Tumour Response to Quercetin, a Bioflavonoid with Some Promises in Therapies

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Quercetin, a well-known bioflavonoid, is commonly found in human diet. This flavonoid along with Genistein gained much attention during the last few years as a potential anticancer drug. The purpose of this study was to investigate the effect of quercetin on tumour growth *in vivo* after determining its toxicological profile. The maximum tolerated dose (MTD) of quercetin was found to be 100 mg/kg. Fifty percent of the S-180 ascitic tumour bearing mice, treated with quercetin with a daily intraperitoneal dose (100 mg/kg) for nine consecutive doses, survived for more than 2 mo. Quercetin did not show significant inhibitory effect on the growth of established mammary tumour inoculated in C3H/J mice. However, increase in tumour size was significantly lower when administered in combination with cyclophosphamide compared to remaining groups. Quercetin was found to be protecting the mouse bone marrow from radiation-induced toxicity by creating hypoxic conditions in the marrow. Hypoxic conditions resulted in elevated LDH levels in quercetin-treated group compared to levels in the control group. Haematological parameters did not show significant difference in treated and control groups. Spleen colony assays suggest quercetin to be nontoxic and that it can be administered at 100 mg/kg dose levels in further studies.

The need for low toxic anticancer compound with novel antiradical and antioxidative/prooxidative mechanism of action is in great demand. Consequently, there has been growing interest in a number of phytochemicals, polyphenolic compounds which have long been recognized to possess many potentially significant benefits, including anticarcinogenic, antioxidant, antiviral and antiproliferative activities[1]. Dietary factors contribute to about one-third of potentially preventable cancers, and the long-term preventive effects of plant-based diets on tumourigenesis, as well as therapeutic advantages, are well documented[2].

In recent years, there has been a growing body of evidence that the major flavonoids (polyphenolics) can inhibit *in vitro* as well as *in vivo* tumour cell growth and induce apoptotic death of cells in different cell lines[3]. Flavonoids, a group of polyphenolic quinoids, are widely distributed in edible plants, edible fruits, leafy vegetables, roots, tubers, bulbs, herbs, spices, legumes, tea, coffee, red wine and beer. We consume these flavonoids through our everyday diet. These flavonoids possess a wide range of both potentially detrimental and protective biological characteristics[3]. Various antioxidant effects have been ascribed to flavonoids, viz., the scavenging of free radicals, the chelation of transition metals and the inhibition of free radicals producing enzymes[4]. In the series tested, the scavenging ability nicely correlated with the number of free aromatic hydroxyl groups. Flavonoids have been found to have a marked selectivity against malignant cell types *in vitro*[5–8]. We reported previously that quercetin (Q) inhibits K-562 leukaemic cell proliferation and induces apoptosis[9]. We initiated antitumour investigation in animal models involving the growth of transplantable tumour to explore the feasibility for further development of this dietary microchemical[11-12]. In this primary study, we investigated the effect of quercetin on eliminating solid tumour growth and increased survival of tumour-bearing animals. We also studied combined antiproliferative effect of quercetin *in vivo* on growth of mammary tumour along with cyclophosphamide, a known cytotoxic drug. In addition, we made attempts to investigate the role of quercetin in protecting mice from radiation-induced injuries.

MATERIALS AND METHODS

Quercetin and dimethyl sulfoxide (DMSO) were purchased from SD Fine Chemicals, Mumbai. A stock

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solution of quercetin (200 mg/ml) in DMSO was prepared. Quercetin is freely soluble in DMSO.

Female C3H/J mice, Swiss mice and Wistar rats of either strain, procured from the animal faculty of Cancer Research Institute, were housed under the following conditions: Room temperature 22°C ±2, relative humidity 55% ±5, dark and light period of 12 h, free access to standard pelleted animal feed and water. Animals were randomly divided into experimental groups.

Toxicological observations:

Six to eight weeks old male Swiss mice were injected intraperitoneally (i.p.) with 25, 50 and 100 mg/kg quercetin daily for 9 consecutive days (n = 6). DMSO stock solution (0.5 ml) of quercetin was taken in Tween 80 (1 ml) and diluted with normal saline to make 10 ml injectable fine suspension and administered in a volume of 0.1 ml per 10 g of body weight. Animals were weighed every 24-48 h and observed every morning and evening for mortality, if any. Results were recorded and expressed as maximum gain in weight compared to controls, median day of death of animals and percentage mortality for each group. The maximum tolerated dose (MTD) was defined as a dose causing significant and reversible body weight loss (5-20%) but no morbidity[13]. Whole blood was collected from the tail vein or by cardiac puncture under light anaesthesia for the determination of peripheral blood counts from three mice of each group after the completion of treatment (day 10).

Collection and processing of blood samples:

Measurements of serum glutamate oxalo transferase (SGOT), serum glutamate pyruvate transferase (SGPT), glucose, urea nitrogen, creatinine, haemoglobin and creatinine kinase were performed using the serum collected from blood of Wistar rats treated for 4 d with quercetin. Control animals were treated with DMSO- Tween 80-saline (0.5:1:8.5). Since use of mice requires accumulation of serum for biochemical parameters, rats were preferred in this study to get sufficient quantity of serum.

Cytoreduction assay:

Mice of the control and quercetin-treated group were sacrificed at the end of the treatment period. Spleens were excised, blotted, weighed and fixed in 10% formalin for 24 h, and ovaries (from the same animals' uteri) were dissected, blotted and weighed.

Spleen colony assay:

Quercetin was administered i.p. daily in Swiss male mice aged 6-8 w old (n = 10) for 9 consecutive days at a dose of 100 mg/kg. Control animals received the same vehicle. A fresh set of 20 normal animals were taken, irradiated (800 rads) once and divided into four groups, each group consisting of five animals. Animals of group I were irradiated control, whereas animals of groups II, III and IV received bone marrow cells (1×10^5 cells/ml) from normal, vehicle- and quercetin-treated animals respectively, by intravenous route. Animals were sacrificed 12 d after bone marrow cells inoculation. Spleen from animals of each group was dissected, and number of colonies on the spleen was counted[14].

Bone marrow protection test:

Swiss mice were divided into four groups; each group consisted of five mice and was treated as follows. Control animals were injected i.p. with 0.2 ml of the vehicle, DMSO- Tween 80- saline (0.5:1:8.5) for 5 d. Quercetin (Q) alone was injected i.p. – 100 mg/kg body weight of quercetin for 5 d. Radiation (RT) alone requires exposure to 800 rads of gamma radiation locally to the abdominal area of mice on day 6. Quercetin with radiation (Q+RT) group animals were injected i.p. for 5 d with 100 mg/kg body weight Q and exposed to 800 rads of gamma radiation after 24 h of drug treatment (on day 6).

After the radiation exposure, animals were sacrificed by cervical dislocation, and bone marrow cells were collected from the femur and tibias by flushing with Iscove's modified Dulbecco's Medium (IMDM). Red blood cells in marrow cells were haemolysed in 0.017 M Tris HCL, pH 7.5, buffer containing 0.8% ammonium chloride. Haemolysed bone marrow suspensions were rinsed twice with PBS, and viability was measured by trypan blue dye exclusion test on day 2 and day 4 during the post-irradiation period[9].

Animal tumour models:

Sarcoma-180 ascitic tumour (2×10^6 cells/animals) was inoculated i.p. into 4-6 w old Swiss mice of either sex. Quercetin at dose levels of 25, 50, 100 mg/kg was administered for nine days, 24 h after tumour inoculation, and deaths were recorded everyday, if any.

Tumour growth delay:

Female C3H/J mice (4-6 w old) were inoculated s.c. with 1×10^6 established mammary tumour cells obtained from a spontaneously developed tumour in the same species. Treatment group received Q at the dose levels of 25, 50, 100 mg/kg by i.p. administration for 9 d, when tumours developed approximately to the volume of 15-20 mm^3.

The control group was treated by daily i.p. injection of 0.2 ml of the drug vehicle. Tumour progression was monitored for 3 w by skin calliper measurements. The ratio of mean tumour volume of treated group to that of control group (T/C) was calculated at 21 days. Maximum tumour regression was defined as the smallest relative tumour volume achieved throughout the treatment course. Tumour growth delay was the time required for the tumour to double in volume. Tumour volume (V) was calculated according to the equation $V = a \times b^2/2$, where a = longest diameter, b = longest diameter at right angles to a.

To increase efficacy of cyclophosphamide (CTX) against the established mammary tumour (Table 5) in C3H/J mice, each set of six mice were stratified into four groups (Control, Q, CTX, Q+CTX). Quercetin (100 mg/kg) alone or along with CTX (50 mg/kg) was administered by i.p. route keeping 3 h gap between the two injections on days 1, 5, 9. Control mice were treated with vehicle by three i.p. injections, 4 d apart from each other. Tumour volume was measured twice-weekly using skin callipers as described above.

RESULTS AND DISCUSSION

Hertog and Hollman have reviewed the potential health effects of quercetin[10]. The compound is a bioflavonoid, which exists in a free or configurated form in many fruits and vegetables. Despite important advances, the physiologically relevant targets of flavonoids and the mechanism by which they exert their effect are not well understood[10]. This has resulted in evaluating the efficacy of flavonoids further in *in vitro* and *in vivo* experimental models for human cancers[5-8]. Quercetin is a known inhibitor of some protein kinases, including protein tyrosine kinases (PTK). We have therefore attempted to assess the efficacy of quercetin in suppressing mouse mammary tumour and increasing the lifespan of S-180 tumour bearing animals after assessing its biological properties.

Toxic side effects of quercetin which have been reported so far include damage to gastrointestinal tract and kidney[10]. To avoid undesirable side effects, we first determined its MTD. Solubility problem arose because of its hydrophobic nature[9]. A fine suspension was prepared from its stock solution, and the MTD was found to be 100 mg/kg in mice dose; there was no apparent decrease in body weight and mortality (Table 1). This dose was used for all further studies.

TABLE 1: PERCENT INCREASE IN BODY WEIGHT IN CONTROL AND TREATED SWISS MICE

Group	Dose (mg/kg)	Day 2	Day 5	Day 9	Day 13
Control	–	5.3±2.1	15.6±1.1	26.1±4.3	36.2±4.6
Quercetin	25	2.2±0.6	5.3±1.2	10.4±1.9*	20.6±2.3**
	50	2.1±0.2	5.3±0.8	6.2±1.3	9.1±0.6*
	100	1.6±0.1	2.1±0.6	3.1±1.1	3.0±1.1

Changes in body weights after i.p. administration of different doses of Q for 9 consecutive days *P < 0.05; **P < 0.01 *vs.* control without Q

Haematological findings do not show any adverse signs of toxicity (Table 2). Although spleen weights were not comparable in both the experimental groups, the overall data indicates quercetin's lack of cytoreduction activity (Table 3). Since Q was administered in the suspension form, there is bound to be slight increase in the weights of some of the organs. Spleen colony assay points out that administration of quercetin repeatedly at its MTD has no adverse effects on the bone marrow cells of treated mice. The number of colonies in IR, control and Q-treated groups was 1, 11.8 and 12.8 respectively.

It is well recognized that a large dose of irradiation can lead to acute or progressive biological effects and thus can be used in clinics to treat patients with malignancies. When used against tumours, radiation also exerts destructive effects on haematopoietic organs such as the spleen, thymus and bone marrow and this damage usually appears with the pronounced syndromes of these systems,

TABLE 2: HAEMATOLOGICAL OBSERVATIONS AFTER TREATMENT WITH QUERCETIN IN MICE

Parameters	Control	Q (100 mg/kg)
Hb g %	15.0±0.32	14.1±0.46
RBC mil/cmm	8.6±0.45	8.1±0.45
PCV %	45.4±3.85	41.4±2.30
TLC	7170±736.21	7500±1241.5
DLC - Neutrophil	23.0±6.24	28.0±6.06
Eosinophil	1.0±0.45	1.0±0.71
Lymphocyte	75.0±6.22	70.0±6.22
Monocyte	1.0±0.71	1.0±0.71

The pattern of changes in blood constituents after *in vivo* treatment with Q (100 mg/kg) for 9 consecutive days. Blood samples were withdrawn from tail vein following the completion of treatment

TABLE 3: RELATIVE SPLEEN, UTERUS AND OVARY WEIGHTS AFTER QUERCETIN ADMINISTRATION IN SWISS MICE

Treatment group	Body organs (mg)		
	Spleen	Uterus	Ovary
Control	74.6	143.06	22.9
Q 50 mg/kg	87.0	152.00	23.0
Q 100 mg/kg	91.3	172.03	23.4

Quercetin was administered i.p. at doses of 50 mg/kg and 100 mg/kg for 9 d. Animals were dissected after the treatment was over. Organs were taken out, blotted and weighed

including leucopoenia, haemorrhage, infection and anaemia. In radiation therapy, how to eliminate the tumour growth and in the meantime possibly enhance the recovery of haematopoietic organs from injuries seemed important. Using flavonoids, we are trying to achieve a significant reduction of tumour growth in experimental tumour systems against radiation-induced damage. In the earlier experiments, we have found that quercetin created hypoxic conditions in tumour tissue (data not shown). Cells in hypoxic state are known to be resistant to chemotherapeutic and radiation treatment[11]. Whether this property can make it a radioprotective agent has remained to be shown. Working on similar lines, we were able to show that quercetin protects mouse bone marrow cells from radiation toxic effect. Bone marrow cell counts of different groups differ according to treatment given. The bone marrow cell counts in radiation-treated group were the lowest, but if the same group received quercetin prior to radiation, there was protection to the level of 50% (Table 4). Serum obtained from quercetin-treated Wistar rats was used for enzymatic studies. Hypoxic conditions created by quercetin resulted in high lactate levels. However, there was no corresponding increase in the levels of acid and alkaline phosphatase (data not shown).

In 1971 quercetin was discovered to moderately inhibit the growth of i.p. implanted P388 leukaemia, increasing the lifespan of the tumour-bearing mice by 27-32%[15]. At that time the level of P388 activity demonstrated by quercetin was not considered sufficient to warrant further investigation. However, taking into consideration the role of dietary micro-chemicals in the treatment of various human tumours, it is now being re-examined by us[5,9,10]. In this study we evaluated the antitumour effect of quercetin on both s.c. implanted mammary tumour in C3H/J mice as well as mice bearing S-180 ascitic tumour. Treatment with quercetin alone did not inhibit the growth of established mammary tumour. However, it retarded the tumour growth in combined group of quercetin+CTX compared to individual group of quercetin and cyclophosphamide. By 2 w after quercetin treatment, retardation of growth became apparent; and by 3 w, difference in mean tumour volume of the individual groups and combined group reached statistically significant levels. By 4 w, when the tumour had enlarged to the extent that necrosis and rupture had occurred, all measurements of size and body weights were discontinued since data obtained beyond this point would not have been meaningful.

The effects of 25, 50 and 100 mg/kg of quercetin on survival of S-180 tumour bearing mice given on days 1-9, i.p., 24 h after S-180 tumour cell transplantation are shown in Table 5. Antitumour effect at 100 mg/kg was significantly higher than with treatments at lower doses. Fifty percent of the animals survived for more than 2 mo. The effects of quercetin were more pronounced when given after 24 h of cell inoculation than after day 5 of the experiment – that is, late treatment (Table 6). It appears that the presence of the C2-C3 double bond and/or attachment of ring B to the chromone structure besides hydroxylation at the 3 position are/is responsible for the antitumour effects shown by quercetin flavonol[5]. Based on the above results, quercetin was co-administered with various cytotoxic drugs. The flavonoids enhanced the antitumour activity of cisplatin (data not shown).

We determined the MTD of quercetin, as well as toxicological profile, using a suitable vehicle for its safe

TABLE 4: PROTECTIVE EFFECT OF QUERCETIN ON MOUSE BONE MARROW AFTER IRRADIATION

Group	Dose	Viability (%) (Day 2)	Viability (%) (Day 4)
Control	––	100	100
Q	100 mg/kg	80.0	93.0
IR	800 (rads)	30.0	45.0
Q+IR	100 mg/kg + 800 rads	85.0	88.0

Bone marrow cells were withdrawn by a syringe, RBCs were discarded and viability of marrow cells was measured by trypan blue dye exclusion test. Q (100 mg/kg) was administered for 5 d prior to irradiation

TABLE 5: ANTINEOPLASTIC ACTIVITY OF QUERCETIN, CYCLOPHOSPHAMIDE AND THEIR COMBINATIONS

Day	Mean tumour volume (mm^3)			
	Control	Q	CTX	Q + CTX
1	136	56.0	93.0	66.0
5	210	133.0	100.0	83.0
9	300	243.0	116.0	111.0
15	333	320.0	156.0	150.0

Mammary adenocarcinoma cells were inoculated s.c. into C3H/J female mice. Treatment started after nodular form of tumour growth was observed and was given i.p. in three intermittent doses

TABLE 6: ANTITUMOUR ACTIVITY OF QUERCETIN IN S-180 ASCITIC TUMOUR

Group	Dose (mg/kg)	Median survival time (day)	Increase in life span (ILS) in %	Long term survival (>60 days)
Control	-	14.0	0.0	0/6
Quercetin	25	18.0	28.5	0/6
	50	27.5*	96.5	0/6
	100	42.0**	200.0	3/6

Comparative survival patterns of tumour-bearing mice treated with Q with different doses. Control group was treated with vehicle only. Quercetin was i.p. administered daily, day 1 to day 9, to tumour-bearing mice. Statistical significance at $*P < 0.05$; $**P < 0.01$ by the student's 't' test

administration, which will be useful for carrying out further experiments on this dietary product. Quercetin on its own did not exert inhibitory effects on *in vivo* tumour systems comprising of established mammary tumour model. The combination of quercetin and CTX arrests the growth of this solid tumour temporarily. However, continuous treatment daily for 9 d was found to be beneficial to the treatment of S-180 ascitic tumour. This non-nutrient appears to be useful as a supplementary product in combination therapy, which may have therapeutic significance. Earlier we have proved that quercetin shows vasoactive properties. This finding, coupled with the present result, suggests that it will be a good candidate for raising attempts to develop new therapeutic radioprotective approaches.

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