and LDL. Cholesterol ester hydroperoxides were generated by exposing human fresh plasma to $Cu^{2+}$to reveal the effect of GA on free radical-induced damage to plasma lipids. GA inhibited LDL oxidation in easily achievable concentrations anddose-dependent manner (0.1 to 1 μM) and also blocked formation of cholesterol ester hydroperoxides in plasma (20 μM). GA also exerted dose-dependent free radical scavenging activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (20 to 50 μM). Cholesterol ester hydroperoxides formed after complete consumption of ascorbic acid consistent with previous findings suggesting that ascorbic acid is the first defense against lipid peroxidation in plasma. The consumption of GA started after the complete consumption of both ascorbic acid and CoQ10, suggesting that both of these antioxidants initially prevented

lipid oxidation. In contrast, other plasma antioxidants (bilirubin, α-tocopherol, uric acid) remained unchanged suggesting that GA is more effective than these antioxidants in hindering formation of plasma lipid peroxidation [82]. Ozgova et al. [83] studied effects of GA on rat liver microsomal lipid peroxidation andhydroxyl radical (·OH) production in NADPH-dependent, 50 μMFe^{+2}–500 mM ascorbate (Fe^{+2}–AA) or 50 μMFe^{+2} systems,respectively. GA inhibited lipid peroxidation in NADPH-dependent and Fe^{+2} –AA systems with IC_{50} values above 30 μM and 80 μM, respectively; without an effect on Fe^{+2} system-associated lipid peroxidation. GA also quenched ·OH radicals in NADPH-dependent and Fe^{+2} –AA systems with IC_{50} values above 40 μM; while it was not capable to chelate Fe^{+2} and was oxidized with Fe^{+3} [83].

Joshi et al. [84] studied effects of GAby employingseveral*in vitro*models, including the isolatedrat liver mitochondria (RLM) and the human erythrocytes. GA scavenged hydroxyl radicals and subsequently blocked formation of the reducing adduct radical(~76%) and oxidizing phenoxyl radical (~24%) and also scavenged organohaloperoxyl radical [84]. Ascorbate repaired phenoxyl radical of GA and redox potential value of GA•/GA couple (0.774 V *vs*NHE) evaluated by cyclic voltammetry was less than those of physiologically important oxidants,which supports the observed antioxidant capacity of GA.

The presence of GAexerted almost complete protection againstγ-radiation in RLM as measured with TBARS and peroxide formation at a concentration of 10 μM with no pro-oxidant effect up to 200 μM. GA also blocked the RLM-protein damage induced by γ-radiation in a concentration dependent manner up to 100 μM, which was assessed by the formation of protein carbonyls and depletion of protein thiol groups. GA efficiently saved antioxidant enzyme superoxide dismutase (SOD) against γ-radiation and also inhibited the γ-radiation induced erythrocyte hemolysis in a concentration dependent manner up to100 μM(~ 75% protection against γ-radiation-induced (coupled with hypotonic shock) hemolysis of human erythrocytes at 100 μM). Lipid peroxidation as assessed by formation of TBARS and peroxides induced by γ-radiation has been reduced by ~93 and ~97%, respectively at 10 μM GA. The authors also suggested that i) the antioxidant and radioprotective properties of GA occured due to its phenoxyl group; ii) both phenolic groups of GA contributed to its antioxidant action; iii) GA also had a reducing power on Fe^{+3} [84].

GA saved against lipid damage via scavenging the lipid radicals (L•/LOO•) and subsequently blocked the oxidizingchain reactions and its radioprotective effect for lipids was much higher than to proteins in aqueous phaseand is almost saturated at 10 μM. GA has an octanol/water partition coefficient value ~40 which indicates that concentration ratio of GA inlipid to aqueous phase reaches ~40 on mixing lipids/membranes to aqueous GA solution. Hence, higherradioprotective effect of GA for lipids wasexplained with high partition coefficient of GA in oil phase resultingin increased concentration in lipids. The reaction of •OH radical with proteins in aqueous phase leads to the formation of protein carbonylsin presence of oxygen, loss of amino acids (cysteine,tryptophan and tyrosine) by oxidation and structural damage of enzymes and GA could scavenge •OH and protein radicals to protect proteins andthree-dimensional structure of SOD [84].

In aqueous solution, the overall rate constants of the dihydroxybenzoic acid DHBA (including GA)-reactions with HOO^- are higher than in non-polar media [85]. This is mostly caused by the sequential double proton loss electron transfer, albeit the hydrogen transfer reactions also accelerates as the polarity of the environment increases; thus, phenoxide anions are much better HOO^- scavengers than the neutral species. The reactivity order in this case is 2,6-DHBA <2,4-DHBA < 3,5-DHBA < 2,5-DHBA (GA)< 3,4-DHBA < 2,3-DHBA, and is directly related to the stability of the phenoxide anions, and to their molar fractions. Taking as a threshold value the rate constants for the HOO^- reaction with polyunsaturated fatty acids, 2,6-DHBA is the single one, among DHBAs, which can not save biomolecules against peroxyl damage in aqueous solution.In such media, 2,4-DHBA, 3,5-DHBA, 2,5-DHBA (GA), 3,4-DHBA, and 2,3-DHBA are predicted to react with HOO-1.2, 20.5, 63.9, 140.6, and 209.1 times faster than Trolox, respectively [85].

Compared to other antioxidants, the three most reactive compounds (2,5-DHBA, 3,4-DHBA and 2,3-DHBA) exert higher peroxyl radical scavenging activity than those of melatonin, caffeine, allicin, thioacrolein, and dopamine; and similar to those of canolol, protocatechuic acid, 2-propenesulfenic acid, and glutathione. Hence, in aqueous solution and at physiological pH, these three DHBA including GA are among the best peroxyl radical scavengers, identified so far [85].

It is also recently suggested that GA blockage of cyclooxygenase (COX, also known as prostaglandin endoperoxide synthase– Figure 7) activity may also associate with its antioxidant activity [86]. Ionization potential of GA is smaller than that of salicylic acid and acetylsalicylic acid, rendering it a stronger single electron donor than either of these two compounds. Hence, it was suggested that GA could inhibit COX via quenching the tyrosyl radical at the active site of COX, which catalyzes the oxygenation and cyclization of arachidonic acid to produce PGG_2 [86]. Yeh and Yen orally administrated GA to rats at a dosage of 100 mg/kg body weight for 14 consecutive days [87]. This treatment increasedmRNA levels and activities of superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalasein the liver and intestines. GA also lowered oxidized glutathione and increased reduced-glutathione and the oxygen radical absorbance capacity in the liver. GA also enhanced total level of Nrf2 (Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2), a transcription factor governing the antioxidant responses [87]. Nrf2 is an important basic leucine zipper (bZIP) protein that controls the expression of antioxidant enzymes whichprotect against oxidative damage triggered by injury and inflammation. Hence, several molecules that induce Nrf2 pathway are actively being studied for treatment of diseases which associate with oxidative stress and GA is a very suitable candidate to induce Nrf2 besides its versatile antioxidant and antiinflammatory effects.

Indeed, studies in TPA-stimulated carcinogenesis models revealed GA-augmentation of many antioxidant pathways. 12-O-Tetradecanoyl phorbol-13-acetate (TPA) is the most active phorbol ester, which is used in two-stage carcinogenicity experiments on mouse skin. Application of TPA to skin enhances lipid peroxidation (malondialdehyde), H_2O_2 levels and reduces catalase, glucose-6-phosphate dehydrogenase, glutathione, glutathione peroxidase, glutathione reductase and glutathione-S-transferase; effects which were efficiently – and dose dependently – reversed with coapplication of GA on the skin [30].

Antioxidant activity of GA may reduce benign tissue damage caused by cancer chemotherapy

Cancer chemotherapeutics kill cells with high proliferative indices; therefore they could damage benign tissues besides killing cancer cells. Cyclophosphamide (CP) is an alkylating chemotherapy agent and immunosuppressive drug, which is widely employed against various cancers and autoimmune diseases. Two active metabolites of CP, acrolein and phosphoramide are closely linked to its chemotherapeutic activity [88]. Chemically active metabolic products of CP cause cytotoxicity via alkylating DNA and protein, and causing crosslinks in DNA. CP also produces carbonium ions that react with the electron-rich centers of nucleic acids and proteins. CP triggers DNA damage, micronuclei induction as well as the production of reactive oxygen species (ROS), which may also cause carcinogenesis [88].

A single intraperitoneal administration of CP (50 mg/kg) in mice increased the MDA level, reduced the glutathione content and antioxidant enzymes (glutathione peroxidase, glutathione reductase, catalase and quinone reductase), and induced DNA strand breaks and micronuclei. When mice were pretreated with GA orally at doses of 50 and 100 mg/kg for 14 consecutive days before the administration of CP, significant reductions in MDA, micronuclei formation and DNA fragmentation and restoration of liver glutathione and antioxidant enzyme activity (glutathione-reductase, glutathione peroxidase,

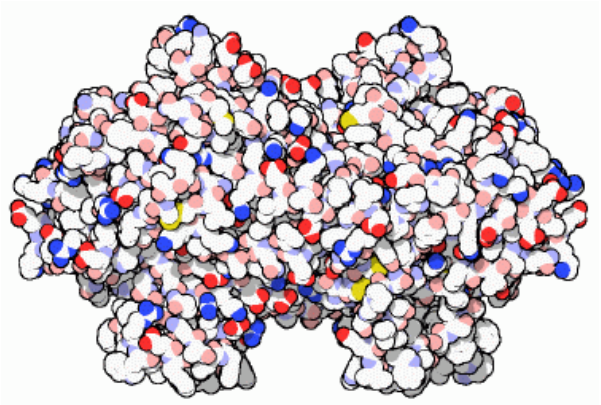

Figure 7: Molecular Structure of Cyclooxygenase

catalase, quinone reductase) were observed. GA also lowered liver damage marker enzymes such as aspartate aminotransferase, alanine aminotransferase and lactate dehydrogenase which were increased after CP treatment [88].

Prooxidant activity of GA at very high levels. Could GA selectively increase chemo-radiotherapy toxicity on tumors while sparing benign tissues?

GA, by virtue of its dihydroxy group in para position, also harbours oxidative features and inhibits glucose-6-phosphate dehydrogenase (G6PDH) and accelerates hemolysis of erythrocyes in G6PDH deficiency *in vitro*. However very high dosages above 5 mM (greater than 770 µg/ml) exerts this efficacy [69]. Hence, it is very unlikely that therapeutic doses of GA would exert such an efficacy in the intact organism. On the other hand, since GA retention occurs in patients with cancer [89]; it may be possible that GA would accumulate within the cancerous tissue and exerts prooxidant activities with high dose and prolonged administration. G6PDH is responsible in maintaining the level of the coenzyme NADPH, which in turn maintains levels the levels of intracellular glutathione. Indeed, GA at high levels (above 3mM) sensitizes Aspergillus fungi to the toxicity of the antifungal agent fludioxonil by perturbing glutathione homeostasis [90]and inhibits erythrocyte glutathione peroxidase at very high doses (7.5 mM) [91].

Glutathione starvation renders tumor cells more sensitive to chemo-radiotherapy [92]. GA at 1 mM sensitizes murine and human leukemia cells to the cell-killing efficacy of merocyanine 540-mediated phototherapy [93]. In the future, it would be logical to evaluate intratumoral glutathione levels with GA exposure in future studies. In the intact organism, treatment of rats even with huge doses of GA (1 g/kg; corresponding to 70 g for an average human subject) did not reduce liver glutathione with single exposure, even slightly augments it with double exposure [94]. Hence, there would be unique opportunity that GA may selectively increase chemo-radiotherapy toxicity in tumors while sparing benign tissues.

A inhibition of inflammatory immunity and boosting of lymphocytes

a) GA Inhibits Cyclooxygenase and 12-Lipoxygenase Enzymes. Relevance for the Anticancer Potential

The pharmacological efficacy of the nonsteroidal antiinflammatory drugs (NSAIDs) is explained mainly with an inhibitory effect on the cyclooxygenase (COX) enzyme, which catalyzes the first step in the conversion of arachidonic acid to prostaglandins and thromboxanes [95]. Aspirin is also thought to work through specific acetylation of a serine hydroxyl function in COX enyzme, which likely resides in proximity to the COX active site; but such an assumption could not explain the irreversible and time-dependent inhibition of COX which is witnessed with a plethora of non-acylating NSAIDs [96]. Holmes et al. [96] proposed that formation of GA from salicylate acid might happen in an inflammatory micromilieu as a consequence of "co-oxidation" coupled to prostaglandin-H-Synthase (PGH-Synthase)-peroxidase activity, which is commenly observed with a variety of phenolic compounds. They presumed that GA in this oxidative micromilieu might undergo further oxidation to potentially reactive quinonoid compounds, which could irreversible inhibit COX via covalent binding. The isolable quinhydrone of GA (a 1:l complex of oxidized and reduced forms) was revealed to augment PGH synthase at low concentrations and block the enzyme at higher concentrations or under conditions of lowered peroxide tone, which is characteristic of antioxidant phenolic compounds in general, including GA [96].

The employed GA concentrations (100-500 µM) were below those generally published to be inhibitory (1-5 mM) and included lower concentrations (100 µM) at which GA has been reported to stimulate COX activity, as similarly witnessed with phenol and other antioxidants acid. At concentrations of GA upto 250 µM, rates of enzyme inactivation upon electrolysis followed pseudo first order kinetics which is consistent with enzyme inactivation being mediated by an oxidation product of GA. This oxidation product must be produced in close proximity to (possibly bound to) the enzyme since simply adding an electrochemically oxidized GA solution to a separate solution of enzyme did not lead to inactivation [96].

A much lower order of inhibition was observed at higher concentrations of GA (above 300 µM) whichwas likely not a function of electrochemical oxidation and attributed to several reasons: i) protection of the COXfrom reactive oxidized species by saturation of potential bindingsites for such species; ii) competitive charge-transfer typeinteractions (e.g. quinhydrone formation) between the oxidizedand reduced forms of GA. The authors stated that the oxidized species of GA which most likely causes this electrochemical inactivation is carboxybenzoquinone, which was previously reported as a product of electrochemical oxidation of GA [96].

In relatively recent years, it is revealed that COX exists as two genetically distinct isoforms. COX-1 is constitutively synthesized as a "housekeeping" enzyme in many tissues and regulates physiological responses such as platelet function and cytoprotection of the gastric epithelia [95]. COX-2 is upregulated by versatile inflammatory agents and conditions, including endotoxin and cytokines and is the isoform mainly responsible for the synthesis of prostanoids involved in pathological processes. Most of the NSAIDs block activity of both COX-I and COX-II, although they vary in their relative potencies against these two isozymes. Whereas the side effects of NSAIDs (e.g.,stomach toxicity, dysfunction in coagulation/platelets) are caused by depresion of COX-I-derived prostanoids, the beneficial pharmacological actions of NSAIDs were attributed to inhibition of COX-2 activity at sites of inflammation. Nonetheless, the assumption that inhibition of prostaglandin biosynthesis mediates the pharmacological effects of NSAIDs has been debated by comparing the actions of salicylate and aspirin. Salicylate does not, unlike its acetylated derivative aspirin, inhibit COX-1 and COX-2 activity *in vitro* but demonstrates a comparable analgesic and anti-inflammatory action as aspirin [95].

Aspirin (IC50 of 5.35 $_{m}$M) suppresses lipopolysaccharide (LPS)-induced and COX-II-dependent synthesis of prostaglandin E_2 (PGE_2) in RAW 264.7 macrophages, whereas no significant inhibition occured upon treatment with sodium salicylate and the salicylate metabolite salicyluric acid at concentrations up to 100 µM. However, the salicylate metabolite GA (10–100 µM) and salicyl-coenzyme A (100 µM), the intermediate product in the formation of salicyluric acid from salicylic acid, significantly inhibited LPS-induced PGE2 production. GA treatment led to a significant inhibition (around 35%) of PGE_2 production at 10 µM, which rose to 62% inhibition at 100 µM. In opposite, γ-resorcylic acid (2,6-dihydroxybenzoic acid) as well as unconjugated coenzyme A failed to affect prostanoidsynthesis, implying that the *para*-substitution of hydroxy groups and the activated coenzyme A thioester are important for COX-II inhibition by GA. None of the salicylate derivatives tested were found to interfere with COX-II expression as assessed by realtime RT-PCR. The authors have underlined that, albeit the aspirin fraction metabolized to GA

is rather small, GA is predominantly formed under inflammatory conditions by neutrophils (which highly produce reactive oxygen species) and also that febrile patients undergoing salicylate treatment excrete higher GA levels [95].

It was hypothesized that aspirin's anticancer effects occur via acetylation-mediated inhibition of COX, as upregulation of COX-II is observed in 80-90% of colorectal cancer; while COX-I expression is unchanged [32]. The findings suggesting that low dose aspirin, employed for its cardioprotective effect, is also efficient in the prevention of colorectal cancer, it was presumed that aspirin's chemopreventive actions occur through sequential steps involving COX-I and COX-II [32]. Moreover, aspirin metabolite GA may also block other proinflammatory enzymes on platelets; for instance, GA a very easily achievable dose (6.2±1.5 μM) significantly blocks platelet 12-lipoxygenase activity [97]. 12-lipoxygenase or with its recent name ALOX12 (Figure 8) is expressed in platelets and neutrophils and its first activity to be shown is the regulation of neutrophil chemotaxis. 12/15-Lipoxygenases (12/15-LOX) are members of the lipoxygenase (LOX) family, which syntheses are induced by the T helper type 2 cytokines, interleukins-4 and -13 [98]. LOX oxygenate free polyenoic fatty acids and ester lipids and even lipid-protein assemblies such as biomembranes and lipoproteins. The primary oxidation products of LOX are either reduced by glutathione peroxidases to corresponding hydroxy derivatives or metabolized into secondary oxidized lipids including leukotrienes, lipoxins and hepoxilins. Knockout and transgenic mouse models revealedmajor roles for 12/15-LOX in inflammatory diseases, including atherosclerosis, cancer and diabetes [98]. While 15-lipoxygenase-1 and 15-lipoxygenase-2 may suppress carcinogenesis, 12-lipoxygenase is found to be dominantly carcinogenic [99].

b) Salicylate is converted to GA when incubated with neutrophils and GA modifies inflammatory cytokine release from peritoneal macrophages

Davis et al. [100] treated human neutrophils in a medium containing 10 mM salicylate and stimulated with phorbol myristate acetate (PMA) for 1 hr. The cell-free supernatant fractions were analyzed by HPLC. Neutrophils ($1x10^6$ cells) produced 55 ± 11 ng of GA and smaller quantities of 2,3-dihydroxybenzoic acid. Inhibitory studies with antioxidants revealed that superoxide dismutase (SOD), heme protein inhibitors, and glutathione inhibited formation of GA, whereas catalase, mannitol, and deferoxamine failed to do so. They proposed that the hydroxylation reaction occured in association with respiratory burst activity triggered by PMA or zymosan and also that the $OH^{\cdot}$ released by activated neutrophils is a plausible candidate for the hydroxylation reaction. Although $OH^{\cdot}$ formation by neutrophils was debated, spin trapping studies exist which demonstrated $OH^{\cdot}$ production through Haber- Weiss or Fenton type reactions requiring iron either inside or outside the neutrophils. Nonetheless, the lack of catalase, mannitol, and deferoxamine to inhibit salicylate hydroxylation casted doubt on the importance of $OH^{\cdot}$ released by these pathways if not completely ruling out this possibility. They suggested that another plausible hydroxylation pathway is the MPO-H_20_2-chloride enzyme system, since HOCI produced by a model MPO enzyme system can react with salicylate compounds. But again, they did not detect GA formation with relevant concentrations of HOC1, MPO plus H_20_2, and cell-free supernatants, yet these findings do not absolutely rule out its involvement in the intact cell [100].

Haynes et al. [101] treated resting and chemically stimulated rat peritoneal monocytes with 1 mM of salicylate and also detected formation of GA. They revealed that GA inhibited IL-1 induced proliferation of peritoneal monocytes and their production of IL-6 *in vitro* with mean IC_{50}concentrations at 200 and 287 μM, respectively. Interestingly, GA stimulated production of IL-1 and TNF-α by rat peritoneal monocytes at mean concentrations of 180 and 172 μM, respectively [101]. IL-6 is an inflammatory cytokine which strongly induces cancer cachexia and (Han et al. 2018) and a recent meta-analysis which analyzed 11583 cancer patients with 23 different cancer types revealed that its enhanced levels correlate with worse prognosis [102]. As its name implies, TNF-α (Tumor Necrosis Factor-α) could trigger robust necrosis of tumors, and biological response modifiers which selectively block TNF-α may increase risk of squamous skin cancer [103]. Hence, GA stimulation of TNF-α and IL-1 production, while suppression of IL-6 may associate with a specific antitumor immune response.

c) GA inhibits neutrophil aggregation and superoxide anion release

GA is an inhibitor of polymorphonuclear leukocyte aggregation and superoxide anion release after challenge with stimuli such as calcium ionophore A 23187 or arachidonic acid, and this inhibition occurs much significant than induced by aspirin or salicylate [1]. Superoxide anion release is inhibited by 97% at 1 mM of GA, equimolar concentrations of aspirin or salicylate were only effective

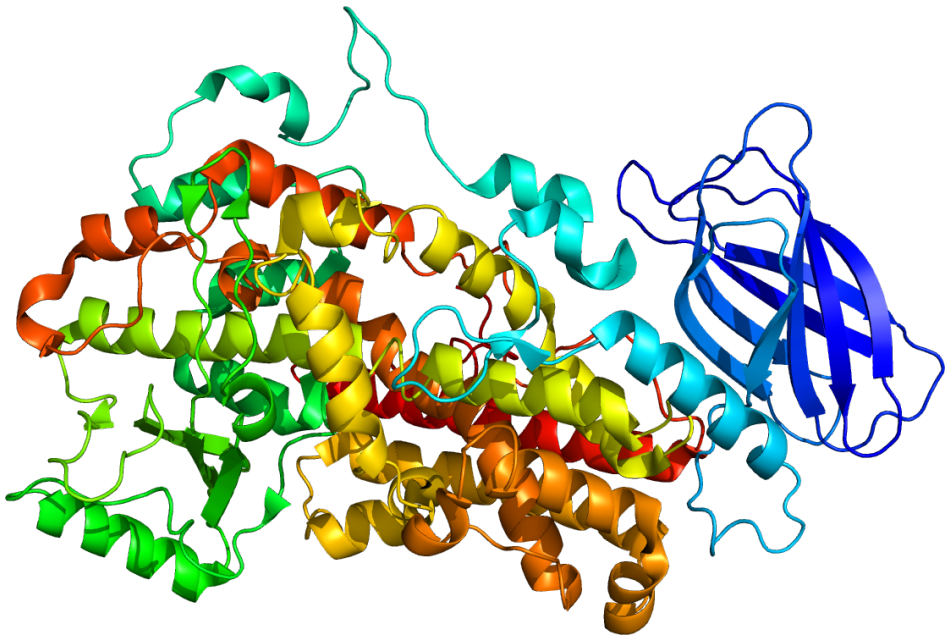

Figure 8: Molecular Structure of 12-Lipoxygenase (ALOX12)

around 40% [1]. As the formation of leukocyte aggregates in the vascular compartment and embolization in capillary networks causes tissue damage in several pathological states, e.g. in myocardial infarction; the authors have suggested that drugs which reduce the activation of leukocytes might be usefulin reducing infarct size [1].

d) GA treatment in clinic causes elevation of lymphocyte counts

Clarke et al. [16] reported that of the 44 patients who had acute rheumatic fever and who were treated with GA compounds, 39 had an average rise of 54% percent and four patients had a fall in their lymphocyte counts. The elevation of the lymphocyte counts was witnessed within the first 10 days after GA therapy had been started and subsequently the lymphocyte counts gradually returned to their former or sometimes lower levels. In some patients, and usually in the latter part of their illness, there was a secondary rise in the lymphocyte counts. Sodium salicylate treatment caused a similar increase in lymphocyte counts [16].

e) GA is also a selective agonist of GPR35, which may explain its selective blockage of inflammatory innate immunity but stimulating lymphocytes

GA is also a selective agonist of GPR35 (Figure 9) [41], a G-Protein Coupled Receptor. GPR35binds the chemokine CXCL17as a chemokine receptor (CXCR8) which is expressed on monocytes, neutrophils, dendritic cells and T cells and lesser on B lymphocytes, iNKTs, eosinophils and basophils [104]. Kynurenic acid, another endogenous ligand of GPR35 acts antiinflammatory while stimulating lymphocyte proliferation [104]. Kynurenic acid activation of the GPR35/CXCR8 exerts antiinflammatory effects through the blockage of the synthesis of early (TNF-α, IFN-γ) and late (HMGB1) inflammatory cytokines and mediators by monocytes and macrophages [104]. GPR35/CXCR8 is expressed in mast cells and some medications used to treat asthma are GPR35/CXCR agonists [105]. GPR35 is highly expressed in intestines, colon tissue and liver and agonism of GPR35 alleviates inflammatory intestinal diseases [105].

f) GA Blockage of xanthine oxidase, hyaluronic acid depolymerization and hyaluronidase activity

Hyaluronic acid, or hyaluronan, is a biological polymer made of the repetition of a unique disaccharidic unit, D-glucuronic acid and D-N-acetylglucosamine, that can reach a molecular mass of 10 million daltons [106]. Hyaluronan is an important connective tissue glycosaminoglycan and its increased biosynthesis is a common feature during tissue remodeling under physiological and pathological conditions [107]. Hyaluronan regulates various cellular functions including cell differentiation and migration through its interactions with hyaladherins [107]. Hyaluronidases are a family of enzymes that catalyse the degradation of hyaluronic acid via cleaving the (1->4)-linkages between N-acetylglucosamine and glucuronate. The three major types of hyaluronidases include two classes of eukaryotic endoglycosidase hydrolases and a prokaryotic lyase-type of glycosidase; and in humans, five functional hyaluronidase exist including HYAL-1, -2, -3, -4 and -5 [108,109]. Via its hydrolysing activity, hyaluronidase lowers the viscosity of hyaluronan and thereby the extracellular matrix and subsequently enhances tissue permeability. There exist contraversial data regarding the effect of hyaluronidase on cancer progression [110]. While low hyaluronidase activity is linked to worse prognosis in pancreatic cancer [111]; HYAL-1 and HYAL-2 expressions are increased colorectal, bladder, prostate, breast and brain malignancies. It is being more and more recognized that the combined overexpression of hyaluronic acid-synthesizing enzymes with hyaluronidases promotes tumorigenic potential [109].

Overall, higher hyaluronidase activity more dominantly may increase tumor aggressiveness and metastasis via enhancing infiltrative capability of invasive tumor cell clones in a microenvironment with reduced viscosity [112]. Moreover, depolymerization of hyaluronic acid may also cause liberation of various growth factors and cytokines involved in signal transduction promoting tumorigenesis [113]. Hyaluronidase degradation of hyaluronic acid also leads to generation of a wide molecular range bioactive oligosaccharides with angiogenic and inflammatory activity and hyaluronidase inhibitors exist which exert anti-inflammatory and anti-cancer activity [114]. The real importance of the intact hyaluronic acid is so important that the naked mole rat do not develop cancer which is linked to their harbouring extremely high-molecular-mass hyaluronan. This variant hyaluronan is over “five times larger” than that in cancer-prone humans and cancer-susceptible laboratory animals [115]. This seminal finding published in Nature [116] also suggest that intact hyaluronic acid is associated with higher resistance to cancer and *vice versa*, hyaluronidase activity which degrades hyaluronic acid shall act more protumorigenic rather than antitumoral.

As suggested above, Meyer and Ragan [10] were the first to show that GA blocks hyaluronidase activity. Later studies that GA not *per se* but rather its oxidation/polymerization products inhibit hyaluronidase, in which “humic acid” like substances play a role

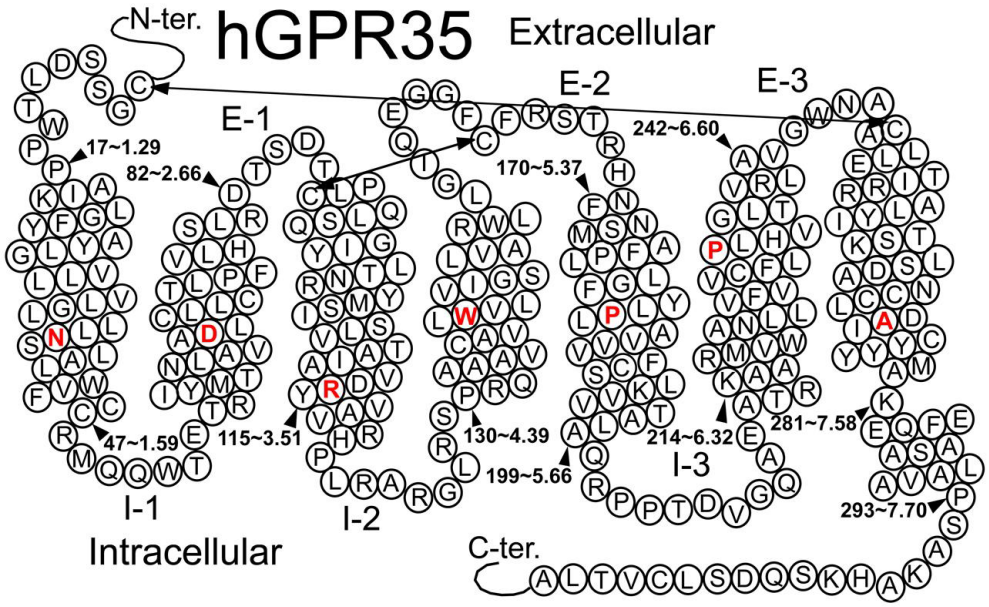

Figure 9: Molecular Structure of GPR35 [105]

[117,118]. Formation of humic acids from salicylic acid proceed through an hydroxyquinone type of intermediate and aerial oxidation of GA could lead formation of humic acid substances [117].

The formation of humic acids from salicylates and gentisates would presumably occur through intermediate formation of hydroxyquinones, which would then take part in a polymeric process in which other suitably constituted phenolic components could participate. Carlin et al. [119] investigated the inhibitory effect of anti-inflammatory drugs on the xanthine oxidase stimulated depolymerization of hyaluronic acid which was evaluated by repeated viscosity measurements. Salicylic and acetylsalicylic, GA and azodisalicylic acid and sulfasalazine inhibit the production of oxygen-derived free radicals by xanthine oxidase, while having little direct activity on hyaluronidase [119].

Cleland et al. [35]demonstrated GA blocked the activity of xanthine oxidase with 0.27 μM of its concentration reduced chemiluminescence of luminol with xanthine oxidase/hypoxanthine to 20% of control values. Overall, despite GA exerts no direct inhibitory activity on hyaluronidase, it could interact with redox reactions leading indirect inhibition of this enzyme *in vivo*.GA avidity to iron and the role of its alkaptonuric oxidation products (very active semiquinones or humic acid like polymers- 2.5μM) seemed to play the major role in blocking hyaluronidase [119]. Further animal tumor experiments would allow determination of the efficacy of GA on tumoral hyaluronic acid and mass spectroscopical analyses would determine intratumoral metabolites of GA, which could block hyaluronidase.

g) GA may decrease eosinophil counts and Leukotriene C4 levels

Clarke et al. [15] frequently analyzed absolute eosinophil counts on 23 acute rheumatic fever patients under GA treatment . A more than 50% percent decline in the absolute eosinophil count was found in these patients within the first 10 days of GA therapy. In most of these patients, the greatest decline in the absolute eosinophil count was observed on the sixth or seventh day of GA treatment . The absolute eosinophil count dropped from 250 and 800 eosinophil cells to zero cells in three patients after seven days of GA therapy and after nine days in another patient [15]. On the other hand, another group failed to demonstrate any effect of GA (upto serum levels of 350 μg/ml) on the eosinophil counts [14]. If GA is indeed capable to reduce eosinophil counts, it may associate with its capability to reduce LTC_4 production(Trautmann et al. 1991), since eosinophils both release LTC_4 and undergo autocrine proliferation in the presence of LTC_4 [120,121]. Reduction of eosinophil activation/LTC4 axis may also contribute to antitumor efficacies in cancer. Tumor-associated eosinophilia was first published in 1893 which is mostlywitnessed in patients with solid tumors and Hodgkin's lymphoma [122]. There exist conflicting data in regard to eosinophil and cancer connection.While tumor-associated eosinophilia was related to better prognosis in head and neck, bladder and prostate and colon cancers; in Hodgkin's lymphoma, oral squamous cell carcinoma, and cervical carcinoma, eosinophils were linked to worse prognosis. Secretory granules of eosinophils contain VEGF that can be secreted upon stimulation with IL-5 and eosinophil supernatants increase endothelial cell proliferation *in vitro* and angiogenesis *in vivo* [122].

Leukotriene C_4 (LTC_4) is a leukotriene which triggers vasoconstriction and bronchoconstriction and extensively involves in allergy and asthma. Synthesis of leukotrienes start with action of 5-lipoxygenase (5-LO) and inhibitors of 5-LOX exerted clear antitumor actions in experimental models of carcinogenesis [123]. In immunocytes causing allergic reactions such as mast cells, its biosynthesis is orchestrated by translocation to the nuclear envelope along with co-localization of cytosolic phospholipase A2 (cPLA2), 5-lipoxygenase (5-LO), 5-LO-Activating Protein (FLAP) and LTC_4 synthase(LTC_4S).In this reaction, the key enzyme is 5-LO, a dioxygenase containing a nonheme-bound ferric central ion, which in the catalytic complex with FLAP forms 5-hydroperoxyeicosatetraenoic acid. Subsequent steps involve LTC_4S or leukotriene A4-hydrolase, which are suggested as potential targets for blocking atherosclerosis and carcinogenesis [124,125]. Noteworthy, GA was found to block LTC4 synthesis in gastric mucosa following ethanol induced gastric toxicity [126]; it would be very tempting to study if could exert

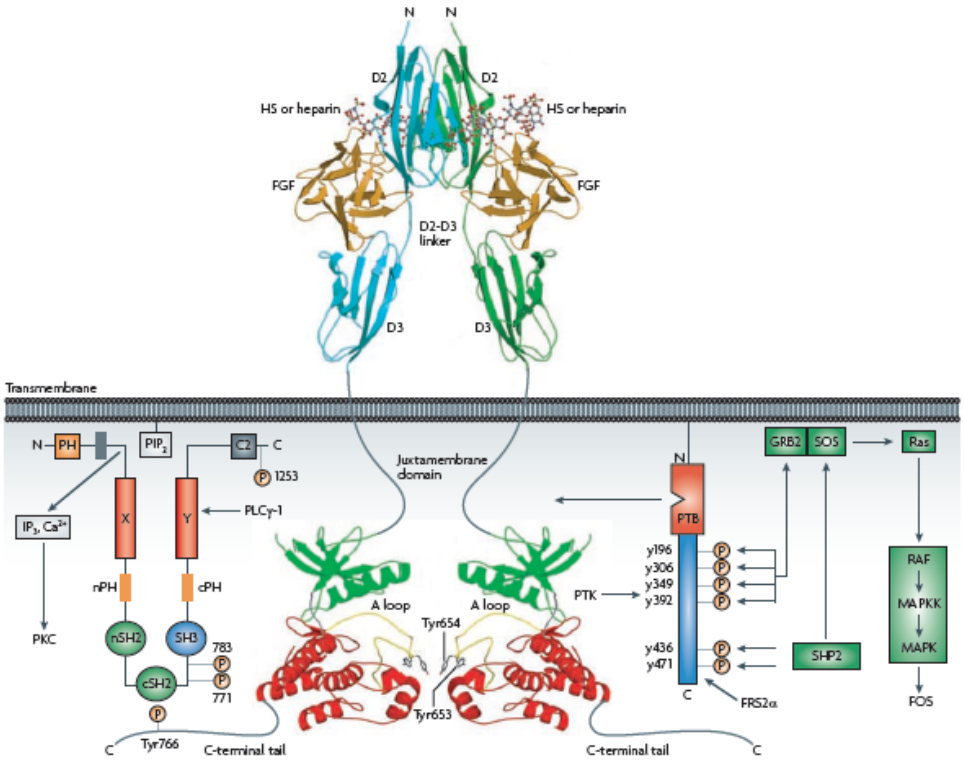

Figure 10: Fibroblast Growth Factor Receptor Signalling

similar activity in cancerous tissues.

GA Inhibition of fibroblastic growth factor (FGF) pathway as a direct route to block cancer and glioblastoma growth

The fibroblast growth factors (FGFs) belong to the largest families of polypeptide growth factors which include 22 FGFs in humans and mice which differ prominently in size and sequence, yet they each contain a core homology region composed of 120 to 130 aminoacid residues [127]. All FGFs bind to heparin with high affinity, but the members of the FGF-19 subfamily (*i.e.*:FGF-15, -19, -21, and -23) have little or no affinity for these glycosaminoglycans. Most FGFs act via binding to membrane tyrosine kinase receptors (FGFRs) and were first purified from bovine brain extracts with their mitogenic and angiogenic features (Figure 10). Their physiological substrate in normal conditions is heparan sulfate, a proteoglycan whoseglycoside moiety is a glycosaminoglycan like heparin. FGFs remaintrapped in the extracellular matrix, from which they are released by heparanasesor other specialized proteins, but their levels may aberrantly increase due to high synthesis and/or release in cancer, where they act as autocrine, paracrine or juxtacrine inducers of tumor promotion. FGFs exert versatile protumorigenic functions by enhancing tumor cell motility, angiogenesis and multiple metastatic pathways (including those involving in degradation of extracellular matrix) and also mediate resistance of cancer cells against chemotherapy and radiotherapy by increasing the apoptotic threshold [127].

GA was discovered to belong to a novel chemical group of FGF inhibitors, which includes homogentisic acid (HGA; 2-(2,5-dihydroxyphenyl)acetic acid) (Figure 5), the toxic agent in alkaptonuria. These compounds inhibit activities of the two prototypical members of the family *in vitro* and *in vivo* and the chemical constraints which define the efficiencyof these aromatic molecules in inhibiting FGF are the *para* conformation of the dihydroxyphenyl group and the functionalization of the aromatic ring by an acidic group. These compounds recognize both the growth factors (Figure 11) and their receptors, displacing heparin from these polypeptides, change the three-dimensional conformation of the growth factor at their receptor, and dissociate the receptor·growth factor signaling complex [127].

The potential inhibition of FGF mitogenic activity by GA was first assayed *in vitro* using FGF-1 and Balb/c 3T3 fibroblasts, where it displayed a half-maximum inhibitory activity (IC_{50}) of ~36 μM. Substitution of carboxylate by sulfonate (2,5-dihydroxybenzene sulfonate (2,5-DHPS), or so called dobesilic acid) caused a dramatic decrease in the IC_{50} (3 μM). The crystallographic datarevealed that

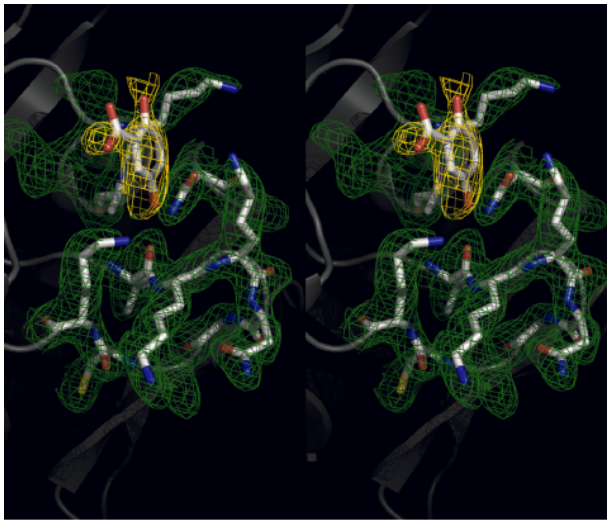

Figure 11: Binding of Gentisic Acid to Fibroblast Growth Factor [127]

2,5-DHPS should also inhibit FGF-2-induced mitogenesis, as the 2,5-DHPS binding site in FGF-1 is highly conserved in FGF-2. Indeed, 2,5-DHPS inhibited FGF 2-driven mitogenesis with an $IC_{50}$of 19.1 μM. Given the homologies in primary structure, it would be also plausible to presume that FGF-3, FGF-4, FGF-8, FGF-9, FGF-16, and FGF-17 could also be inhibited by 2,5-DHPS and GA, as there is substantial conservation in the binding site between FGF-1 and each of these other family members [127].

The researchers than investigated the effect of 2,5-DHPS on angiogenesis of FGF-1 or FGF-2 soaked gelatin sponges and on the growth and angiogenesis of C6 rat glioma. 2,5-DHPS was effective both orally and intraperitoneally to block angiogenesis of FGF-soaked sponges. Robust vascularization is a major characteristic of high grade gliomas; FGF-1 and FGF-2 are abundant in mostglioblastomas, the former is associated to malignant astrocytes,the latter is associated with the matrix surrounding neovascularization. Hence, the investigators inoculated rats with C6 glioblastoma cells and five days afterimplantation, when the tumors protruded throughout theskin, they treated rats with either vehicle only or 2,5-DHPS (100 mg/kg). After 10 days of treatment, they surgically removed the tumors and defined their volume. 2,5-DHPS treatment blocked the growth of C6 rat glioma by around 50% [127].

GA inhibition of cyclin dependent kinases important in cancer growth

Cyclin dependent kinases (CDKs) are master regulators of the cell cycle, and get activated by binding with temporally expressed cyclins during different cell cycle steps. CDK1 and CDK2 are activated via binding to cyclins A and B and provide the progression through G2 and M phases [32]. In many malignancies, CDK regulation is disturbed and cyclins are over expressed. Using *in vitro* kinase assays, Dachineni et al. have shown that GA and GA isomers 2,3-dihydroxybenzoic acid (2,3-DHBA), 2,4-dihydroxybenzoic acid and 2,6-dihydroxybenzoic acid could block CDK1 enzyme activity [32]. However, aspirin, salicylic acid, benzoic acid, 3,4-DHBA, 3,5-DHBA, and 5-amino-salicylic acid were incapable to inhibit CDK1 activity, which indicates that the DHBAs with a common -OH group at the 2nd carbon is presumably essential for the inhibitory effect on CDK1. The authors had suggested that salicylic acid metabolites generated through intracellular CYP450 enzymes within the colonic epithelia, or formed by gut microflora may involve in the chemopreventive effect of aspirin against colon cancer via inhibition of CDKs.

Agonism of vasoactive intestinal peptide pathway. Importance for glioblastoma

In a guinea pig isolated trachea model, GA was shown to induce relaxation via acting as an agonist of Vasoactive Intestinal Peptide (VIR)-receptor [128]. There exist conflicting data on the effects of VIP on glioma growth. Some authors proposed that VIP acted proliferative on glioma, since VIP accelerated rat C6 glioma growth *in vitro* [129] and expression of nuclear VIP-receptor correlates with grade in human glial tumors [130]. Nonetheless, there exist substantial opposite evidence suggesting that VIP might act antiproliferative in human [131] ; and serum starved rat C6 glioma cells [132]. Moreover, more recent studies showed prominent antiinvasive potential of VIP on human glial tumor cells both in normoxia [133]; and hypoxia [134]. We think that 3-dimensional spheroid cultures of human glioma may reveal the net effect of GA on VIP-associated pathways in glioma invasion.

GA Inhibition of OAT3 (Organic Anion Transporter-3)/ SLC22A8 (Solute Carrier 22A8) involved in brain efflux of anticancer drugs

GA is a specific inhibitor of human OAT3 (Organic Anion Transporter-3)/SLC22A8 (Solute Carrier 22A8) (Figure-12) [135,136]; and Roct, rat homologue of OAT3 is expressed in rat brain and choroid plexus [137]. Organic anion transporters regulate the elimination of versatile endogenous and exogenous molecules, xenobiotics and their metabolites including cyclic nucleotides, prostaglandins, and dicarboxylates. OAT3 is inhibited by

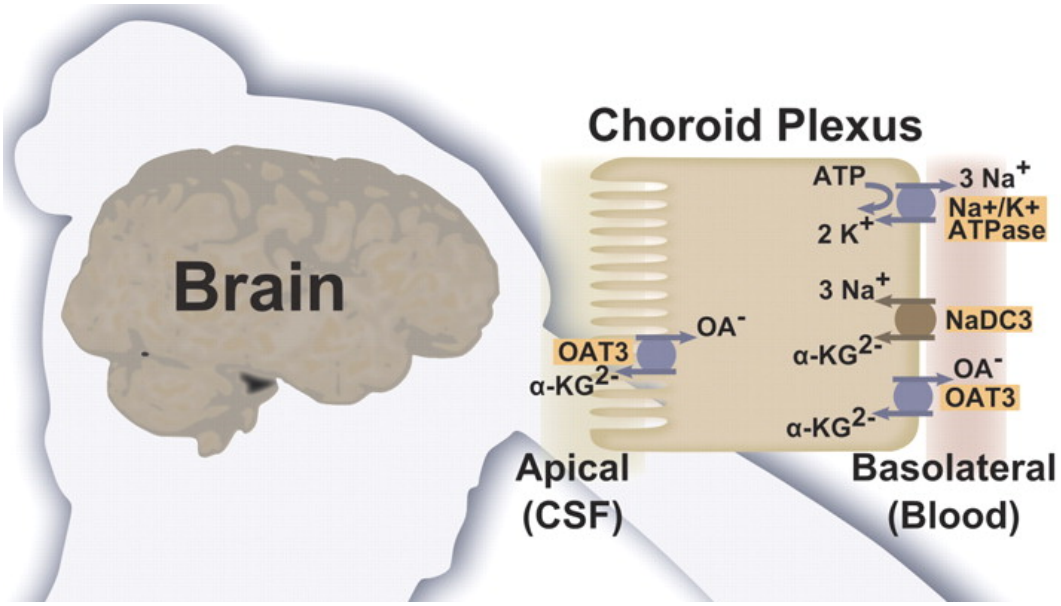

Figure12: OAT3 (OrganicAnion Transporter-3)/SLC22A8Functioning in the Brain Tissue [135]

bendamustine, which induces lymphoma cell apoptosis and superior efficacy of the bendamustine over chlorambucil in the treatment of chronic lymphoid leukemia is attributed to inhibition of OAT3 [138]. OAT3 is an important component of blood brain barrier and reduces accumulation of drugs in the CNS via active efflux; and OAT3 inhibitors increases CNS concentration of several drugs including bumetanide and N-acetyl-cysteine [139,140]. Oat3 mediates the uptake of amphipathic and hydrophilic organic anions, at the brush border surface of the choroid plexus to eliminate xenobiotics and endogenous waste from the cerebrospinal fluid (CSF) together with convective flow mediating CSF turnover [141]. OAT3 also involves in plasma [142] and cerebral [143] elimination of methotrexate. The central nervous distrubition of the HIV reverse transcriptase inhibitor, zalcitabine (2'3'-dideoxycytidine, ddC) in guinea pig brain involves organic anion transporters (OAT1/OAT3-like) [144]; and nucleoside analogues reduce risk of liver cancer even when employed after curative treatment of viral hepatitis [145]. Overall, OAT3 inhibition by GA may also help to achieve CNS-accumulation of anticancer chemotherapeutics and nucleoside analogues sufficient to block brain tumor growth.

GA enhancement of cholesterol efflux transporters ABCA1 and ABCG1. Potential ınterference with cancer metabolism

GA was recently discovered to upregulate the levels of cholesterol efflux transporters ABCA1 and ABCG1 in retinal pigment epithelium cells [146]. There exist many evidence that high levels of cholesterol promotes carcinogenesis, whereas lowering cholesterol reduces risk of cancer. Low serum cholesterol level and usage of statins is associated with a lowered risk of aggressive prostate cancer, whereas ABCA1 gene promoter is hypermethylated in this type of cancer causing reduction of ABCA1 protein and elevation of cholesterol [148]. A similar situation is detected in ovarian cancer cells, ABCA1 is hypermethylated in association with enhanced tumor growth in vitro and *in vivo* [149]. In cancer cells, cholesterol levels are mostly increased and ABCA1 deficiency enhances mitochondrial cholesterol, inhibits release of mitochondrial pro-apoptotic molecules, and increases cancer cell survival [150]. On the other hand, we shall admit that there exist studies which showed that ABCG1 may promote tumor growth in experimental models of melanoma and lung cancer [151,152].

Neuroprotective Activity

Above, we had indicated that GA ia selective agonist of GPR35/CXCR8 [41], GPR35/CXCR8 is highly expressed in rat dorsal root ganglion (DRG) but modestly in rat brain, cerebrum, and spinal cord [105]. GPR35/CXCR8 expression occurs in embryonic mouse corpus striatum mediale and hypothalamus and its agonists suppress neuronal activity on CA1 field of adult rat hippocampus. GPR35/CXCR8 is expressed in cultured astrocytes and in rat nociceptive DRG neuronsanda GPR35 agonist, pamoic acid exerts prominent antinociceptive activity in mice in a visceral pain model [105]. Therefore, it would not be illogical that GA would attenuate nociceptive pain.

Conclusions

Aspirin and its metabolites still provide very interesting resources to discover novel treatment strategies for cancer. Of course, we do not anticipate that either aspirin or its metabolites could be "a cure of cancer"; nonetheless, they may act as significant adjuvants by enhancing antitumoral potency and reducing systemic side effects of classical treatment modalities (chemotherapy, radiotherapy). As we have discussed in detail, GA is a very likely candidate to act as such an adjuvant. Currently, we have shown that GA is capable to directly block in vitro growth of C6 glioblastoma at concentrations, which could be easily achieved with clinical dosages of aspirin, which we will send for publication in a short time. In future, we plan to assess antitumoral efficacy and antitoxicity of combined preparates of aspirin with its metabolite GA in several cancer models.

References

1. Lorico A, Masturzo P, Villa S, Salmona M, Semeraro N, et al. Gentisic acid:an aspirin metabolite with multiple effects on human blood polymorpho-nuclear leukocytes. Biochem Pharmacol 1986 Jul;35(14):2443-2445.
2. Bojić M, Sedgeman CA, Nagy LD, Guengerich FP. Aromatic hydroxylation of salicylic acid and aspirin by human cytochromes P450. Eur J Pharm Sci. 2015 Jun;73:49-56.
3. Grootveld M, Halliwell B. Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo. Identification of hydroxylated derivatives of salicylate in human body fluids. Biochem J. 1986 Jul 15;237(2):499-504.
4. Cleland LG, Lowthian PJ, Imhoff D, Bochner F, Betts WH, et al. Plasma and synovial fluid gentisate in patients receiving salicylate therapy. J Rheumatol. 1985 Feb;12(1):136-139.
5. Exner M, Hermann M, Hofbauer R, Kapiotis S, Speiser W, et al. The salicylate metabolite gentisic acid, but not the parent drug, inhibits glucose autoxidation-mediated atherogenic modification

of low density lipoprotein. FEBS Lett. 2000 Mar;470(1):47-50.

6. Gorsuch MT. Clinical and laboratory investigation of sodium gentisate as an anti-rheumatic treatment. Med Womans J. 1950 Sep;57(9):9-23.
7. Astill BD, Fassett DW, Roudabush RL. The metabolism of phenolic antioxidants. 4. The metabolites of gentisic acid in the dog. Biochem J. 1964;90(1):194-201.
8. Rosenberg EF, Krevsky DA, Kagan BM. Laboratory and clinical experience with sodium gentisate in rheumatic disease. Ann Intern Med. 1952 Jun;36(6):1513-1519.
9. Kleinsorge H, Pohl W. [Gentisic acid in therapy of rheumatic polyarthritis]. Med Klin. 1953 Jul 17;48(29):1038-1040.
10. Meyer K, Ragan C. The Antirheumatic Effect of Sodium Gentisate. Science. 1948 Sep;108(2802):281.
11. Meade BW, Smith MJ. The Estimation of Sodium Gentisate in Plasma and Urine. J Clin Pathol. 1951a May;4(2):226-230.
12. Consden R, Stanier M. Metabolism of gentisic acid. Biochem J. 1951 Jan;48(1):xiv.
13. Roseman S, Dorfman A. The determination and metabolism of gentisic acid. J Biol Chem. 1951 Sep;192(1):105-114.
14. Meade BW, Smith MJ. Salicylate gentisate, and circulating eosinophils. Lancet.1951b Apr;1(6658):773-774.
15. Clarke NE, Mosher RE. Phenolic compounds in the treatment of rheumatic fever. II. The metabolism of gentisic acid and the ethanolamide of gentisic acid. Circulation. 1953 Mar;7(3):337-344.
16. Clarke NE, Mosher RE, Clarke CN. Phenolic compounds in the treatment ofrheumatic fever. I. A study of gentisic acid derivatives. Circulation. 1953 Feb;7(2):247-257.
17. Sandler G. The use of sodium gentisate in acute rheumatic fever. J R Army Med Corps. 1957 Jan;103(1):27-32.
18. Batterman RC, Kryle LS, Dann S. The analgesic action of sodium gentisate. N Y State J Med. 1951 May;51(9):1152-1154.
19. O'Brien JR. Effect of anti-inflammatory agents on platelets. Lancet. 1968 Apr;1(7548):894-895.
20. Kuhn H, Bergmann E. [New medicinal treatment of rheumatism]. Med Klin. 1954 Jan;49(2):76-78.
21. Greenaway JC, Bark DH, Juchau MR. Embryotoxic effects of salicylates:role of biotransformation. Toxicol Appl Pharmacol. 1984 Jun;74(1):141-149.
22. Gutknecht J. Aspirin, acetaminophen and proton transport through phospholipid bilayers and mitochondrial membranes. Mol Cell Biochem. 1992 Sep;114(1-2):3-8.
23. Boyd LJ, Lombardi AA, Svigals CS. Sodium gentisate in the treatment of chronic arthritis. Bulletin NY Med Coll. 1950 Jan;13:91-96.
24. Spizizen J, Kenney JC, Hampil B. Biochemical studies on the phenomenon of virus reproduction. III. The inhibition of coliphage T2r$^+$ multiplication by sodium salicylate and sodium gentisate. J Bacteriol. 1951 Sep;62(3):323-329.
25. Lee HI, León J, Raskin I. Biosynthesis and metabolism of salicylic acid. Proc Natl Acad Sci U S A. 1995 May;92(10):4076-4079.
26. Belles JM, Garro R, Fayos J, Navarro P, Primo J, et al. Gentisic Acid As a Pathogen-Inducible Signal, Additional to Salicylic Acid for Activation of Plant Defenses in Tomato. Molecular Plant-Microbe Interactions. 1999 Mar;12(3):227-235.
27. Bellés JM, Garro R, Pallás V, Fayos J, Rodrigo I, et al. Accumulation of gentisic acid as associated with systemic infections but not with the hypersensitive response in plant-pathogen interactions. Planta. 2006 Feb;223(3):500-511.
28. Campos L, Granell P, Tárraga S, López-Gresa P, Conejero V, et al. Salicylic acid and gentisic acid induce RNA silencing-related genes and plant resistance to RNA pathogens. Plant Physiol Biochem. 2014 Apr;77:35-43.
29. Radtke J, Linseisen J, Wolfram G. Phenolic acid intake of adults in a Bavarian subgroup of the national food consumption survey. Z Ernahrungswiss. 1998 Jun 37(2):190-197.
30. Sharma S, Khan N, Sultana S. Study on prevention of two-stage skin carcinogenesis by Hibiscus rosa sinensis extract and the role of its chemical constituent, gentisic acid, in the inhibition of tumour promotion response and oxidative stress in mice. Eur J Cancer Prev. 2004a Feb;13(1):53-63.
31. Cavalcante FML, Almeida IV, Düsman E, Mantovani MS, Vicentini VEP. Cytotoxicity, mutagenicity, and antimutagenicity of the gentisic acid on HTC cells. Drug Chem Toxicol. 2018 Apr;41(2):155-161.
32. Dachineni R, Kumar DR, Callegari E, Kesharwani SS, Sankaranarayanan R, et al. Salicylic acid metabolites and derivatives inhibit CDK activity:Novel insights into aspirin's chemopreventive effects against colorectal cancer. Int J Oncol. 2017 Dec;51(6):1661-1673.
33. Paterson JR, Blacklock C, Campbell G, Wiles D, Lawrence JR. The identification of salicylates as normal constituents of serum:a link between diet and health? J Clin Pathol. 1998 Jul;51(7):502-505.
34. Soleas GJ, Diamandis EP, Goldberg DM. Wine as a biological fluid:history, production, and role in disease prevention. J Clin Lab Anal. 1997;11(5):287-313.
35. Cleland LG, Whitehouse MW, Betts WH. Gentisate, a salicylate metabolite with antioxidant properties. Drugs Exp Clin Res. 1985;11(8):463-467.
36. Sharma S, Khan N, Sultana S. Modulatory effect of gentisic acid on the augmentation of biochemical events of tumor promotion stage by benzoyl peroxide and ultraviolet radiation in Swiss albino mice. Toxicol Lett. 2004 Nov;153(3):293-302.
37. Huang HC, Syu KY, Lin JK. Chemical composition of Solanum nigrum linn extract and induction of autophagy by leaf water extract and its major flavonoids in AU565 breast cancer cells. J Agric Food Chem. 2010 Aug 11;58(15):8699-8708.
38. Chung IM, Lim JJ, Ahn MS, Jeong HN, An TJ, et al. Comparative phenolic compound profiles and antioxidative activity of the fruit, leaves, and roots of Korean ginseng (Panax ginseng Meyer) according to cultivation years. J Ginseng Res. 2016 Jan;40(1):68-75.
39. Lee J, Ha SJ, Lee HJ, Kim MJ, Kim JH, et al. Protective effect of Tremella fuciformis Berk extract on LPS-induced acute inflammation via inhibition of the NF-κB and MAPK pathways. Food Funct. 2016 Jul;7(7):3263-3272.
40. Sitarek P, Skała E, Toma M, Wielanek M, Szemraj J, et al. Transformed Root Extract of Leonurus sibiricus Induces Apoptosis through Intrinsic and Extrinsic Pathways in Various Grades of Human Glioma Cells. Pathol Oncol Res. 2017 Jul;23(3):679-687.
41. Deng H, Hu H, Fang Y. Multiple tyrosine metabolites are GPR35 agonists. Sci Rep. 2012 Apr;2:373.
42. Kanda M, Watanabe H, UchiyamA S, Nakata Y, Sakamoto Y. Formation of gentisic acid from homogentisic acid. VI. J Biochem. 1964 Jan;55(1):78-80.
43. Fulenwider JT, Nordlinger BM, Faraj BA, Ivey GL, Rudman D. Deranged tyrosine metabolism in cirrhosis. Yale J Biol Med. 1978 Nov-Dec;51(6):625-633.
44. Chen Q, Espey MG, Sun AY, Pooput C, Kirk KL, et al. Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice. Proc Natl Acad Sci U S A. 2008 Aug;105(32):11105-11109.
45. Devireddy LR, Hart DO, Goetz DH, Green MR. A mammalian siderophore synthesized by an enzyme with a bacterial homolog involved in enterobactin production. Cell. 2010 Jun;141(6):1006-1017.
46. Liu Z, Ciocea A, Devireddy L. Endogenous siderophore 2,5-dihydroxybenzoic acid deficiency promotes anemia and splenic iron overload in mice. Mol Cell Biol. 2014a

Jul;34(13):2533-2546.

47. Correnti C, Richardson V, Sia AK, Bandaranayake AD, Ruiz M, et al. Siderocalin/Lcn2/NGAL/24p3 does not drive apoptosis through gentisic acid mediated iron withdrawal in hematopoietic cell lines. PLoS One. 2012;7(8):e43696.
48. Liu Z, Reba S, Chen WD, Porwal SK, Boom WH, et al. Regulation of mammalian siderophore 2,5-DHBA in the innate immune response to infection. J Exp Med. 2014 Jun;211(6):1197-1213.
49. Siddaraju MN, Dharmesh SM. Inhibition of gastric $H(^+)$,$K(^+)$-ATPase and Helicobacter pylori growth by phenolic antioxidants of Curcuma amada. J Agric Food Chem. 2007 Sep;55(18):7377-7386.
50. Mink S, Roy Chowdhury SK, Gotes J, Cheng ZQ, Kasian K, et al. Gentisic acid sodium salt, a phenolic compound, is superior to norepinephrine in reversing cardiovascular collapse, hepatic mitochondrial dysfunction and lactic acidemia in Pseudomonas aeruginosa septic shock in dogs. Intensive Care Med Exp. 2016 Dec;4(1):24.
51. Roof BS, Turner JC. Protein interactions of gentisic acid and certain of its oxidation products. J Clin Invest. 1955 Nov;34(11):1647-1652.
52. Trnavská Z, Sit'aj S, Grmela M, Malinský J. Certain intermediary metabolites and the formation of fibrils from collagen solutions. Biochim Biophys Acta. 1966 Oct;126(2):373-381.
53. Xu XD, Shao SX, Jiang HP, Cao YW, Wang YH, et al. Warburg effect or reverse Warburg effect? A review of cancer metabolism. Oncol Res Treat. 2015;38(3):117-122.
54. Gentric G, Mieulet V, Mechta-Grigoriou F. Heterogeneity in Cancer Metabolism:New Concepts in an Old Field. Antioxid Redox Signal. 2017 Mar;26(9):462-485.
55. Wilde L, Roche M, Domingo-Vidal M, Tanson K, Philp N, et al. Metabolic coupling and the Reverse Warburg Effect in cancer:Implications for novel biomarker and anticancer agent development. Semin Oncol. 2017 Jun;44(3):198-203.
56. Anderson NM, Mucka P, Kern JG, Feng H. The emerging role and targetability of the TCA cycle in cancer metabolism. Protein Cell. 2018 Feb;9(2):216-237.
57. Sajnani K, Islam F, Smith RA, Gopalan V, Lam AK. Genetic alterations in Krebs cycle and its impact on cancer pathogenesis. Biochimie. 2017 Apr;135:164-172.
58. Deng P, Haynes CM. Mitochondrial dysfunction in cancer:Potential roles of ATF5 and the mitochondrial UPR. Semin Cancer Biol. 2017 Dec;47:43-49.
59. Kalyanaraman B, Cheng G, Hardy M, Ouari O, Bennett B, et al. Teaching the basics of reactive oxygen species and their relevance to cancer biology:Mitochondrial reactive oxygen species detection, redox signaling, and targeted therapies. Redox Biol. 2017 Dec;15:347-362.
60. Urra FA, Muñoz F, Lovy A, Cárdenas C. The Mitochondrial Complex(I)ty of Cancer. Front Oncol. 2017 Jun;7:118.
61. Troncone M, Cargnelli SM, Villani LA, Isfahanian N, Broadfield LA, et al. Targeting metabolism and AMP-activated kinase with metformin to sensitize non-small cell lung cancer (NSCLC) to cytotoxic therapy:translational biology and rationale for current clinical trials. Oncotarget. 2017 Apr;8(34):57733-57754.
62. Kim SY. Cancer Energy Metabolism:Shutting Power off Cancer Factory. Biomol Ther (Seoul). 2018 Jan;26(1):39-44.
63. Hines WJ, Bryant C, Smitch MJ. Effects of salicylate, gamma-resorcylate and gentisate on oxidase enzyme systems from guinea-pig liver mitochondria. Biochem Pharmacol. 1963 Oct;12:1109-1116.
64. Hines WJ, Bryant C. The effects of salicylate on guinea-pig testis mitochondria compared with the effects of aging and repeated washing. Biochem Pharmacol. 1966 Jan;15(1):119-121.
65. Hines WJ. Gentisate and guinea-pig testis metabolism. J Pharm Pharmacol. 1966;18(4):256-257.
66. Biswas S, Lunec J, Bartlett K. Non-glucose metabolism in cancer cells--is it all in the fat? Cancer Metastasis Rev. 2012 Dec;31(3-4):689-698.
67. Liu Y. Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. Prostate Cancer Prostatic Dis. 2006;9(3):230-234.
68. Glasgow JF, Middleton B, Moore R, Gray A, Hill J. The mechanism of inhibition of beta-oxidation by aspirin metabolites in skin fibroblasts from Reye's syndrome patients and controls. Biochim Biophys Acta. 1999 May;1454(1):115-125.
69. Shahidi NT, Westring DW. Acetylsalicylic acid--induced hemolysis and its mechanism. J Clin Invest. 1970 Jul;49(7):1334-1340.
70. Dull BJ, Salata K, Van Langenhove A, Goldman P. 5-Aminosalicylate:oxidation by activated leukocytes and protection of cultured cells from oxidative damage. Biochem Pharmacol. 1987 Aug;36(15):2467-2472.
71. Liu ZC, McClelland RA, Uetrecht JP. Oxidation of 5-aminosalicylic acid by hypochlorous acid to a reactive iminoquinone. Possible role in the treatment of inflammatory bowel diseases. Drug Metab Dispos 1995;23(2):246-250.
72. Bigler J, Whitton J, Lampe JW, Fosdick L, Bostick RM, et al. CYP2C9 and UGT1A6 genotypes modulate the protective effect of aspirin on colon adenoma risk. Cancer Res. 2001 May;61(9):3566-3569.
73. Blakeborough MH, Owen RW, Bilton RF. Free radical generating mechanisms in the colon:their role in the induction and promotion of colorectal cancer? Free Radic Res Commun. 1989;6(6):359-367.
74. Semba M, Inui N. Inhibitory effects of radical scavengers on diacylglycerol-promoted transformation in BALB/3T3 cells. Toxicol Lett. 1991 May;56(3):299-303.
75. Martín V, Herrera F, Carrera-Gonzalez P, García-Santos G, Antolín I, et al. Intracellular signaling pathways involved in the cell growth inhibition of glioma cells by melatonin. Cancer Res. 2006 Jan;66(2):1081-1088.
76. Liou JS, Chen CY, Chen JS, Faller DV. Oncogenic ras mediates apoptosis in response to protein kinase C inhibition through the generation of reactive oxygen species. J Biol Chem. 2000 Dec;275(50):39001-39011.
77. Thomas WJ, Thomas DL, Knezetic JA, Adrian TE. The role of oxygen-derived free radicals and nitric oxide in cytokine-induced antiproliferation of pancreatic cancer cells. Pancreas. 2002 Mar;24(2):161-168.
78. Yeh CT, Shih PH, Yen GC. Synergistic effect of antioxidant phenolic acids on human phenolsulfotransferase activity. J Agric Food Chem. 2004 Jun;52(13):4139-4143.
79. Pecci J, Foye WO. The avidity of salicylic, gentisic, and salicyluric acids for heavy metal cations. J Am Pharm Assoc Am Pharm Assoc. 1960 Jul;49:411-414.
80. Broinizi PRB. Avaliacao da atividade antioxidante dos compostos fenolicos naturalmente presentes em subprodutos do pseudofruto de caju (Anacardium occidentale L.). [Evaluation of the Antioxidant Activity of Phenolic Compounds Naturally Contained in By-products of the Cashew Apple (Anacardium occidentale L.]. Ciencia e Tecnologia de Alimentos. 2007;27 (4):902–908.
81. Hermann M, Kapiotis S, Hofbauer R, Seelos C, Held I, et al. Salicylate promotes myeloperoxidase-initiated LDL oxidation:antagonization by it's metabolite gentisic acid. Free Radic Biol Med. 1999 May;26(9-10):1253-60.
82. Ashidate K, Kawamura M, Mimura D, Tohda H, Miyazaki S, et al. Gentisic acid, an aspirin metabolite, inhibits oxidation of low-density lipoprotein and the formation of cholesterol ester hydroperoxides in human plasma. Eur J Pharmacol. 2005 Apr;513(3):173-179.

83. Ozgová S, Hermánek J, Gut I. Different antioxidant effects of polyphenols on lipid peroxidation and hydroxyl radicals in the NADPH-, Fe-ascorbate- and Fe-microsomal systems. Biochem Pharmacol. 2003 Oct;66(7):1127-1137.

84. Joshi R, Gangabhagirathi R, Venu S, Adhikari S, Mukherjee T. Antioxidant activity and free radical scavenging reactions of gentisic acid:in-vitro and pulse radiolysis studies. Free Radic Res. 2012 Jan;46(1):11-20.

85. Perez-Gonzalez A, Galano A, Alvarez-Idaboy A. Dihydroxybenzoic acids as free radical scavengers:mechanisms, kinetics, and trends in activity. New J Chem. 2014 Jan;38:2639-2652.

86. Borges RS, Castle SL. The antioxidant properties of salicylate derivatives:A possible new mechanism of anti-inflammatory activity. Bioorg Med Chem Lett. 2015 Nov;25(21):4808-4811.

87. Yeh CT, Yen GC. Induction of hepatic antioxidant enzymes by phenolic acids in rats is accompanied by increased levels of multidrug resistance-associated protein 3 mRNA expression. J Nutr. 2006 Jan;136(1):11-15.

88. Nafees S, Ahmad ST, Arjumand W, Rashid S, Ali N, et al. Modulatory effects of gentisic acid against genotoxicity and hepatotoxicity induced by cyclophosphamide in Swiss albino mice. J Pharm Pharmacol. 2012 Feb;64(2):259-267.

89. Morel A, Josserand A, Chappet J. Sodium gentisate retention in a cancerous organism;possible relation with collagen pathology. C R Seances Soc Biol Fil. 1952 Sep;146(17-18):1341-1342.

90. Kim JH, Campbell BC, Mahoney N, Chan KL, Molyneux RJ, et al. Enhancement offludioxonil fungicidal activity by disrupting cellular glutathione homeostasis with 2,5-dihydroxybenzoic acid. FEMS Microbiol Lett. 2007 May;270(2):284-290.

91. Hopkins J, Tudhope GR. The effects of drugs on erythrocytes in vitro:Heinz body formation, glutathione peroxidase inhibition and changes in mechanical fragility. Br J Clin Pharmacol. 1974 Jun;1(3):191-195.

92. Mitchell JB, Russo A. The role of glutathione in radiation and drug induced cytotoxicity. Br J Cancer Suppl. 1987 Jun;8:96-104.

93. Anderson MS, Kalyanaraman B, Feix JB. Enhancement of merocyanine 540-mediatedphototherapy by salicylate. Cancer Res. 1993 Feb;53(4):806-809.

94. Sproull DH. Salicylate and liver glutathione. Br J Pharmacol Chemother. 1961Apr;16(2):180-187.

95. Hinz B, Kraus V, Pahl A, Brune K. Salicylate metabolites inhibit cyclooxygenase-2-dependent prostaglandin E(2) synthesis in murine macrophages. Biochem Biophys Res Commun 2000 Jul;274(1):197-202.

96. Holmes TJ Jr, Vennerstrom JL, John V. Inhibition of cyclooxygenase by electrochemical oxidation of gentisic acid. J Biol Chem. 1985 Nov;260(26):14092-14095.

97. Radomski M, Michalska Z, Marcinkiewicz E, Gryglewski RJ. Salicylates and 12-lipoxygenase activity in human washed platelets. Pharmacol Res Commun. 1986 Nov;18(11):1015-1030.

98. Kühn H, O'Donnell VB. Inflammation and immune regulation by 12/15-lipoxygenases. Prog Lipid Res. 2006 Jul;45(4):334-356.

99. Fürstenberger G, Krieg P, Müller-Decker K, Habenicht AJ. What are cyclooxygenases and lipoxygenases doing in the driver's seat of carcinogenesis? Int J Cancer. 2006 Nov;119(10):2247-2254.

100. Davis WB, Mohammed BS, Mays DC, She ZW, Mohammed JR, et al. Hydroxylation of salicylate by activated neutrophils. Biochem Pharmacol. 1989 Nov;38(22):4013-4019.

101. Haynes DR, Wright PF, Gadd SJ, Whitehouse MW, Vernon-Roberts B. Is aspirin a prodrug for antioxidant and cytokine-modulating oxymetabolites? Agents Actions. 1993 May;39(1-2):49-58.

102. Lippitz BE, Harris RA. Cytokine patterns in cancer patients: A review of the correlation between interleukin 6 and prognosis. Oncoimmunology. 2016 May;5(5):e1093722.

103. Chen Y, Friedman M, Liu G, Deodhar A, Chu CQ. Do tumor necrosis factor inhibitors increase cancer risk in patients with chronic immune-mediated inflammatory disorders? Cytokine. 2018 Jan;101:78-88.

104. Małaczewska J, Siwicki AK, Wójcik RM, Kaczorek E, Turski WA. Effect of oral administration of kynurenic acid on the activity of the peripheral blood leukocytes in mice. Cent Eur J Immunol. 2014 Apr;39(1):6-13.

105. Shore DM, Reggio PH. The therapeutic potential of orphan GPCRs, GPR35 and GPR55. Front Pharmacol. 2015 Apr 15;6:69.

106. Nusgens BV. [Hyaluronic acid and extracellular matrix:a primitive molecule?]. Ann Dermatol Venereol. 2010 Apr;137 Suppl 1:S3-S8.

107. Heldin P. Importance of hyaluronan biosynthesis and degradation in cell differentiation and tumor formation. Braz J Med Biol Res. 2003 Aug;36(8):967-973.

108. Stern R. Hyaluronidases in cancer biology. Semin Cancer Biol. 2008 Aug;18(4):275-280.

109. McAtee CO, Barycki JJ, Simpson MA. Emerging roles for hyaluronidase in cancer metastasis and therapy. Adv Cancer Res. 2014;123:1-34.

110. Stern R. Hyaluronan metabolism:a major paradox in cancer biology. Pathol Biol (Paris). 2005 Sep;53(7):372-382.

111. Cheng XB, Sato N, Kohi S, Yamaguchi K. Prognostic impact of hyaluronan and its regulators in pancreatic ductal adenocarcinoma. PLoS One. 2013 Nov;8(11):e80765.

112. Csóka TB, Frost GI, Stern R. Hyaluronidases in tissue invasion. Invasion Metastasis. 1997 Jan;17(6):297-311.

113. Girish KS, Kemparaju K. The magic glue hyaluronan and its eraser hyaluronidase: a biological overview. Life Sci. 2007 May 1;80(21):1921-1943.

114. Girish KS, Kemparaju K, Nagaraju S, Vishwanath BS. Hyaluronidase inhibitors:a biological and therapeutic perspective. Curr Med Chem. 2009;16(18):2261-2288.

115. Tsepilov RN, Beloded AV. Hyaluronic Acid--an "Old" Molecule with "New" Functions:Biosynthesis and Depolymerization of Hyaluronic Acid in Bacteria and Vertebrate Tissues Including during Carcinogenesis. Biochemistry (Mosc). 2015 Sep;80(9):1093-1108.

116. Tian X, Azpurua J, Hine C, Vaidya A, Myakishev-Rempel M, et al. High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. Nature. 2013 Jul;499(7458):346-349.

117. Forrest J, Overell BG, Petrow V, Stephenson O. Some observations on the inhbition of the actıon of hyaluronidase on hyaluronic acid by gentisic acid and its oxidation products. J Pharm Pharmacol. 1952 Sep;4(1):231-242.

118. Roseman S, Dorfman A. Effect of gentisic acid and related compounds on bovine testicular hyaluronidase. J Biol Chem. 1952 Nov;199(1):345-355.

119. Carlin G, Djursater R, Smedegard G, Gerdin B. Effect of anti-inflammatory drugs on xanthine oxidase and xanthine oxidase induced depolymerization of hyaluronic acid. Agents Actions. 1985 Jul;16(5):377-384.

120. Alvarez J. [Leukotriene modifiers in the treatment of asthma]. Rev Alerg Mex. 1999 May-Jun;46(3):72-77.

121. Minoguchi K, Adachi M. [Leukotriene modifiers]. Nihon Rinsho. 2001 Oct;59(10):1979-1985.

122. Galdiero MR, Varricchi G, Seaf M, Marone G, Levi-Schaffer F, et al. Bidirectional Mast Cell-Eosinophil Interactions in Inflammatory Disorders and Cancer. Front Med (Lausanne). 2017 Jul;24;4:103.

123. Chen X, Sood S, Yang CS, Li N, Sun Z. Five-lipoxygenase pathway of arachidonic acid metabolism in carcino-genesis and cancer chemoprevention. Curr Cancer Drug Targets. 2006 Nov;6(7):613-622.

124. Peter H, Lýdia B. [Modulation of leukotriene pathway - potential

targets]. Ceska Slov Farm. 2012 Aug;61(3):101-106.

125. Mashima R, Okuyama T. The role of lipoxygenases in pathophysiology;new insights and future perspectives. Redox Biol. 2015 Dec;6:297-310.
126. Trautmann M, Peskar BM, Peskar BA. Aspirin-like drugs, ethanol-induced rat gastric injury and mucosal eicosanoid release. Eur J Pharmacol. 1991 Aug;201(1):53-58.
127. Fernández IS, Cuevas P, Angulo J, López-Navajas P, Canales-Mayordomo A, et al. Gentisic acid, a compound associated with plant defense and a metabolite of aspirin, heads a new class of in vivo fibroblast growth factor inhibitors. J Biol Chem. 2010 Apr;285(15):11714-11729.
128. Cunha JF, Campestrini FD, Calixto JB, Scremin A, Paulino N. The mechanism of gentisic acid-induced relaxation of the guinea pig isolated trachea:the role of potassium channels and vasoactive intestinal peptide receptors. Braz J Med Biol Res. 2001 Mar;34(3):381-388.
129. Sokolowska P, Nowak JZ. Effects of PACAP and VIP on cAMP-generating system and proliferation of C6 glioma cells. J Mol Neurosci. 2008 Nov;36(1-3):286-291.
130. Barbarin A, Séité P, Godet J, Bensalma S, Muller JM, et al. Atypical nuclear localization of VIP receptors in glioma cell lines and patients. Biochem Biophys Res Commun. 2014 Nov;454(4):524-530.
131. Vertongen P, Camby I, Darro F, Kiss R, Robberecht P. VIP and pituitary adenylate cyclase activating polypeptide (PACAP) have an antiproliferative effect on the T98G human glioblastoma cell line through interaction with VIP2 receptor. Neuropeptides. 1996 Oct;30(5):491-496.
132. D'Amico AG, Scuderi S, Saccone S, Castorina A, Drago F, et al. Antiproliferative effects of PACAP and VIP in serum-starved glioma cells. J Mol Neurosci. 2013 Oct;51(2):503-513.
133. Cochaud S, Meunier AC, Monvoisin A, Bensalma S, Muller JM, et al. Neuropeptides of the VIP family inhibit glioblastoma cell invasion. J Neurooncol. 2015 Mar;122(1):63-73.
134. Maugeri G, Grazia D'Amico A, Reitano R, Magro G, et al. PACAP and VIP Inhibit the Invasiveness of Glioblastoma Cells Exposed to Hypoxia through the Regulation of HIFs and EGFR Expression. Front Pharmacol. 2016 May;7:139.
135. Emami Riedmaier A, Nies AT, Schaeffeler E, Schwab M. Organic aniontransporters and their implications in pharmacotherapy. Pharmacol Rev. 2012;64(3):421-449.
136. Wang L, Sweet DH. Potential for food-drug interactions by dietary phenolic acids on human organic anion transporters 1 (SLC22A6), 3 (SLC22A8), and 4 (SLC22A11). Biochem Pharmacol. 2012 Oct;84(8):1088-1095.
137. Ohtsuki S, Kikkawa T, Mori S, Hori S, Takanaga H, et al. Mouse reduced in osteosclerosis transporter functions as an organic anion transporter 3 and is localized at abluminal membrane of blood-brain barrier. J Pharmacol Exp Ther. 2004 Jun;309(3):1273-1281.
138. Hagos Y, Hundertmark P, Shnitsar V, Marada VV, Wulf G, et al. Renal human organic anion transporter 3 increases the susceptibility of lymphoma cells to bendamustine uptake. Am J Physiol Renal Physiol. 2015 Feb 15;308(4):F330-F338.
139. Töllner K, Brandt C, Römermann K, Löscher W. The organic anion transport inhibitor probenecid increases brain concentrations of the NKCC1 inhibitor bumetanide. Eur J Pharmacol. 2015 Jan;746:167-173.
140. Hagos FT, Daood MJ, Ocque JA, Nolin TD, Bayir H, et al. Probenecid, an organic anion transporter 1 and 3 inhibitor, increases plasma and brain exposure of N-acetylcysteine. Xenobiotica. 2017 Apr;47(4):346-353.
141. Kusuhara H, Sugiyama Y. Efflux transport systems for organic anions and cations at the blood-CSF barrier. Adv Drug Deliv Rev. 2004 Oct;56(12):1741-1763.
142. Narumi K, Sato Y, Kobayashi M, Furugen A, Kasashi K, et al. Effects of proton pump inhibitors and famotidine on elimination of plasma methotrexate:Evaluation of drug-drug interactions mediated by organic anion transporter 3. Biopharm Drug Dispos. 2017 Dec;38(9):501-508.
143. Li L, Agarwal S, Elmquist WF. Brain efflux index to investigate the influence of active efflux on brain distribution of pemetrexed and methotrexate. Drug Metab Dispos. 2013 Mar;41(3):659-667.
144. Gibbs JE, Thomas SA. The distribution of the anti-HIV drug, 2'3'-dideoxycytidine (ddC), across the blood-brain and blood-cerebrospinal fluidbarriers and the influence of organic anion transport inhibitors. J Neurochem. 2002 Feb;80(3):392-404.
145. He L, Liu X, Zhao Y, Zhang S, Jiang Y, et al. Efficacy of Nucleot(s)ide Analogs Therapy in Patients with Unresectable HBV-Related Hepatocellular Carcinoma:A Systematic Review and Meta-Analysis. Dis Markers. 2017;2017:7075935.
146. Ananth S, Gnana-Prakasam JP, Bhutia YD, Veeranan-Karmegam R, Martin PM, et al. Regulation of the cholesterol efflux transporters ABCA1 and ABCG1 in retina in hemochromatosis and by the endogenous siderophore 2,5-dihydroxybenzoic acid. Biochim Biophys Acta. 2014 Apr;1842(4):603-612.
147. Guo Y, Su ZY, Zhang C, Gaspar JM, Wang R, et al. Mechanisms of colitis-accelerated colon carcinogenesis and its prevention with the combination of aspirin and curcumin:Transcriptomic analysis using RNA-seq. Biochem Pharmacol. 2017 Jul;135:22-34.
148. Lee BH, Taylor MG, Robinet P, Smith JD, Schweitzer J, et al. Dysregulation of cholesterol homeostasis in human prostate cancer through loss of ABCA1. Cancer Res. 2013 Feb;73(3):1211-1218.
149. Chou JL, Huang RL, Shay J, Chen LY, Lin SJ, et al. Hypermethylation of the TGF-β target, ABCA1 is associated with poor prognosis in ovarian cancer patients. Clin Epigenetics. 2015 Jan;7(1):1.
150. Smith B, Land H. Anticancer activity of the cholesterol exporter ABCA1 gene. Cell Rep. 2012 Sep;2(3):580-590.
151. Sag D, Cekic C, Wu R, Linden J, Hedrick CC. The cholesterol transporter ABCG1 links cholesterol homeostasis and tumour immunity. Nat Commun. 2015 Feb;6:6354.
152. Tian C, Huang D, Yu Y, Zhang J, Fang Q, et al. ABCG1 as a potential oncogene in lung cancer. Exp Ther Med. 2017 Jun;13(6):3189-3194.