Ruta 6 selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: A novel treatment for human brain cancer

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Abstract. Although conventional chemotherapies are used to treat patients with malignancies, damage to normal cells is problematic. Blood-forming bone marrow cells are the most adversely affected. It is therefore necessary to find alternative agents that can kill cancer cells but have minimal effects on normal cells. We investigated the brain cancer cell-killing activity of a homeopathic medicine, Ruta, isolated from a plant, *Ruta graveolens*. We treated human brain cancer and HL-60 leukemia cells, normal B-lymphoid cells, and murine melanoma cells *in vitro* with different concentrations of Ruta in combination with $Ca_3(PO_4)_2$. Fifteen patients diagnosed with intracranial tumors were treated with Ruta 6 and $Ca_3(PO_4)_2$. Of these 15 patients, 6 of the 7 glioma patients showed complete regression of tumors. Normal human blood lymphocytes, B-lymphoid cells, and brain cancer cells treated with Ruta *in vitro* were examined for telomere dynamics, mitotic catastrophe, and apoptosis to understand the possible mechanism of cell-killing, using conventional and molecular cytogenetic techniques. Both *in vivo* and *in vitro* results showed induction of survival-signaling pathways in normal lymphocytes and induction of death-signaling pathways in brain cancer cells. Cancer cell death was initiated by telomere erosion and completed through mitotic catastrophe events. We propose that Ruta in combination with $Ca_3(PO_4)_2$ could be used for effective treatment of brain cancers, particularly glioma.

Introduction

The many modalities of cancer treatments, including surgery, chemotherapy, radiotherapy, immunotherapy, and gene therapy, are all directed towards killing tumor cells or preventing cell proliferation. Although conventional chemotherapies have traditionally been used to treat patients with various types of cancer, their side effects and damage to normal cells have been of monumental concern. Blood-forming bone marrow cells are the first cells to be adversely affected by chemotherapy, leading to a decline in the number of peripheral blood cells. It is therefore highly desirable to search for alternative chemical agents that can effectively destroy cancer cells but have minimal or no side effects on normal cells.

Extracts of the perennial plant *Ruta graveolens* Linn (family-Rutaceae) have been used in traditional homeopathy (1). Constituents of the plant include volatile oils, coumarin, yellow glucosid, alkaloids, and Rutin. Rutin ($C_{27}H_{30}O_{16}\cdot 3H_2O$), the main active compound (Fig. 1), and its glycone, first isolated from the leaves of *R. graveolens*, are well known protectors against nuclear exposures and capillary bleedings (2,3). Rutin is commonly used in the treatment of bone injuries, bacterial infection, poor eye-sight, gout, rheumatism, and hysteria. An extract from *R. graveolens* has also shown mutagenic activity when tested in *Salmonella* (4). Medicine in ancient Greece and Rome also employed it as an abortifacient (5). Laboratory studies in adult albino mice have shown the protection Ruta provides against the clastogenic effects induced by X-radiation (6). Ruta 6 (10^{-12} concentration), which is a diluted potency of the mother tincture (Ruta Q), a plant extract homeopathic drug (see Materials and methods), has also been effective in the treatment of cysticercosis (7). In addition Ruta 6, in combination with calcium phosphate [$(Ca_3PO_4)_2$] 3x(10^{-3} concentration), has shown potent antitumor activity in patients with brain cancer (present data). Although the molecular mechanisms and/or the targets by which Ruta 6 produces its biological effects remain unknown, it effectively kills the cancer cells, especially human brain cancer cells, protects B-lymphoid cells from hydrogen peroxide (H_2O_2)-induced damage, and shows mitogenic effects on normal peripheral blood lymphocytes (PBLs) in culture (present data).

Telomeres, which are repeated DNA sequences (T2AG3)n present at both ends of chromosomes, act as 'guardians' of the genome (8). Telomere sequences also serve as survival factors in human and murine solid tumors of various histopathologic origins by amplifying telomeric DNA (9). On the other hand, telomere erosion induced by chemotherapeutic

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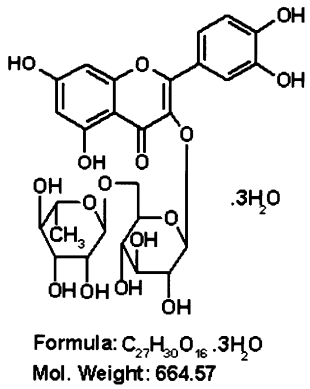

Figure 1. Chemical structure of Rutin, the active compound in *Ruta graveolens*.

drugs and different plant and animal products, or even present in spontaneously regressing swine melanomas, has been shown to cause mitotic catastrophe and induction of apoptosis (8,10-13).

Two of us (P.B. and P.B.) have used Ruta 6 and $Ca_3(PO_4)_2$ combination therapy to treat 15 patients diagnosed with advanced intracranial malignant brain cancer at the PBH Research Foundation, Kolkata, India. The other two authors (S.P. and A.S.M.) have performed *in vitro* experiments to study the effects of Ruta 6 and $Ca_3(PO_4)_2$ on human and murine cancer cells and normal human peripheral blood lymphocytes at The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA. The purpose of our *in vivo* and *in vitro* studies was threefold: a) to demonstrate that Ruta 6 + $Ca_3(PO_4)_2$ combination therapy can eliminate intracranial cancer cells, by either inducing cell death or preventing further proliferation; b) to explore the molecular mechanism of cell death by Ruta 6 + $Ca_3(PO_4)_2$ treatment of brain cancer cells *in vitro*; and c) to demonstrate the protective effects, if any, on normal human peripheral blood lymphocytes in culture. Our *in vivo* results show successful elimination of brain cancer cells from patients who received Ruta 6 and $Ca_3(PO_4)_2$ combination therapy for advanced disease. Induction of cancer cell death *in vitro* was via telomere erosion. The protection of normal lymphocytes in cell cultures was by induction of mitogenic activity.

Materials and methods

Preparation of Ruta. The alcohol extract of the plant *Ruta graveolens*, Ruta 6 (10^{-12} concentration), prepared from the mother tincture Ruta Q as described below, and the *Calcarea phosphorica* (calcium phosphate) 3x (10^{-3} concentration) that was prescribed to the brain cancer patients for oral consumption and used in all *in vitro* experiments were obtained from the Holistic Remedies Pvt. Ltd, Mumbai, India (in collaboration with Bioforce A.G. Switzerland). Ruta Q, the mother tincture extracted from *R. graveolens* according to homeopathic pharmacopia, was diluted to Ruta 1 by adding 1 ml of Ruta Q to 99 ml of absolute ethyl alcohol. One milliliter of Ruta 1 when added to 99 ml of alcohol made Ruta 2. Similarly, Ruta 6 was prepared by performing more serial dilutions.

To treat the various cell lines, we prepared the doses of Ruta as follows: a) Ruta 6: 70 ml of Ruta 6 was evaporated in a Petri dish in an incubator at 37˚C to approximately 100 µl, and 10 ml of RPMI medium was added to this. The plate was further incubated at 37˚C to evaporate the remaining alcohol. Low dose, 2 ml of the above medium containing Ruta 6 + 35 mg of $Ca_3(PO_4)_2$ was used to treat cells in 10 ml of medium. High dose, 3 ml from the above medium containing Ruta 6 + 35 mg of $Ca_3(PO_4)_2$ was used to treat cells in 10 ml of medium; b) Ruta 1: 20 ml of Ruta 1 was evaporated to approximately 100 µl, and 2 ml of RPMI medium was added to this. The plate was further incubated to evaporate the remaining alcohol. Of this medium 1 ml containing Ruta 1 + 35 mg of $Ca_3(PO_4)_2$ was used to treat cells in 10 ml of medium; c) Ruta Q: 2 ml of Ruta Q was evaporated as described, and 2 ml of medium was added to this. Of this medium 1 ml containing Ruta Q + 35 mg of $Ca_3(PO_4)_2$ was used to treat cells in 10 ml of medium.

The dosage of Ruta 6 prescribed for our patients was two drops (about 100 µl) in a teaspoonful (about 5 ml) of drinking water taken orally twice a day. The usual dose of $Ca_3(PO_4)_2$ prescribed was 5 grains (~0.324 g) taken orally twice a day.

Clinical features of patients with intracranial brain cancers. The 15 patients (9 male, 6 female) with intracranial brain cancers who were treated with Ruta 6 + $Ca_3(PO_4)_2$ at the PBH Research Foundation, Kolkata, India, had been diagnosed with glioma (9 patients), meningioma (3 patients), craniopharyngioma (1 patient), neurinoma (1 patient), and pituitary tumors (1 patient). Diagnoses were based on radiology and/or histopathology. Most of these cases were at the advanced stage of the disease when homeopathic treatment was started in Kolkata, India. The patients gradually improved, as indicated by serial computed tomography scans and clinical examinations. The major complaints before treatment were headache, problem with vision, paralysis, convulsive seizures, vomiting, trembling of extremities, loss of memory, numbness, insomnia, and loss of taste. The age range was from 10 to 65 years, and the time required for cure/symptom-free state/static condition was 3 months to 7 years.

Cell lines used. The human malignant glioma cell line MGR1 (kindly provided by Dr F. Ali-Osman), the human promyelomonocytic leukemia cell line HL-60, the murine metastatic melanoma K1735 clone X-21 (kindly provided by Dr I.J. Fidler), two normal human B-lymphoid cell lines (2164P and 3590P), and two normal peripheral blood samples (from a healthy male donor and a healthy female donor) were used in these studies. Approximately 3-5x10^6 cells from each of these lines were seeded in T-75 plastic culture flasks in 10 ml of RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco BRL, New York, NY) and incubated at 37˚C in an atmosphere of 5% CO_2 and 95% air. Whole blood (1 ml) was cultured in 9 ml of RPMI-1640 medium, with or without phytohemagglutinin (PHA), and Ruta 6 and $Ca_3(PO_4)_2$ for 72 h at 37˚C.

Treatment of normal B-lymphoid and brain cancer MGR1 cells with Ruta and hydrogen peroxide (H_2O_2). To examine whether Ruta induced synergistic cytotoxicity in MGR1 brain cancer and protection of normal cells exposed to H_2O_2, cells from both lines were treated with various doses of Ruta

alone (Ruta 6-low and high dose, Ruta 1, and Ruta Q), H_2O_2 alone (0.2 μM), or a combination of Ruta and H_2O_2 for 24 h. Hydrogen peroxide, diluted with sterile distilled water, was used as a potent clastogen to treat brain cancer and normal human B-lymphoid cells. Control cultures received no drug or H_2O_2. The cultures were harvested as described later.

Pretreatment of peripheral blood lymphocytes (PBLs) with Ruta. To examine whether Ruta acts as a mitogen and a non-clastogen in normal cells, PBL cultures from two normal healthy donors were set up in RPMI-1640 medium supplemented with 10% fetal bovine serum. The first culture tube received the usual concentration of PHA (~1 mg/10 ml). The second tube did not receive any PHA. The third, fourth, and fifth tubes received doses of Ruta 6-low, Ruta 6-high, and Ruta Q. The sixth tube received PHA plus Ruta 6-high dose, all added at the time of culture initiation. All the cultures were incubated at 37˚C, and the cells were harvested after 72 h following the standard air-drying techniques.

Cell harvesting and cytological preparations. All drug-treated and control MGR1 cell cultures, B-lymphoid and PBL cultures were treated with colcemid (0.04 μg/ml) for 45 min at 37˚C and then processed for chromosomal preparations (14). All air-dried slides were coded and then stained in Giemsa for the evaluation of mitotic index; frequency of normal, tetraploid and endoreduplicated cells; and for any other obvious mitotic catastrophes, including chromosome- and chromatid-type abnormalities.

Quantitative fluorescence in situ hybridization (Q-FISH). The coded slides were processed for Q-FISH analysis using the Cy 3-labeled peptide nucleic acid (PNA) telomere probe obtained from Dako Corporation (Carpinteria, CA) following the manufacturer's protocol. The slides were examined using a Nikon photomicroscope equipped with a cooled charged-coupled device (CCD) camera. The telomeric signals in interphase nuclei (100-200 from each sample) were quantified by using a Metaview Imaging System software version 3.6a (Universal Imaging Co., Westchester, PA). The percent telomeric area with respect to nuclear area was measured in pixels for mean and median amounts of telomeric DNA present in each sample.

Determination of subdiploid population by the FACS analysis. Control and drug-treated normal B-lymphoid and MGR1 cancer cells were washed with cold phosphate-buffered saline (PBS). Approximately $1x10^6$ cells from each set of experiments were resuspended in 0.5 ml of a propidium iodide (PI) solution (50 μg/ml PI, 0.1% Triton X-100, and 0.1 sodium citrate in PBS). These cells were incubated in PI solution at 4˚C in the dark for 24 h and then the fluorescence was read on the Coulter Epics (R) XL cell counter (Beckman Coulter, Brea, CA). The percentage of cells with hypodiploid DNA content was calculated using the multi-graph program.

Results

Outcome of brain cancer patients treated with Ruta 6 + $Ca_3(PO_4)_2$. The combination therapy of Ruta 6 and $Ca_3(PO_4)_2$ was very effective in the treatment of intracranial brain cancers. Of the 9 patients with glioma, 8 (88.9%) showed complete regression, and the other patient showed partial regression. Two of the three patients with meningioma showed prolonged arrest of their tumors and the third had complete regression. The one patient with craniopharyngioma and the one patient with pituitary tumors both showed complete regression, and the 1 patient with neurinoma has had prolonged arrest of her tumor as determined by computed tomographic scan images (data not shown).

In our *in vitro* experiments, we studied whether Ruta 6 + $Ca_3(PO_4)_2$ could induce cell death in human (HL-60 and MGR1 glioma) and murine (K 1735 clone X-21) cancer cells and provide chemo-protection for normal human PBLs and B-lymphoid cells, by inducing mitotic catastrophe and erosion of telomeric DNA selectively in the cancer cells and by inducing cell proliferation in the normal cells. Although these cancer cells showed different degrees of sensitivity to Ruta treatment *in vitro*, the bulk of the data presented here will be on the human MGR1 glioma cells. Of the two human B-lymphoid cell lines established from two normal individuals (one male, one female) and used in the Ruta treatment experiments, data on only one cell line will be presented. The PBL cultures from the two normal healthy volunteers (one male, another female) showed induction of mitosis with normal chromosome morphology when PHA was replaced by Ruta in their blood culture medium.

Ruta 6 + $Ca_3(PO_4)_2$ induces mitotic catastrophe in cancer cells. Human MGR1 glioma cancer cells were treated with different concentrations of Ruta + $Ca_3(PO_4)_2$ (Ruta 6, 1, and Q) for 24 h at 37˚C, and cytological preparations were studied for mitotic catastrophes. We evaluated various mitotic catastrophes, including the frequency of metaphases with aberrations (chromatid- and chromosome-types, fragments, pulverization and telomeric associations), mitotic index (MI), endoreduplication, and tetraploidy. Fig. 2 contains metaphase spreads from control and Ruta 6-treated brain cancer cells. It shows normal chromosome morphology (Fig. 2A), select endoreduplicated chromosomes with telomeric associations (TAs), chromatid- and chromosome-type aberrations (Fig. 2B), and an endoreduplicated metaphase with severe chromosome fragmentation (Fig. 2C). Metaphases with the configurations shown in Fig. 2B and C were not observed in control MGR1 cells. In 24 h-treated cells (Ruta 6-high), 64.3% of the metaphases were abnormal as compared with only 8% in control cells (Table I). There was a dose-dependent increase in the number of metaphases with chromosome aberrations. A similar result was obtained with the K 1735 clone X-21 murine melanoma cells in which the control cells showed TA in 2.4% of metaphases. However, 24.4% of the metaphases in treated cells showed TAs with dicentric morphology and acentric fragments. Most of these abnormalities were present in either endoreduplicated or tetraploid cells, but they were rarely present in a metaphase spread with one stem line chromosome number (as shown for MGR1 in Fig. 2A).

Ruta in combination with H_2O_2 induces synergistic effects on MGR1 glioma cancer cells. Human MGR1 glioma cancer cells were plated (~2 million per flask) in four T-75 culture

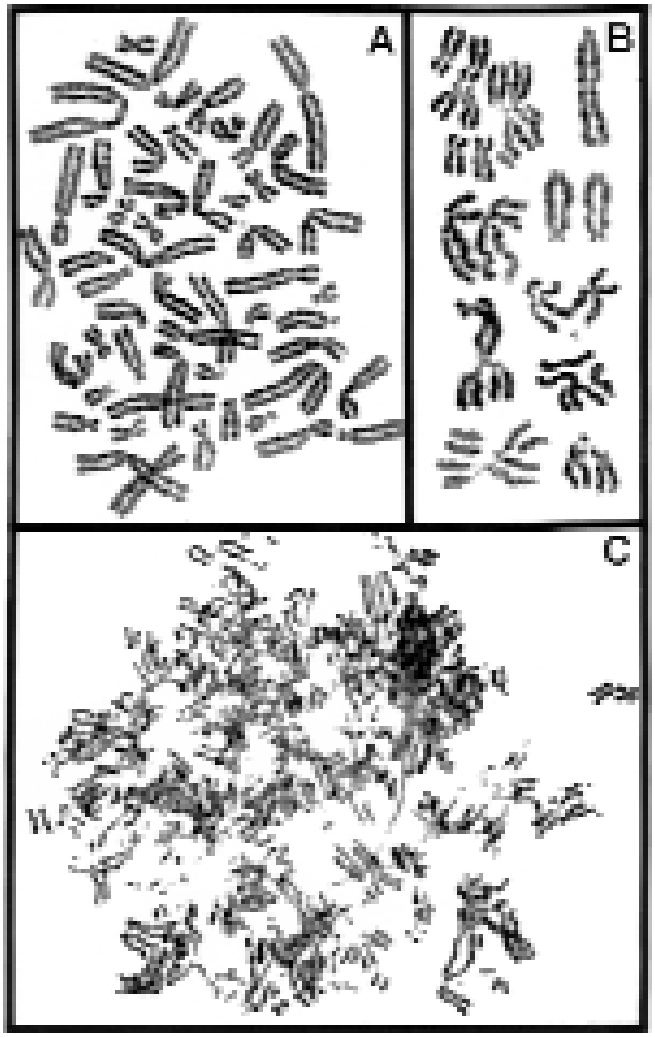

Figure 2. Metaphases from control and Ruta 6-treated MGR1 human brain cancer cells showing mitotic catastrophe: A, normal metaphase spread from a control culture; B, endoreduplicated partial metaphase spread showing dicentrics, chromatid breaks, and tri-radial configurations; and C, an endo-reduplicated metaphase with extensive chromosome fragmentations from Ruta-treated cultures.

Table I. Frequency of normal and abnormal metaphases in human MGR1 brain cancer cells treated with Ruta + $Ca_3(PO_4)_2$ for 24 h.

Experiment no.	Dose	Mitotic index (%)	% Normal metaphases		% Metaphases with aberrations
			1S	2S	
SP4262	Control	15.8	90.0	2.0	8.0
SP4363	Ruta 6 (low dose)	10.3	42.8	7.6	49.5
SP4364	Ruta 6 (high dose)	9.6	30.7	4.9	64.3
SP4267	Ruta 1	12.2	22.8	1.3	75.9
SP4293	Ruta Q	0.9	0.0	0.0	100.0

Note: The description of doses is given in Materials and methods section.

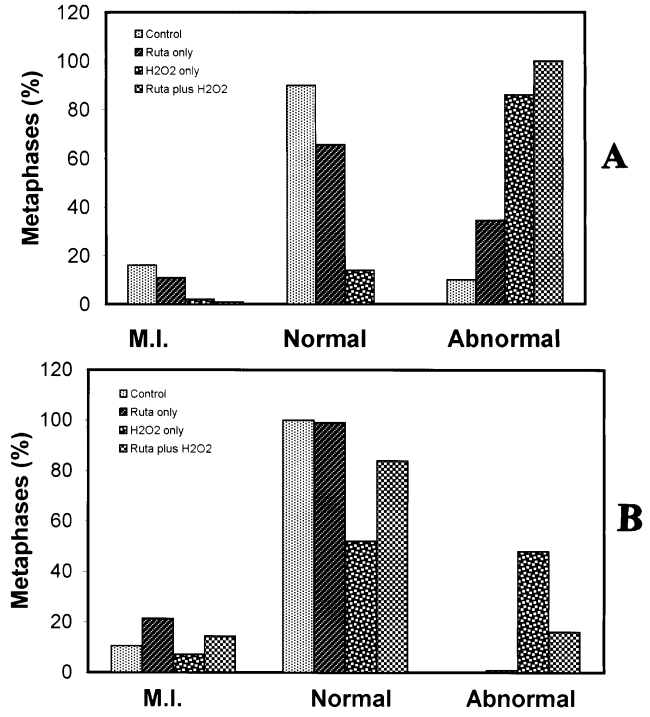

Figure 3. Histograms showing percentages of mitotic index (MI) and normal and abnormal metaphases of human brain cancer and B-lymphoid cells treated for 24 h with Ruta 6-high dose only, H_2O_2 only and in combination: A, human MGR1 brain cancer cells showing higher percentages of abnormal metaphases in H_2O_2- and Ruta 6-treated cells; B, normal human B-lymphoid cells showing more normal metaphases in Ruta-treated cultures and protection by Ruta 6 against H_2O_2.

flasks and were allowed to attach. Of these, the control flask received no treatment. The second flask was treated with Ruta 6-high. The third flask was treated with H_2O_2 (0.2 μM) alone. The fourth flask was treated with Ruta 6-high and H_2O_2 (0.2 μM) together. All treatments in this set of experiments were performed for 24 h.

Following treatment, the MI and percent of metaphases with normal and abnormal chromosome morphology were scored under an oil immersion objective lens. H_2O_2 induced clean chromatid- and chromosome-type aberrations in the brain cancer cells (data not shown). The percentages of metaphases with normal and abnormal spreads are shown in Fig. 3A. In the cells treated with Ruta 6 and H_2O_2 in combination, 100% of the metaphase spreads showed structural abnormalities. In more than 100 metaphases examined from 3 to 4 slides, not a single spread showed normal chromosome morphology. Cells treated with H_2O_2 alone showed more chromosome aberrations than did metaphases of the cells treated with Ruta alone (Fig. 3A). Cells treated with the combination of Ruta 6 + H_2O_2 showed a significantly higher percentage of metaphases with aberrations than for any other treatment. The bulk of these aberrations were chromatid-type breaks and TAs because of the loss of telomeric DNA. Mitotic indices were highest in the control and lowest in the combination-treated cells (Fig. 3A). From these experimental results, it appears that Ruta 6 provides no protection from H_2O_2-induced damage in MGR1 glioma cancer cells. Rather, it has synergistic damaging effects on MGR1 cancer cells.

Table II. Frequency of metaphases with aberrations in a B-lymphoid cell line treated in medium either with or without Ruta + $Ca_3(PO_4)_2$ and H_2O_2 for 24 h.

Experiment no.	Dose	% Normal metaphases		% Metaphases with aberrations
		1S	2S	
SP4338	Control	92.0	8.0	0.0
SP4341	Ruta 6 (high dose)	91.4	3.8	4.8
SP4345	0.2 μM H_2O_2	46.0	6.0	48.0
SP4342	Ruta 6 + 0.2 μM H_2O_2 (high dose)	79.0	2.0	9.0
SP4343	Ruta 1	91.2	4.9	3.9
SP4344	Ruta 1+ 0.2 μM H_2O_2	87.4	3.9	8.7

Table III. Induction of mitoses in two peripheral blood samples incubated in medium either with or without Ruta [Ruta 6 + $Ca_3(PO_4)_2$] and PHA for 72 h.

Treatments	Mitosis	Chromosome abnormality
RPMI-1640 + PHA	+	-
RPMI-1640 only	-	-
RPMI-1640 + Ruta 6 (high dose)	+	-
RPMI-1640 + PHA + Ruta 6 (high dose)	+	-

+, Metaphases present; -, Metaphases and chromosome abnormalities absent.

Ruta protects human B-lymphoid cells against H_2O_2-induced chromosome damage. Eight culture flasks were set up (~5 million cells/flask) using a B-lymphoid cell line derived from a normal healthy individual. Three cultures were exposed to Ruta alone (Ruta 6-low, Ruta 6-high and Ruta 1, respectively), the fourth to H_2O_2 (0.2 μM) alone, and the fifth, sixth, and seventh to a combination of Ruta (Ruta 6-low, Ruta 6-high, and Ruta 1, respectively) and H_2O_2; the eighth flask was used as a control. As with the MGR1 glioma cancer experiments, B-lymphoid cells were also evaluated for MI and the percentage of normal and abnormal metaphases in each set of experiments. As shown in Fig. 3B, the MI value was elevated in cells treated with Ruta alone compared with the control value. The mitotic catastrophe value, if any, was almost similar in the control and Ruta only-treated B-lymphoid cells. There were, however, no metaphases with chromosome aberrations in cells treated with Ruta alone. The B-lymphoid cells treated with combined Ruta 6 + H_2O_2 and cells receiving only H_2O_2 showed a significant difference in the frequency of metaphases with aberrations. A reduction of >50% of metaphases with aberrations in the cells receiving the combination treatment indicated a protection from H_2O_2 insults to B-lymphoid cells by Ruta treatment (Table II). These results indicate that instead of inducing aberrations in B-lymphoid cells, Ruta stimulates mitosis and also protects the cells from H_2O_2-induced damage.

Ruta induces mitogenic activity in normal human blood lymphocytes. As shown in Table III, PHA alone and the combination of Ruta 6 and PHA stimulated cell division in both samples of normal human blood lymphocytes, which showed mitotic spreads as expected. The cultures that received neither PHA nor Ruta 6 did not show any metaphase spreads. However, the cell cultures exposed to Ruta 6 alone showed metaphases in both peripheral blood samples, although reduced in frequency as compared with PHA-stimulated cultures. All metaphases in Ruta 6 only-stimulated cultures from both donors showed normal chromosome morphology (data not shown). The MI in lymphocytes exposed to a combination of Ruta 6 + PHA was not significantly different from that for the PHA only-treated cell cultures. From these observations, we conclude that Ruta 6 acts as a mitogen for normal human lymphocytes and induces no aberrations in their chromosomes.

Effects of Ruta on telomere dynamics in MGR1 glioma cancer and normal B-lymphoid cells. Human MGR1 glioma and normal B-lymphoid cells exposed to Ruta 6 alone for 24 h showed significantly different values when quantification of telomeric DNA was compared by the Q-FISH technique. As shown in Fig. 4, there was no reduction in telomeric signals in interphase nuclei of the control (Fig. 4A) and Ruta 6-treated B-lymphoid cells (Fig. 4B). However, there was a significant difference in the amount of telomeric DNA in interphase nuclei of the untreated control (Fig. 4C) and Ruta 6-treated (Fig. 4D) human brain cancer cells. Ruta 6-treated brain cancer cells showed a drastic reduction of telomeric DNA as compared with the untreated control. From the Q-FISH results, it appears that Ruta 6 treatment is detrimental to brain cancer cells but not to normal B-lymphoid human cells. The differential loss of telomeric DNA in brain cancer and normal B-lymphoid cells may explain why, in the former, more metaphases showed mitotic catastrophe as compared with an insignificant or no amount of mitotic abnormality in the latter cells.

Determination of subdiploid population by the FACS analysis. To determine whether Ruta treatment induced apoptosis in human brain cancer cells and protected B-lymphoid cells from apoptosis, we subjected MGR1 cancer cells, normal B-lymphoid cells exposed to Ruta 6-high dose, added every day in cultures for 72 h, and untreated control cells to flow cytometry. Fig. 5 shows the representative histograms obtained after 72 h of continuous treatments. MGR1 brain cancer cells treated for 24 and 48 h showed duration-dependent G1 arrest (data not shown). Ruta 6 induced reproducible and significant levels of cell death in brain cancer cells, as reflected by a G1 DNA content of 40.8%

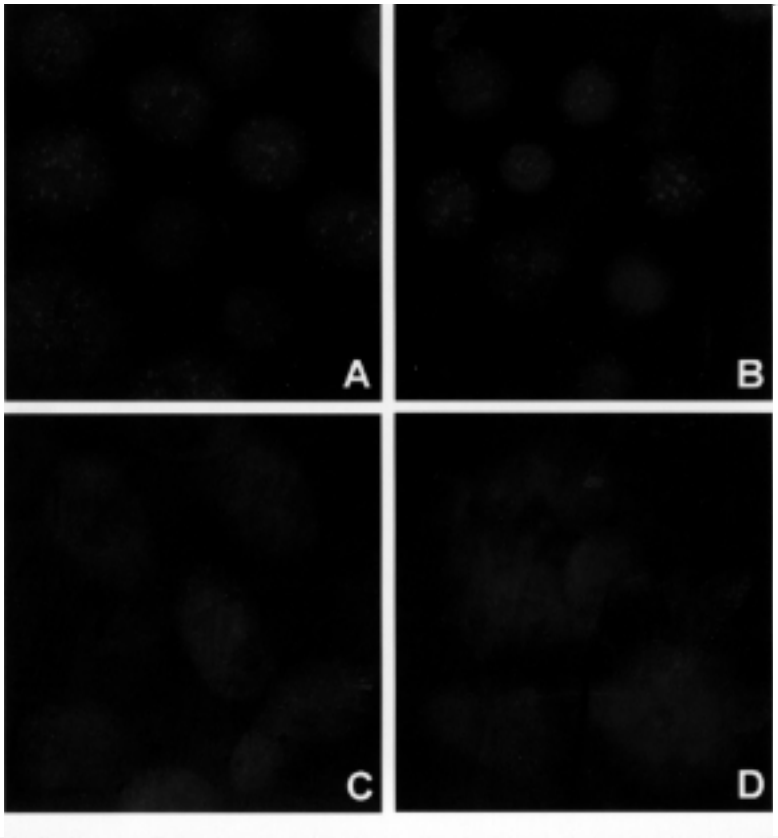

Figure 4. FISH preparations of interphase cells from a human B-lymphoid cell line and MGR1 brain cancer either untreated or treated with Ruta 6 + $Ca_3(PO_4)_2$ are stained with DAPI for DNA (blue), and telomeric DNA labeled with rhodamine (red). B-lymphoid control cells (A) and Ruta 6-treated cells (B) both show no reduction in telomeric signals. Untreated control (C) and Ruta-treated (D) human brain cancer cells show significant difference in telomeric signals. Large nuclei from Ruta-treated cells show reduced telomeric signals. All microphotographs were taken at the same magnification.

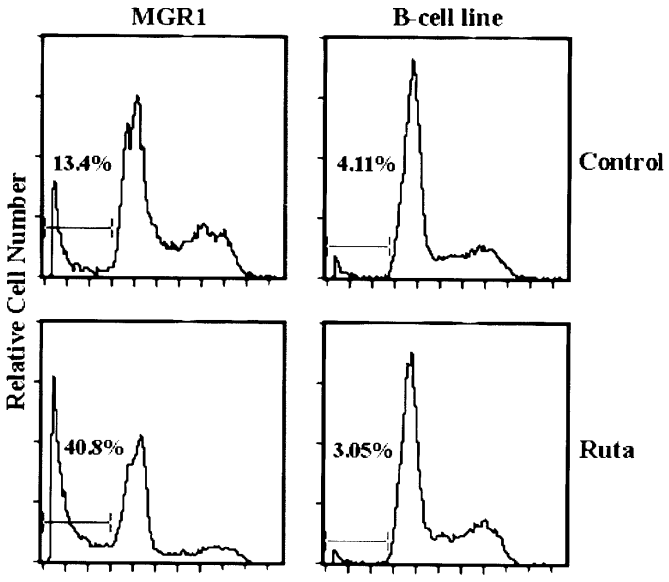

Figure 5. FACS analyses of MGR1 brain cancer cells and normal B-lymphoid cells for apoptosis after treatment with Ruta 6. Both cell types were treated for 72 h with the same dose of Ruta. Treated and control cells of MGR1 and B-lymphoid cultures were harvested and then stained with propidium iodide and subjected to flow cytometric analysis. The proportion of cells with subdiploid DNA content in each treatment is indicated in the histograms. Similar results were obtained in two independent experiments.

cells compared with 13.4% for the control. In contrast, the subdiploid G1 DNA values for B-lymphoid cells differed little between Ruta 6-treated and control cells, with values of 4.11% and 3.05%, respectively. The FACS analysis data correlated well with the results obtained with mitotic catastrophe frequency. These results further imply that Ruta 6 induces death-signaling pathways in human glioma brain cancer cells, both *in vivo* and *in vitro*, and survival-signaling pathways in normal B and T lymphocytes.

Discussion

In the present study, we found that a combination of Ruta 6 and $Ca_3(PO_4)_2$ taken orally can either block the progression of or completely regress human glioma brain cancers, with minimal or no side effects. The patients diagnosed with glioma, when treated with Ruta 6, showed better results compared with patients having other types of intracranial cancers. Although the number of patients in our group was small, the outcome of homeopathic treatment was highly encouraging and novel.

How Ruta inhibits the growth of human glioma brain cancer cells or induces complete regression, is currently not known. To shed light on this phenomenon, we performed a number of *in vitro* experiments using human and murine cancer cells, human normal B-lymphoid cells, and normal

PBLs in culture. Our results indicate the following: a) although Ruta is cytotoxic to human and murine cancer cells, it is more damaging to human glioma brain cancer cells than to HL-60 leukemia cells (data not shown); b) Ruta induces cell division in normal human PBLs when grown in supplemented RPMI-1640 without PHA; c) Ruta does not induce chromosome aberrations in normal B-lymphoid cells or PHA-stimulated T lymphocytes in culture; d) Ruta does not protect human glioma brain cancer cells from genetic damage induced by H_2O_2; e) Ruta protects B-lymphoid cells from H_2O_2-inflicted damage as measured by a reduced number of metaphases with chromosome aberrations; f) Ruta induces severe telomere erosion in MGR1 brain cancer cells but has no effect on B-lymphoid cells and normal lymphocytes, as measured by Q-FISH; g) preferential killing of glioma brain cancer cells by Ruta is apparently mediated through the loss of telomeric DNA, followed by the arrest of cells in the G2/M phase, induction of endomitosis and fragmentation of DNA, leading to cell death; h) FACS analysis indicates that Ruta induces cell death in a dose- and duration-dependent manner in human MGR1 brain cancer cells, followed by saturation effects. However, Ruta protects B-lymphoid cells and PHA-stimulated T lymphocytes, even acting as a mild mitogen in such cultures.

Rutin, the active ingredient of Ruta, is known for its anti-oxidant and anti-inflammatory activities and also for reducing oxidative damage in a rodent model (15,16). In addition, Ruta is also known to protect from DNA strand breaks and to prevent mutagenesis (17,18). $Ca_3(PO_4)_2$ was added in our *in vivo* and *in vitro* experiments because it activates phospholipase, which cleaves phosphalidylinositol biphosphate, a membrane-bound molecule that activates protein kinase C. The cleavage product brought about by phospholipase triggers an influx of calcium ions into the cell, which help transfer the cytoplasmic nuclear factor of activated T cells into the nucleus via calmodulin- and calcineurin-associated enzymes. Calcineurin modulates the induction of tumor necrosis factor α, a potent activator of NF-κB, which ultimately leads cells to apoptosis (19-21) and/or spontaneous regression or prolonged arrest of tumor cells (22). NF-κB is a transcription factor and plays a critical role in the immune system. The other possibility could be that Ruta induces deamidation (removal of an amide group) of the antiapoptotic protein Bcl-x_L in human brain cancer cells but not in normal B and T lymphocytes. Deamidation is known to occur in a regulatory domain of Bcl-x_L which renders inactivation of this protein. This may result in the cancer cells becoming more sensitive to cell death than normal cells (23).

The Ruta 6 and $Ca_3(PO_4)_2$ combination was capable of protecting normal B-lymphoid cells against H_2O_2-induced chromosome damage by reducing the level of damage >50%. However, the combination treatment on MGR1 glioma cancer cells showed synergistic cytotoxic effects with no protection of cancer cells. Even the MI in Ruta-exposed B-lymphoid cells was higher (21.4%) compared with the control (10.4%), showing its mitogenic effect on normal cells. In addition, the MI in H_2O_2 only-treated B-lymphoid cells was 7.1% compared with 14.4% in cells treated with Ruta 6 + H_2O_2. These results strongly suggest that Ruta 6 + $Ca_3(PO_4)_2$ treatment is mitogenic and nonclastogenic in normal cells but antimitotic and apoptogenic in human MGR1 glioma cancer cells.

How glioma brain cancer cells are killed or checked from further proliferation and how normal cells are protected by Ruta is not known. Telomeres, which protect individual chromosomes and the entire genome, are reduced in Ruta 6-treated cancer cells but not in normal B-lymphoid cells (Fig. 4). FACS analysis data of Ruta 6-treated cells showed the accumulation of more subdiploid cells in MGR1 glioma cancer cells (40.8%) than in B-lymphoid cells (3%), suggesting that more brain cancer cells were being killed (Fig. 5). In a series of publications, we have shown that erosion of telomeres is the earliest chromatin event that leads to a cascade of apoptotic machinery in spontaneously regressing swine melanoma and/or drug-induced cell death in cancer cells (8-13,24,25). Irrespective of the as-yet-unknown protective mechanism(s) operating in normal B-lymphoid cells, it is clear from our *in vivo* and *in vitro* observations that this Ruta has the novel property of preferentially killing human glioma brain cancer cells and protecting normal body cells. Overall, our results show that plant-derived Ruta 6 and $Ca_3(PO_4)_2$, when taken orally, can induce regression of human glioma brain cancers *in vivo*. This might be achieved by the induction of telomere loss in cancer cells as shown in our *in vitro* experiments with glioma-derived brain cancer cells. In contrast to conventional chemotherapy that kills not only cancer cells but also normal cells, the Ruta 6 + $Ca_3(PO_4)_2$ combination kills glioma brain cancer cells selectively and protects normal lymphocytes by inducing cell division in blood-forming cells. This homeopathic medicine could be prescribed for optimum treatment of brain cancers in general, and gliomas in particular, as well as possibly reducing severe side effects and protecting blood-forming cells in these patients.

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