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From epidemiology to treatment: Aspirin's prevention of brain and breast-cancer and cardioprotection may associate with its metabolite gentisic acid

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ABSTRACT

Background: Epidemiological studies indicate that aspirin consumption reduces the risk of tumors, which is especially relevant for colonic adenoma and carcinoma. Similar observations were made for glial brain tumors and breast cancers, yet the results are inconsistent. Gentisic acid (GA) is a minor catabolite of aspirin; yet humans carrying CYP2C9-variants incapable to catabolize aspirin to GA do not benefit from aspirin in prevention against colonic adenoma. GA blocks binding of Fibroblastic Growth Factor to its receptor and its sulphonate metabolite dobesilic acid blocks growth of C6 glioblastoma *in vivo*. GA is also an endogenously produced siderophore in mammalians for the transport of iron, a trace element which stimulates tumor growth and enhances anthracycline cardiotoxicity.

Materials and Methods: In this study, we assessed whether GA exerts direct antitumor activity on C6 glioma cells *in vitro* (cytotoxicity, colony growth, 3H-thymidine labeling analysis of DNA synthesis); and whether it can modify growth of Ehrlich breast ascites carcinoma (EAC) and solid tumors (EST) *in vivo*. GA and antitumoral trace element selenium block 12-lipoxygenase activity and aspirin's paradoxical inflammatory effects are seen in selenium-deficient humans; thus, we also investigated antitumor interactions between GA and sodium selenite. Lastly, we evaluated whether GA could protect against doxorubicin cardiotoxicity due to its function to chelate iron.

Results: Clinically achievable doses of GA blocked growth, colony formation and DNA synthesis of C6 glioma *in vitro* with high significance. GA enhanced the survival of EAC-bearing mice at a dosage of 0.4 mg/mice/day, in which 33% of the treated animals survived more than 3-weeks, when all untreated mice succumbed to their tumors. Selenium decreased EST volumes initially, yet increased tumor volumes at later stages in surviving mice. GA alone reduced solid tumor growth and did not modify selenite antineoplasticity initially, but blocked the late tumor-stimulating effects of selenite. Lastly, doxorubicin-induced cardiac myofibrillary and endothelial damage and hyalinization necrosis were attenuated with GA treatment.

Conclusions: GA highly merits to be studied in further animal models as an anticancer and chemoprotective drug.

1. Introduction

Conflicting findings exist regarding effects of aspirin on the risk of brain and breast cancers. Some studies demonstrated that usage of aspirin is associated with reduced risk of high grade glial tumors [28,70,80], yet other studies failed to demonstrate such a protective efficacy [5,23]. Similarly; while some studies showed that aspirin had no effect on the risk of breast cancer [80]; others reported reduction of breast cancer risk with regular aspirin intake [51,78,79]. These inconsistent results are not easily explicable; yet we propose that reduced catabolism of aspirin to gentisic acid (GA, 2,5-dihydroxybenzoic acid) may be responsible. The first cancer-chemopreventive efficacy of aspirin (acetylsalicylic acid) is demonstrated in colon cancer; and inflammatory colitis enhances risk of colon cancer which can be blocked by aspirin [37]. Aminosalicylic acid (5-ASA) is also used in the treatment of inflammatory colitis and is a scavenger of the oxidants released by neutrophils in inflammatory bowel disease [49]. Oxidation of 5-ASA by HOCl and reactive intermediates were defined by mass spectral

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analysis which revealed the formation of iminoquinone and quinone intermediates including GA [49]. GA is a byproduct of aspirin catabolism and in carriers of the *CYP2C9* variant alleles, salicylate conversion to GA is hampered, causing in a reduced inhibition of prostaglandin synthesis and lack of risk reduction against colon adenoma [8].

GA may also involve in aspirin's chemopreventive efficacy against breast and brain cancers and polymorphisms of enzymes involving in aspirin catabolism to GA may cause different epidemiological results. Some preliminary clues support our proposal. *Solanum nigrum* Linn (SN) plant has been used in South Asian folk medicine for anticancer effects, which contains high amounts of GA and its extract induces autophagic death of human AU565 breast cancer cells [40]. In a similar manner, extracts of *Leonurus sibiricus* L. rich in GA exerts antiproliferative and apoptotic effect on high grade glioma cell cultures [69]. More than 65 years have passed, since Morel et al. demonstrated that GA retention occurs in patients with cancer [56]; but only a handful studies exist on the anticancer potencies of this simple and cheap agent. In this study, we assessed antitumoral efficacies of GA on C6 glioma and Ehrlich mammary carcinoma and also determined its interaction with selenium's antitumoral potential and cardiotoxicity of anthracycline.

For the study, Ehrlich ascites and solid tumor models were selected because Ehrlich carcinoma cells are still considered as breast carcinoma and respond to tamoxifen treatment, despite they are derived from an old tumor model [59,74,76]. Moreover, they are very suitable to assess anticancer activity of antiinflammatory compounds because intraperitoneal injection of Ehrlich tumor cells induces prostaglandin E_2 synthesis, neutrophil and macrophage infiltration [32], iNOS and COX-1 synthesis and induction of the synthesis of MCP-1 and IL-1β, which are pro-inflammatory and protumorigenic cytokines [33]. Similarly, C6 glioma cells significantly produce PGE_2 *in vitro*, which is sensitive to aspirin and indomethazine suppression [41]; and additionally, their *in vivo* growth can be suppressed with aspirin treatment [2].

2. Materials and Methods

2.1. Cell culture, assessment of growth inhibition and drug treatments

C6 glioma cells obtained from American Type Culture Collection (ATCC) were maintained using RPMI-1640 (Biological Industries, Haemek, Israel) with 15% heat inactivated fetal calf serum (FCS), 0.2 mM glutamine, 50 μg/ml neomycin and 100 μg/ml streptomycin. The flasks were kept in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C. We used early passages (4–7 t h) of C6 cells after obtaining the cell line. Cells prepared in 5 ml of RPMI-1640 were plated into a six-well plate in 1×10^5/ml concentration with 100% vitality. GA was obtained from Sigma-Aldrich (85707 – Sigma, purity > 99%, CAS Number 490-79-9) and dissolved in pure water. GA was given in equal volumes of 100 μl into plates, and after 24, 48, 72 and 96 h, cells were harvested with trypsin-EDTA C (Biological Industries, Haemek, Israel). Vital cells were identified by trypan blue exclusion and counted on a Thoma chamber. To reveal changes of the cell proliferation, only vital cells are compared between different groups.

2.2. Colony growth

Stock agar (Gibco, 152–00391 M) solution of 0.3% concentration was prepared at 45 °C. Four volumes of RPMI-1640 was rapidly mixed with one volume of stock agar solution and 1 ml of this solution was filled into each well of a six-well plate and used as an underlayer. Subsequently, plates were incubated at 4 °C for 15 min. For each well 3×10^3, 100% vital cells were suspended in 1 ml RPMI-1640 media and mixed with 2 ml of (4 vol. RPMI + 1 vol. Stock agar solution mix). One millilitre of this suspension was put onto each under layer of six-well plates. Drugs were added in 100 μl final volume to the corresponding groups. Six-well plates were then kept in an electronic incubator under a humidified atmosphere of 4.5% CO_2 and air mix for 1 week. Following 1 week of incubation, colonies containing more then 50 cells were counted under an inverted microscope. Colony inhibition was calculated with the following formula: Clonogenic Cell Death % = 100 × (1-colony number of drug treated group/colony number of control group).

2.3. S-Phase Fractions assased by ^{3}H-thymidine-labelling index

Half an hour before the end of each 24-h period of monitoring plating efficacy, cells were incubated with 1 μCi/ml ^{3}H-thymidine (Amersham, England, sp. act. 185 GBq/mmol, 5 Ci/mmol). Following 30 min of incubation, cells were treated with trypsin and smear slides were prepared. Then slides were fixed with Carnoy's fixative (a 3:1 ratio of ethanol/glacial acetic acid) and unbound radioactive materials were washed twice with 2% perchloric acid at 4 °C for 30 min. Cover slips were coated with gel emulsion film (Ilford K2, England). After 3 days of exposure at 4 °C, autoradiograms were washed with D-19 developer and slides were evaluated after Giemsa staining. With 100 × 12.5 magnification 3×10^3 cells were evaluated in 100 areas, cells with at least five grain in their nuclei were considered to be labelled.

2.4. Determination of the antitumor efficacy of GA in Ehrlich Ascites Tumor model

All animal experimentations were carried out according to the guidelines of the Declaration of Helsinki. Male Balb/C mice (10-weeks-old, 20–25 g) were purchased from the Experimental Animal Research Center-Cerrahpasa Faculty of Medicine (Istanbul), and kept there at least 1 week before use. The animals were fed with a standard pellet diet and drinking water *ad libitum*. Ehrlich Ascites Tumor (EAT) cell lines were obtained from the same center and were propagated as transplantable ascites tumors in female Balb/C mice. EAT grows both in female and male mice, yet EAT is maintained in female mice to allow that female hormones would stimulate the growth of this tumor origined from a mammary carcinoma. Male mice were used to assess the effects of drug treatments to avoid the likely effects of fluctuating female sex steroids (eg. due to menstrual cycle) which may differ between different experimental groups.

For ascites tumor model, tumor cells (EAT) aspirated from the peritoneal cavity of mice were washed with saline and 1×10^6 tumor cells were given intraperitoneally to four group of animals (14 mice per group). Treatments were began following intraperitoneal tumor inoculation. In the first (control) group, mice (n = 14) were given saline solution by gavage every day. GA was dissolved in pure water and given by intragastric gavage. In the experimental groups, GA treatments were applied at dosages of 0.1, 0.2, 0.4 and 0.8 mg/day to second, third and fourth group of mice, respectively. There were 14 mice in each dosage groups. The mice weighed around 20 g and the average weights of mice did not differ between these groups; hence, these treatments roughly corresponded to 5, 10, 20 and 40 mg/kg/day of GA. Animals were observed and weighed everyday for the development of ascites tumor; and deaths due to tumor burden were recorded. Weight changes were calculated as percentage changes by comparison to the mean weight of animals in the same groups.

2.5. Determination of the antitumor efficacy of GA and sodium selenite in ehrlich solid tumor model

For the solid tumor model, one million EAT cells were injected in to the right hind limb of male Balb/C mice. Formation of solid tumors were monitored until the 10 t h day of inoculation and mice were distributed to different experimental groups which had similar size of tumors which did not differ significantly from each other. Animals in group-I were kept as control, which received the vehicle. Control groups (n = 14) were given saline solution intraperitoneally every day. Selenium group animals (n = 15) were given 40 μg/per mice (2 mg/kg)