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Effect of the Bioflavonoid Morin on HEp-2 Cells

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Bioflavonoids are plant compounds which aid in disease resistance (Marini-Bettolo and Monache 1977) and attract pollinating insects (Thompson et al. 1972). Flavonoids have been shown to affect biological systems variably, having antibacterial, antiviral (Miski et al. 1983), antifungal (Donnelly 1977), mutagenic (Brown 1980), antineoplastic and/or enzyme inhibitory properties (Pritsos et al. 1985; Bohmont et al. 1987; Hodnick et al. 1986; Duval et al. 1988). Antibacterial activity against *Mycobacterium phlei* was demonstrated in five 5,6,7-trisubstituted falvones which were extracted from *Gompherena martiana* and *G. boliviana* (Pomilio et al 1992). The flavonoid cirsimaritin, which was extracted from the leaves of *Salvia palaestina*, was demonstrated to have high activity against *Staphylococcus aureus*, *S. epidermitis*, *Echerichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (Miski et al. 1983). Antiviral activity was discovered in baicalin, a flavonoid extracted form *Scutellaria baicalensis georgi*, which inhibited human T and B cells via inhibition of the reverse transcriptase of infected cells (Baylor et al. 1992). Further, flavonoid inhibition of reverse transcriptase activity in Moloney murine leukemia infected cells has been reported (Chu et al. 1992). For example , a number of flavonoids were shown to inhibit the activity of succinoxidase (Hodnick et al. 1986) and other flavonoids have been reported to inhibit mitochondrial respiration via inhibition of NADH oxidase, succinoxidase and ATPase (Bohmont et al. 1987). Mitochondrial enzymes are not the only enzymes affected by flavonoids. The flavonoid dichlone interferes with liver glycolysis in rats via inhibition of glutathione reductase (Pristos et al. 1985). Antineoplastic activity has been demonstrated in the cytotoxic flavonoid eupatorin which displayed cytotoxic activity against human carcinoma of the nasopharynx (Kupchan et al. 1979). Three flavonoids with inhibitory activities against Epstein-Barr virus early antigen induction by a tumor promoter have been isolated (Murakami 1992). Further, twelve flavonoids which inhibited antineoplastic activity against adenocarcinoma 755, Friend virus leukemia, Lewis lung carcinoma and Walker carcinosarcoma 256 have been reported (Edwards et al 1979).

Morin [2',3,4',5,7-pentahydroxyflavone or 2-(2,4-dihydroxyphenyl)-3,5,7-

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trihydroxy-4H-1-benzopyran-4-one] is a commercially available, naturally occurring flavonoid. It is the coloring agent of *Chlorophora tinctoria*, old fustic, and *Maclura ponifera*, the osage orange (Windholz 1976). Morin is an inhibitor of NADH oxidase and succinoxidase as well as the ATPase of isolated beef heart mitochondria (Bohmont et al. 1987). It is suggested that the inhibition of mitochondrial enzymes by flavonoids may contribute to their cytotoxic and antineoplastic activities (Hodnick et al. 1986, and Pisani et al. 1986). Since morin affects a number of mitochondrial enzymes, we were interested in its effect on a line of malignant cells. Therefore, the purpose of this study was to determine the effect of morin treatment on the growth of HEp-2 cells and their cytochrome oxidase activity.

METHODS AND MATERIALS

HEp-2 (Human Epidermoid #2) cells were obtained from the American Type Culture Collection (ATCC), type CCL23. This cell line was derived from the primary epidermoid carcinoma of the larynx of a 57 year-old male and was established in culture around 1952 (Toolan 1954). The HEp-2 cell line was probably contaminated with the cells of another line, HeLa, sometime between 1962 and 1966 and as a consequence contain certain HeLa marker chromosomes.

HEp-2 cells were grown in Corning 75 cm^3 tissue culture flasks containing the following medium: 80 ml sterile, double deionized water; 10 ml 10x Minimum Essential Medium (MEM) with Earle's salts (GIBCO); 10 ml newborn calf serum (GIBCO); 1 ml 200 mM L-glutamine (GIBCO); and 5 ml sterile filtered 10% $NaHCO_3$. The cells were maintained at 37°C in a National carbon dioxide incubator which was infused with pure, sterile CO_2 as necessary to maintain culture media pH between 7.4 and 7.8 via a bicarbonate/carbonic acid buffering system. EDTA-trypsin (GIBCO) was used to remove the cells from the flask.

In order to obtain a 1.0 mM concentration of morin, it was necessary to dissolve 31.735 mg morin in 5 ml of 10% $NaHCO_3$ before adding the other medium components. The medium was then vacuum filtered through a 0.2 μ M Nalgene filter for sterilization. Osmolalities and pH of control and experimental media were measured by the Advanced TM Micro-Osmometer Model 3MO and an Orion pH meter respectively.

Growth curves were obtained for cells grown in control medium and for those grown in medium containing 1.0 mM morin. Data was collected daily over four days according to NCI guidelines, the cell culture screening test system for toxicity (Cancer Chemotherapy National Service Center 1972). Growth was measured each day by Coulter counter (cell number), Coomassie G-250 protein assay reagent (Pierce Chemical Co.) using bovine serum albumin as the standard, and viability counts with Trypan Blue vital dye (Patterson 1979). The IC_{50} (the concentration that would inhibit cell growth and reduce the cell number by half on day four) was determined by growing cells in media containing varying concentrations of morin (0-control, 0.011, 0.05, 0.1,

0.5, and 1.0 mM). At the end of four days, growth was measured by the aforementioned parameters.

Homogenates of all HEp-2 cells were prepared by removing the cells from the flasks with control medium. Coulter counts and viability counts were performed before the cells were centrifuged at 1300 X g for 5 min. The supernatant was removed and the pellets were resuspended in cold phosphate buffered saline. Centrifugation and resuspension were repeated twice. The cell suspensions were then homogenized in ice with a Brinkman Polytron Type PT1020 3500 homogenizer at setting 8 for two 3-4 min. intervals at which time the homogenates were observed with an inverted phase microscope. The process was repeated until approximately 90% of the cells appeared to be ruptured. Protein concentrations were quantified as described earlier.

Cytochrome C from horse heart (Sigma Chemical Co. Type VI) was used as the substrate and was prepared as a 4.6×10^{-5} M solution in 0.03 M phosphate buffer. Reduction of the substrate was achieved by the addition of a small amount of sodium dithionite crystals and indicated by the development of a bright pink color. Excess sodium dithionite was removed by passing the solution through a Sephadex column (Einarsdottir et al. 1988).

The activity of cytochrome oxidase was measured by following the oxidation of cytochrome C at 550 nm (Smith 1955) for 5 min. with the kinetics program in a Gilford Response Spectrophotometer. One or two drops of saturated potassium ferricyanide was then added to the reaction cuvette to complete oxidation of the substrate. The optical density was read at 550 nm to obtain the 10^3 extinction coefficient. In order to determine the effect of morin on the cytochrome oxidase activity of cells in culture, HEp-2 cells were exposed to 0.5 mM morin for four days. The treated cells and control cells were removed form the culture flasks, washed with sterile phosphate buffered saline, homogenized and assayed for cytochrome oxidase activity. To determine the effect of morin on the mitochondria after removal of the cell membrane barrier, untreated HEp-2 cells were homogenized and exposed to both 0.1 μm and 0.05 μm morin immediately before assay for cytochrome oxidase activity. All data were statistically analyzed to determine the standard errors based on a t-score and the 95% confidence limits. Sample size for growth and toxicity studies was three. Enzyme assay sample size was four.

RESULTS AND DISCUSSION

The osmolalities of the control and experimental culture media were 360 mOsm/kg H_2O and 352 mOsm/kg H_2O respectively. The pH of the control and experimental media ranged from 7.4 - 7.8 over a four-day incubation period prior to addition of cells and during the growth assays. Morin had no apparent effect upon the pH of the media. Viability counts of HEp-2 cells indicated that a minimum of 89% of the cells were viable for each assay. There was a significant increase in cell growth in the control series by day 4 compared to initial (day 0) cell count protein value (Fig. 1 and 2). There were

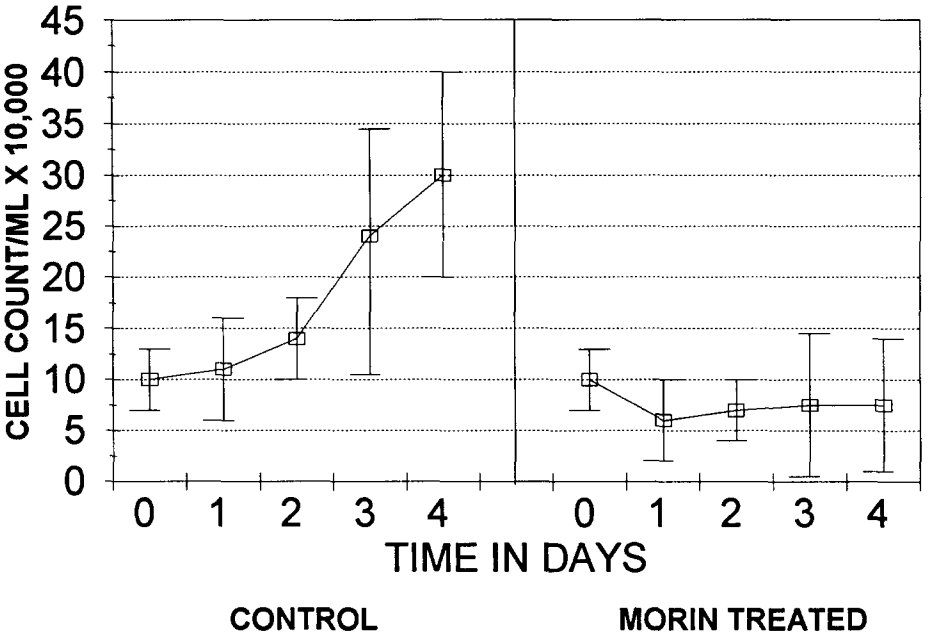

Figure 1. Effect of 1.0 mM morin on the growth of HEp-2 cells as measured by cell number. Values represent the mean +/- 95% confidence limits for three assays.

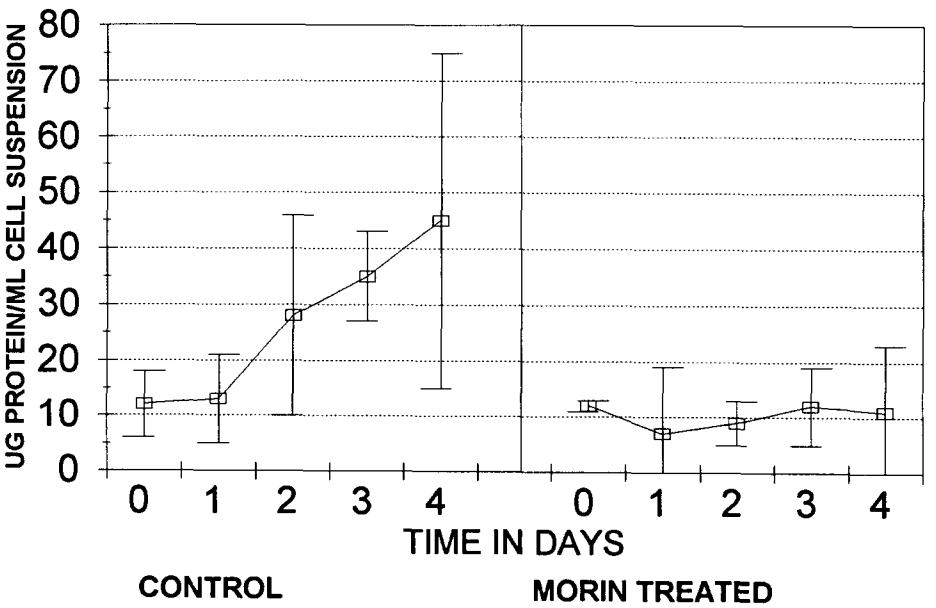

Figure 2. Effect of 1.0 mM morin on the growth of HEp-2 cells as measured by Coomassie protein assay. Values represent the mean +/- 95% confidence limits for three assays.

no significant differences in HEp-2 cell growth between untreated controls and cells treated with 1.0 mM morin on days 1, 2 or 3 as measured by cell count, however, there was a significant difference between the groups on day 4 (Fig. 1). There were no significant differences in HEp-2 cell growth between the untreated and treated groups as measured by protein concentration on days 1, 2 or 4. However, there was a significant difference between the untreated and treated cells on day 3 (Fig. 2). The day 4 IC_{50} for morin was 0.5 mM. The mean number of HEp-2 cells exposed to 0.5 mM morin was reduced from 3.0 x 10^5/ml cells to 1.5 x 10^5/ml cells by day 4. The mean protein concentration was reduced from 55 µg/ml to 29 µg/ml by day 4.

The cytochrome oxidase studies on the HEp-2 cells treated with morin in culture revealed no significant differences between the activity of untreated cells and cells treated with 0.5 mM morin (Table 1). In the cytochrome oxidase studies on untreated cells which were homogenized and then assayed for cytochrome oxidase activity, there was a significant difference between the activity of untreated HEp-2 cell homogenates and homogenates treated with 0.1 and 0.05 µm morin (Table 1).

Table 1. Cytochrome oxidase activity in intact and ruptured HEp-2 cells treated with morin. Values expressed as the mean specific activity +/- 95% confidence limits (n=4).

Treatment	**Mean**	**95% Confidence Limits**
Intact		
Control	1.33	0.72-1.95
Morin (0.5 mM)	1.37	0.74-2.00
Ruptured		
Control	1.96	1.33-2.58
Morin (0.05 µM)	0.85*	0.54-1.15
(0.1 µM)	0.67**	0.52-0.82

Significant difference at the 0.05* or 0.01** level of confidence

Cell number and protein concentration were equally useful as measurements of growth, producing curves with similar patterns, and indicating inhibition by morin. Growth patterns varied between treated and untreated groups. The untreated cells displayed a growth pattern similar to all untreated groups: a lag phase with a small increase in cell number (1.7% average) and protein concentration (1.5% average) at day 1; a sharp increase occurred on days 2 and 3 in cell number (53.6% average) and protein concentration (69.9% average); and a smaller increase in cell number (27.6% average) and protein concentration (25.0% average) by day 4 which denoted the commencing of a planer phase with no increase in cell number or protein concentration. The treated cells, however, showed a slight drop in cell number (28.7% average) and protein concentration (24.0% average) on day 1 with a return to day 0 values for the remaining days. These values clearly

indicate an initial cytotoxic effect on HEp-2 cells by morin with a static effect on growth.

The IC_{50} of morin for HEp-2 cells indicates that the line is able to withstand relatively high concentrations of this compound. This cell line is characteristically resistant to changes in temperature, medium and pH. Initially, we encountered a problem of solubility of morin in the reaction medium which was solved by increasing $NaHCO_3$ from 0.2% to 0.5%. The change in $NaHCO_3$ concentration had no effect on cell growth, further indicating the ability of this cell line to resist changes in medium.

As stated earlier, the inhibition of mitochondrial enzymes by flavonoids is conjectured to contribute to their cytotoxic and antineoplastic activities (Hodnick et al. 1986). Morin is known to affect mitochondrial enzymes including the cyotchrome oxidase of HEp-2 cells. It would appear that morin would be quite cytotoxic to the HEp-2 cells; however, we have found that the IC_{50} for HEp-2 is quite high. We speculate that either morin never reached the mitochondria of the intact HEp-2 cells or that only very small concentrations of intact morin reached it and that these had very little effect on the organelle. It is possible that the morin was metabolized to non-cytotoxic metabolites via microsomal enzymes before reaching the mitochondria.

It is also possible that the cell membrane acts as a barrier between the morin and the mitochondria. Our studies reveal that the cyotchrome oxidase activity was significantly affected in cells ruptured before exposure to morin compared to cells which had been cultured with morin. There was no inhibition of cytochrome oxidase activity in HEp-2 cells following treatment in culture for 4 days at 0.5 mM morin. When non-treated cells were ruptured, a 43% inhibition in cytochrome oxidase activity occurred in the ruptured cells treated with 0.5 μm morin compared to untreated ruptured cells. This suggests that the intact cell membrane provides a least a 120,000 fold resistance to morin.

We have considered the possibility that HEp-2 cells can metabolize morin via microsomal enzymes. Further studies into morin and its effect on the HEp-2 cell line will need to include studies of the effect of HEp-2 microsomal enzymes on morin. Although we have not investigated this system with regard to morin, because of the difference in enzyme activity between intact cells cultured with morin and ruptured cells treated with morin, we believe the cell membrane is a major factor contributing to the high IC_{50} in this cell line. This study suggests that morin could be used as a growth inhibitory agent on HEp-2 cells in studies where avoidance of extreme cytotoxicity is desirable. The use of concentrations of morin lower than 0.5 mM would slow growth without totally jeopardizing the cell population.

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