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Evaluation of Toxicity and Antitumour Effects of a Hydroxypropyl β-Cyclodextrin Inclusion Complex of Quercetin

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The general toxicity and antineoplastic activity of a hydroxypropyl beta cyclodextrin complex were investigated recently by us. Complexed quercetin at the large lethal dose of 400 mg/kg was not found to be toxic. (Free quercetin can be administered only at smaller doses because of its poor aqueous solubility). The maximum tolerated dose corresponding to the LD10 was > 400 mg/kg for hydroxypropyl beta cyclodextrin complex of quercetin obtained after a single intraperitoneal application which proved to be less toxic than quercetin. *In vitro* experiments have shown that hydroxypropyl beta cyclodextrin complex of quercetin induces apoptosis in both K-562 and B16F10 melanoma cells. Therapeutic experiments in C3H/J mice implanted with mammary adenocarcinoma cells resulted in significantly increased effectiveness of hydroxypropyl beta cyclodextrin complex of quercetin compared to free quercetin. We observed no apparent toxicity to bone marrow of irradiated Swiss mice previously administered hydroxypropyl beta cyclodextrin complex of quercetin for a week. This suggest that hydroxypropyl beta cyclodextrin complex was able to protect bone marrow cells from lethal effect of radiation. When the cytotoxicities of quercetin and its complexes were compared on erythrocytes of rat and rabbits, no significant differences were observed. The ability to selectively target quercetin via its cyclodextrin inclusion complex against cancer growth could improve the therapeutic effectiveness of cyclodextrin preparations as well as reduce adverse side effects associated with quercetin. The new cyclodextrin inclusion complex appears to have high potential for the treatment of leukemias and possibly also for solid tumors.

The solubility of poorly water-soluble drugs is found to be increased by their dispersion in water-soluble carriers. The most commonly used carriers are long chain polymers like polyethylene glycol and polyvinyl pyrrolidine[1]. From the safety point of view naturally occurring polymers such as proteins or polysaccharides may be good and ideal candidates[2]. However, all of these efforts are hampered by the difficulty of targeting the infiltrating cells. The problems of tumour targeting therapy include toxicity to normal organs, limited capacity of the target and failure of the therapeutic agents to reach intracellular sites of action[3]. The search for new carrier molecules that mav transfer the active moiety efficiently, maintaining the selectivity for neoplastic cells is very active[4,5]. In this particular situation the markedly high targeting efficiency, the large capacity of the target and possibly its rapid internationalization make cyclodextrin a very promising vehicle to carry radioisotopes, drugs, toxins or therapeutic agents in the diagnosis and or therapy for various tumours[6,7].

Naturally occurring polyphenolic antioxidants are receiving increasing attention in recent years. Several studies demonstrate, the cancer preventing effects of polyphenolic anti-

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oxidants like flavonoids[8,9]. The therapeutic potential of flavonoids shown by recent research and the presence of these compounds in our diet makes necessary their understanding that is not only leading to new drug discoveries but also influence our drinking and dietary habits. Quercetin, a polyphenolic flavonoid, an antioxidant by nature and extremely hydrophobic, is a component of onion, one of the common dietary items. Quercetin shows several biological effects[10]. *In vitro* it inhibits the growth of several human and animal tumour cell lines[11-13].

Previously we reported the antitumour activity of a β-cyclodextrin inclusion complex of quercetin[14] (QBCD). The β-cyclodextrin inclusion complex did not result in impressive tumour regressions in several animal tumour models, promoting preparation of other inclusion complexes. Hydroxypropyl-β-cyclodextrin (HP-β-CyD) inclusion complex of quercetin (HPQ) is the newly made preparation with unique properties that include good water solubility, nontoxic and possess antiproliferative activity. In this paper, we present our study that evaluated the effect of HP-β-CyD inclusion complex of quercetin on the proliferation rate, apoptosis in K-562 erythroleukemia and B16F10 melanoma cells, as well as toxicity and tumour growth inhibition in animal tumour models.

MATERIALS AND METHODS

Quercetin was purchased from S. D. Fine Chemicals, Mumbai. Hydroxy propyl β-cyclodextrin was obtained from Sigma Chemical Co. St. Louis, MO, USA. All tissue culture biologicals were purchased from Gibco Laboratories, Grand Island, NY, USA. A stock solution of quercetin (200 mg/ml) in dimethyl sulphoxide was prepared and stored at -20°. The tumour cell lines used in these experiments were obtained from NCCS, Pune.

Preparation of inclusion complex:

The preparation of technique was based on the modified procedure described earlier[15]. The flavonoid quercetin and HP-β-CyD were mixed in de-ionized water (1:5 molar ratio). A small quantity of ammonium hydroxide (0.5 ml 35% ammonium hydroxide per liter water) was added to solubilize quercetin. The whole solution was stirred mechanically for 2 h. Care was taken to avoid photodegradation during magnetic stirring. The solution was frozen overnight in a refrigerator below 0° and lyophilized over a period of 30 h using freeze drier. The dried powder was passed through sieve (60 # mesh) and stored in a desiccator until further action.

Determination of content uniformity:

The inclusion complex (HPQ, 50 mg) was weighed accurately and dissolved in 100 ml de-ionized water in a volumetric flask. An aliquot from this solution was further diluted to get a 10 μg/ml solution. The absorbance was measured spectrophotometrically at 355 nm and the concentration was estimated using the standard curve obtained earlier. The estimation of quercetin was done in triplicate.

Cell lines:

The K-562 erythroleukemic and B16F10 melanoma cell lines were grown in IMDM medium supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (100 units/ml potassium penicillin GI, 50 μg/ml streptomycin sulphate). Cells were grown at 37° in a water-jacket incubator in a humidified 5% CO_2 atmosphere. In all experiments described below the B16F10 melanoma cells were incubated for 24 h to allow cell attachment before adding the inclusion complex.

Cell proliferation assay:

HPQ was dissolved in phosphate buffered saline (PBS) at a concentration of 10 mg/ml. K-562 and B16F10 cells were exposed to different concentrations (1, 5, 10, 25, 50, 100, 200 μg/ml) of HPQ for various time intervals. Viable cells were counted in a hemocytometer by trypan blue (0.2%) dye exclusion technique. The survival fraction in treated cultures was expressed as percentage of controls in which an equal volume of PBS was added.

DNA synthesis inhibition:

For the determination of DNA synthesis, the cells (10^6 cells/ml) were grown in 35 mm petridishes and treated with HPQ for 72 h at 37°. The cells were pulse labeled with 0.5 μCi/ml [^{3}H] thymidine (Specific activity: 1 mCi/ml) for 4 h, washed with ice-cold PBS and ice-cold 6% trichloroacetic acid to remove unincorporated acid-soluble lable and lysed with 0.1 N NaOH (1 ml) overnight at room temperature. The 0.5 ml of the lysate was mixed with 4 ml scintillation cocktail and counted in liquid scintillation counter.

Morphological assessment:

Aliquot of cell suspensions were taken onto microscope slides and cytospined. Cells were fixed with ice-cold anhydrous methanol for 6 min at -20°, fixed cells were then stained with Wright-Giernsa dye and examined under a light microscope. Cells were also fixed with 1% formaldehyde (metha-

nol free) in PBS for 15 min at 4° and permeabilized with 70% cold ethanol for a minimum of 1 h at -20°. Cells were stained with Hoechst 33258 dye in PBS (10 μg/ml) followed by examination by fluorescence microscopy.

DNA gel electrophoresis:

To assess the pattern of DNA cleavage caused by quercetin and HPQ, agarose gel electrophoresis was performed as described[16]. In brief, control and drug treated cells ($2x10^6$ cells) were lysed with 0.5 ml lysis buffer. The DNA was dissolved in TE buffer. Ten units of Rnase was added to the samples. The samples were subjected to electrophoresis on a 1.2% agarose gel in TBE buffer at 50 V for 3 h. The resulting DNA fragmentation pattern was visualized on an UV transilluminator.

Tumour growth inhibition experiments:

C3H/J mice were injected with mammary adenocarcinoma tumour cells in 0.2 ml of sterile PBS s.c. ($5x10^7$ cells), and separated into two groups (6 animals/group). When the tumours were approximately 4 mm in diameter mice were treated with three weekly doses of 400 mg/kg HPQ (i.p. on days 1, 5, 9). Tumours were measured thrice weekly after treatment and tumour volumes were calculated as $0.5\ a^2 \times b$, where a and b are minor and major axes of the tumour. Mice were monitored routinely for weight loss and survival times were noted[17].

Bone marrow protection test:

Normal Swiss mice (20 in number) were divided into four groups: Group I- control; Group II - HPQ (400 mg/kg) administered i.p., on days 1-5; Group III - irradiation, 800 rads on day 6; Group IV-HPQ (400 mg/kg) on days 1-5 and irradiation on day 6. Bone marrow was removed from the femur on two different days (day 2 and 4) after whole body irradiation. Bone marrow cell suspension was obtained by flushing femur shafts with Eagle's medium. Bone marrow cells were counted by a hemocytometer.

***In vitro* hemolytic activity:**

Blood samples were withdrawn by cardiac puncture of rat heart. HPQ was incubated at different concentrations (10-40 μg/ml) with freshly collected blood, for 60 min at 37°. Aliquots of the supernatants obtained after centrifugation (1500 g for 30 min) were diluted 1:100 in 0.9% NaCl and the concentration of hemoglobin was determined in a spectrophotometer by calculating the difference between the absorbance at 575 nm and 561 nm. Total hemolysis (100%) was obtained by incubation of erythrocytes in water containing 0.02% Triton X-100 at 1:1 (v/v) ratio.

RESULTS AND DISCUSSION

This is the first ever study done with cyclodextrin inclusion complexes of quercetin. Earlier we reported our findings on QBCD. Since there were no therapeutic gains the QBCD complex was discarded. Even then cyclodextrins are effective carriers of drugs which enabled us to make an additional inclusion complex of quercetin i.e. HPQ.

TABLE 1: GROWTH INHIBITORY EFFECT OF THE INCLUSION COMPLEX ON CANCER CELLS.

Cell line	IC_{50}[a] (μg/ml) Exposure time		
	24h	48h	72h
K-562	42±2.0	38±1.6	28±1.4
B16F10	52±2.9	45±3.2	36±1.7

[a]IC_{50} is the concentration (μg/ml) of compound which caused a 50% decrease in cells in culture. Values were calculated from dose - response curve in triplicate with standard errors < 10%.

Initial studies on K-562 and B16F10 cell lines established its *in vitro* cytotoxicity. IC50 values on these cell lines differ according to the origin and sensitivity of each cell line (Table 1). Thymidine incorporation in both K-562 and B16F10 melanoma cells was decreased in a dose related manner by the addition of HPQ for a 24 h period (fig. 1). On prolonged exposure to the complex the cells entered programmed cell death and died due to the induction of apoptosis (fig. 2). Different sensitivities to HPQ-induced apoptosis have also been demonstrated. HPQ-induced significant apoptosis in K-562 cell line and the response was higher. Therefore, HPQ-induced cytotoxicity in target K-562 and B16F10 tumour cells proceeds via activation of events characteristic of programmed cell death. Cell growth inhibition by HPQ, which was resulted from cell death was characterized morphologically. Morphological apoptosis was observed under light microscope, which was further confirmed by dual fluorescence and electron microscopy. These findings are compatible with evidence implicating DNA fragmentation as was seen in B16F10 cells. A clear DNA fragmentation was observed at 72 h after treatment with HPQ indicating that HPQ induces apoptotic cell death in these cancer cells like quercetin the parent compound. Activation of Ca^{2+}/Mg^{2+} dependent endonuclease appears as the underlying mechanism of the cytotoxic response to HPQ. In K-

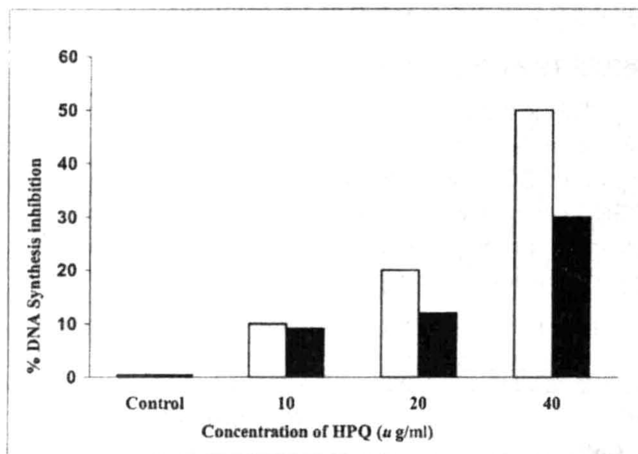

Fig. 1: The effect of HPQ on DNA synthesis of K-562 and B16F10 cells.

K □), B16F10(■) cells were seeded at 1x10⁶ cells/ml in HPQ supplemented medium and incubated for 3 days at 37° in 5% CO_2. DNA synthesis was determined using (^3H) thymidine incorporation assay. The c.p.m. of the control was assigned a value of 100 and any decrease at each of the test concentrations was calculated as a percentage of this value. After 72 h each of the cell lines showed a significant dose related decrease in DNA synthesis.

562 leukemic cells endonuclease activity was found to cause high molecular weight internucleosomal DNA cleavage therefore ladder formation was not observed in this tumour cell line. It is to be noted here that DNA fragmentation is only another hallmark of apoptosis which is considered to be the result of activation of endogenous endonuclease[18].

In the preliminary animal study all the Swiss mice survived after administration of HPQ at a dose of 400 mg/kg i.p. After the administration of HPQ at a dose of 400 mg/kg i.p. there was a gradual increase in body weight during the three weeks of observation (The reported organ toxicity of quercetin or its degradation products to the gastrointestinal tract can contribute to a lower increase in body weight of mice after administration of quercetin). Similarly after administration of HPQ nephrotoxicity associated with the parent compound quercetin was not observed (unpublished data). Thus it was shown that the complex is free of total toxicity and acute nephrotoxicity and that the maximum tolerated dose (MTD) of the complex was found to be more than 400 mg/kg, which is quite high, compared to quercetin (100 mg/kg) and QBCD (50 mg/kg). A purposeful change in the pharmacokinetics of flavonoids by linking them to a drug carrier is one of the possibilities of decreasing the toxicity.

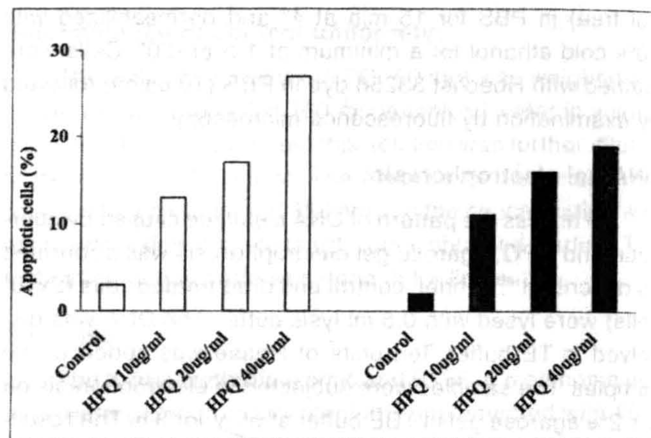

Fig. 2: The percentage of apoptotic cells induced by HPQ in K-562 and B16F10 cells.

K-562(□), B16F10 (■) apoptotic cells were measured by morphological changes in cells exposed to HPQ under a microscope. Around 200 cells showing apoptotic characteristics were scored in each field. Total cells in 10 fields were counted. Results shown reflect the means for three separate experiments.

In relation to toxicological observations, HPQ did not cause significant lysis of rat and rabbit erythrocytes which was found to be less than 20%. For all samples, percent hemolysis <2% indicated lack of hemolytic activity.

At its MTD and following intermittent schedule of administration HPQ exerted moderate antitumour effect in C3H/J mice bearing mammary adenocarcinoma. It is obvious that the antitumour effects of the inclusion complex will be more pronounced if daily treatment schedule is followed than the intermittent schedule of treatment. Our results indicated that inhibition of the growth of mammary adenocarcinoma transplantable tumour by HPQ was slightly more than free quercetin (fig. 3).

Modulation of cellular radiosensitivity or radio-tolerance is important in radiation therapy of cancer[19]. Previous work in this laboratory showed that quercetin, QBCD protect bone marrow from the damages of sublethal irradiation[14]. The results obtained with HPQ are similar (Table 2). Using an adequate concentration of HPQ, radiation induced hematopoietic damage is reduced. However, a more significant bone marrow protection than that of quercetin can be obtained with the inclusion complexes and may actually have an important role as a bone marrow protecting agents of quercetin. The flavonoid being known to be vasoconstrictor creates an hypoxic environment in tissues. Hypoxic cells are

TABLE 2: EFFECT OF INCLUSION COMPLEX TREATMENT ON THE SURVIVAL OF MOUSE BONE MARROW CELLS AFTER IRRADIATION.

Group	Viability [a] (%) (day 2)	Viability [a] (%) (day 4)
HPQ (400 mg/kg)	78.8	81.3
IR (800 rads)	37.5	44.4
HPQ + IR (400 mg/kg + 800 rads)	68.8	71.4

[a]The bone marrow protective activity was determined by measuring the number of bone marrow (BM) cells remaining after treatment, relative to control. The values are the mean of two different experiments.

always resistant to radiation treatment. This explains the radioprotection obtained with flavonoids.

The diverse effects of HPQ viz. the inhibition of cellular proliferation, induction of apoptosis, the inhibition of mammary adenocarcinoma as shown by our studies were contributed partially by the inhibition of the enzyme, protein tyrosine kinase. Clinical application of tyrosine kinase targeted therapy is a new and a growing field[20].

Considering its antiproliferative effect, radioprotection, nontoxic nature and improved drug solubility, it can be suggested that the biodegradable HP-β-CyD complex which was investigated is advantageous. HPQ appears to be more effective than free quercetin and merits further attention.

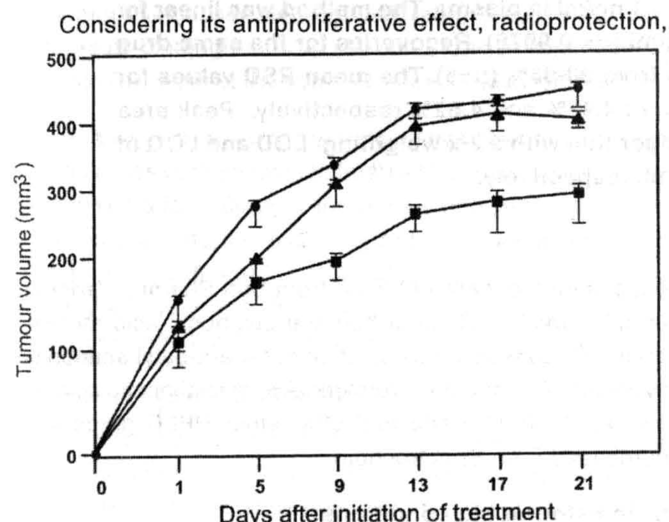

Fig. 3: *In vivo* antitumoral activity of quercetin and its complex on mouse mammary adenocarcinoma.

Tumours were induced in C3H/J mice by s.c. inoculation of adenocarcinoma cells. Q (100 mg/kg) and HPQ (400 mg/kg) were administered i.p. on every fourth day (i.p./ days 1-5-9). Groups of six female mice were used for each experiment. Average tumour volumes of control (●), quercetin (▲) and HPQ (■) are shown. Differences in tumour volumes between control and treated mice were statistically significant at the end of treatment ($p < 0.05$). Bars SD.

REFERENCES

1. Torchilin V.P. and Trubetskoy V.S., **Adv. Drug. Delivery Rev.** 1995, 16, 141.
2. Lynch, W.E., Sartiano, G.P. and Ghaffar, A., **Amer. J. Hematol.**, 1980, 9, 249.
3. Gregoriadis, G., **Nature,** 1977, 265, 407.
4. Gregoriadis, G., **Lancet,** 1981, 2, 241.
5. Gabizon, A.A., **Clin Cancer Res.,** 2001, 7, 223.
6. Tsai, L., Milic, J. and Stupar, M., **Arch. Oncol.,** 1998, 6, 125.
7. Mishra, P.R., Mishra, M., Namdeo, A. and Jain, N.K., **Indian J. Pharm. Sci.,**1999, 61, 93.
8. Kuo, S.M., **Crit. Rev. Oncol.,** 1997, 8, 47.
9. Galvez, Lopez-Lazaro, M., Martin - Cordero C., Toro, M.V. and Ayuso, M.J., **Anticancer Res.,** 2001, 21, 1546.
10. Jiyunm, L., Tai, Z. and Hopkins, S.J., **Drug Future.,** 1997, 22, 720.
11. Indap, M.A., Bhosle, S.C., Miranda, M. and Rao, S.G.A., **Indian Drugs.,** 1998, 35, 128.
12. Kang, T.B. and Liang, N.C., **Biochem. Pharnmacol.,** 1997, 54, 1013.
13. Rong, Y., Yang, E.B., Zhang, K. and Mack, P., **Anticancer Res.,** 2000, 20, 4339.
14. Indap, M.A., Bhosle, S.C., Vavia, P.R. and Tayade, P.T., **Indian Drugs.,** 1998, 35, 545.
15. Masashi, K., Naoki, N. and Nagai, T., **Chem. Pharm. Bull.,** 1975, 23, 3062.
16. Couldwell, W.T., Hinton, D.R., He, S., Chen, T.C., Sebat, I., Weiss, M.H. and Law, R.E., **FEBS Lett.,** 1994, 345, 43.
17. Gerar, R.I., Greenberg, W.H., MacDonald, M.M., Schumache, A.M. and Abbot, B.J., **Cancer Chemother. Rep.,** 1972, 3, 1.
18. Peitsch, M.C., Mannherz, H.G. and Tschopp, J., **Trends Cell Biol.,** 1994, 4, 37.
19. Kalechman, Y., Albeck, M., Oron, M., Sobelman, D., Gurwith, M., Sehgal, S.N. and Sredni, **J. Immunol.,** 1990, 145, 1512.
20. Donato N.J. and Talpaz, M., **Clin. Cancer Res.,** 2000, 6, 2965.