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Enhanced Anti-tumour Effects of Acriflavine in Combination with Guanosine in Mice

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Abstract

The anti-tumour activity of acriflavine in combination with guanosine has been evaluated in solid or ascitic tumour-implanted animal models. Guanosine is known to potentiate the anti-tumour effects of some chemotherapeutic agents.

Administration of acriflavine (15 mg kg^{-1} day^{-1}, i.m., 14 days) to ICR mice subcutaneously implanted with Ehrlich carcinoma resulted in ~30% inhibition in tumour growth. In contrast, minor tumour growth inhibition was observed in animals treated with guanosine at the same daily dose. Treatment of animals with both acriflavine and guanosine (AG60, 1 : 1, w/w) at 30 mg kg^{-1} resulted in ~65% inhibition in tumour growth rate. Whereas treatment with acriflavine or guanosine resulted in 70% or 30% decrease in tumour weight, respectively, treatment of tumour-implanted mice with AG60 (30 mg kg^{-1}) resulted in a 96% decrease in tumour weight, relative to control, 14 days after tumour-cell implantation. Dose-related inhibition in tumour growth rate was also observed in animals treated with AG60, with maximum (65%) inhibition noted at a dose of 30 mg kg^{-1} (ED50 23 mg kg^{-1}). Suppression of body weight increase and elevated plasma glucose levels by acriflavine or AG60 indicated that glucose utilization might be impaired. The anti-tumour effect of AG60 was also determined in CDF1 mice intraperitoneally implanted with Ehrlich ascitic tumour. Ehrlich ascitic tumour proliferation was completely suppressed by AG60 (30 mg kg^{-1}, i.p.). Microscopic analyses of intraperitoneal touch-prints revealed that AG60 was more effective in suppressing tumour proliferation than acriflavine alone. Fluorescent microscopic examinations demonstrated that acriflavine avidly bound with Yac-1 cell plasma membrane, leading to morphological changes in the cells, such as bleb formations, swelling and ballooning. The time-related changes in tumour cell morphology by acriflavine or AG60 might represent energy depletion, followed by osmotic lysis as a result of cationic influx. Enhanced anti-tumour activity of acriflavine in combination with guanosine might be explained by the blocking of nutrient transport through selective acriflavine binding with plasma membrane and by concomitant guanosine perturbation of cellular ATP production.

This study demonstrates that guanosine enhances the anti-tumour effects of acriflavine against a variety of cancer cells without serious adverse effects, providing a preclinical basis for potential application of this combination against cancer proliferation.

The development of drugs that target tumour cell membranes might hold promise in inhibiting tumour growth. Blocking the transfer of nutrients, oxygen transport and signal transduction would be a promising way of inhibiting tumour growth and suppressing metastatic activity by inhibiting platelet aggregation of metastatic cells and reducing the adhesion of tumour cells to the vascular endothelium (Kohn & Liotta 1995).

Acriflavine, which is trypanocidal (Macadam & Williamson 1974), has been shown to be mutagenic to viruses and bacteria. Acriflavine treatment results in amorphous DNA masses, nucleolar membrane disappearance and irregular fragmentation of nucleolar material in *Trypanosoma rhodesiense*. In addition, the binding of this compound to the plasma membrane might lead to the induction of respiratory deficiency in tumour cells.

The anti-tumour effects of acridines have been demonstrated in a variety of systems. Although the primary step of diacridine inhibition would be on the synthesis of RNA, DNA and proteins, statistical correlation strongly indicates that the anti-neoplastic action of acridines is most closely correlated with the plasma membrane (Bose et al 1966; Canellakis & Chen 1979). It has been suggested that certain acridines including acriflavine and diacridines inhibit cellular transformation by altering tumour-cell surface membranes (Chakraborty et al 1980) and that the anti-tumour effects of diacridines correlate best with their effects on the plasma membrane.

It has been shown that the membrane binding of acriflavine leads to the inhibition of protein kinase C through interaction with regulatory lipid cofactors or the regulatory domain of the protein. Some of the anti-tumour effects of acriflavine might be a result of the inhibition of protein kinase C (Hannun & Bell 1988). Because acriflavine can potently bridge with phospholipid, owing to the amino functional groups in the 3 and 6 positions of the parent compound, acriflavine was employed in this study as a potential blocker of nutrient transport such as glucose utilization.

Other studies have shown that either guanosine or guanosine 5′-monophosphate potentiates anti-tumour effects of certain

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chemotherapeutic agents. The anti-tumour activity of 5′-deoxy-5-fluorouridine can be potentiated in combination with guanosine or guanosine 5′-monophosphate without increasing toxicity to the host, as has been demonstrated in cultured cells and in tumour-xenoplanted animals (Iigo et al 1987). Guanosine monophosphate, one of the two end-products in de-novo purine nucleotide synthesis, exerts feedback inhibition of IMP synthesis, resulting in inhibition of AMP production. Accumulation of excess guanosine 5′-monophosphate inhibits the biosynthesis of ribose phosphate pyrophosphate. Resultant depletion of AMP, which can be synthesized from ribose phosphate pyrophosphate, would affect cellular ATP production through feedback product inhibition.

This study was designed to establish whether anti-tumour effects of acriflavine can be enhanced by combination treatment with guanosine, on the basis of the assumptions that certain acridines, including acriflavine, interact with the plasma membrane and modify the permeability of the cells (Bose et al 1966; Canellakis & Chen 1979) and that guanosine perturbs cellular production of ATP preferentially in rapidly growing cells.

Materials and Methods

Materials

Acriflavine (acriflavine neutral) comprising 3,6-diamino-10-methylacridium chloride and 3,6-diaminoacridine in the ratio 2 : 1, and guanosine (guanosine hydrate) were purchased from Aldrich (Milwaukee, WI, USA). Other reagents used for cell culture were obtained from Sigma (St Louis, MO, USA).

Animals

Male ICR mice and CDF1 mice, 20 ± 2 g, were obtained from Daehan Laboratory Animals (Seoul, Korea) and maintained at a temperature between 20 to 23°C with a relative humidity of 50% under 12-h light/dark cycle. Animals were caged under the supply of filtered pathogen-free air and food (Jeilgedang, Korea) and water were freely available.

Tumour transplantation and treatment regimen

Transplantable tumour cells were maintained via regular passage involving subcutaneous or intraperitoneal implantation of tumour cells in the Central Research Laboratory, Taerim Pharmaceutical Co. Evaluation of anti-neoplastic activity was conducted in animals bearing subcutaneous or intraperitoneal xenografts. The response of subcutaneous xenografts was assessed for inhibitory effects on solid tumour growth. Briefly, Ehrlich carcinoma cells (1×10^7 cells mouse^{-1}) were subcutaneously transplanted into the left inguinal area with an inoculation volume of 50 μL. Beginning 24 h after implantation, the mice were under consecutive daily treatment with drug(s) for 14 days. Daily tumour growth was measured with vernier callipers (Mitutoyo, Japan). The tumour volume was calculated according to the formula width^2 × length/2, as described elsewhere (Corbett et al 1977). Experiments on tumour growth inhibition were routinely performed after 14 days, at which time the tumour volume was on average 0·9–1·1 cm^3 without treatment.

To examine anti-tumour activity against ascitic tumour growth, ICR mice were used for Ehrlich ascitic tumour cell implantation (1×10^7 cells). CDF1 mice were employed for intraperitoneal implantation of L1210 (1×10^5) or P388 cells (1×10^6) and subjected to the treatment regimen. The effects of anti-tumour agents on ascitic tumour cell proliferation were evaluated in tumour-bearing mice after intraperitoneal injections of acriflavine, guanosine or both acriflavine and guanosine, 1 : 1 (w/w) herein abbreviated as AG60. The weight of cells in ascitic fluid was measured 10 days post-treatment. Briefly, mouse intraperitoneum was washed with normal saline (2 mL) and the washed fluid was centrifuged at 3000 *g* for measurement of the volume of packed cells. Ascitic tumour cells were histochemically assessed from the touch-prints of intraperitoneal exudate under light microscopy using Papanicolaou's staining.

Dose selection study

Male ICR mice, 20 ± 2 g, were used to determine the LD50 for AG60, i.e. the dose which killed 50% of the mice. A single dose, 30–120 mg kg^{-1} of AG60 was administered intramuscularly to groups of animals. The mortality was determined after 14 days.

Cell culture

Yac-1 mouse leukaemia cells were used to examine the morphological changes of tumour cells after in-vitro treatment with acriflavine, guanosine or AG60. Yac-1 cells were cultured in RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal bovine serum, streptomycin (100 μg mL^{-1}) and penicillin (100 unit mL^{-1}).

MTT cytotoxicity assay

The conversion of the tetrazolium salt 3-((4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by the cells was used for assay of cytotoxicity against cell proliferation. The MTT assay was based on metabolic reduction of MTT, as modified previously (Mosmann 1983). Yac-1 cells were cultured in 96-microtitre plate wells ($2{\cdot}5 \times 10^3$ well^{-1}) in the presence of RPMI-1640 complete growth medium, 10% foetal bovine serum and the agent of interest. After 24-h incubation, the cells were also incubated with MTT (0·5 mg mL^{-1}) for 4 h at 37°C to enable the production of formazan. Acidified isopropanol (0·04 M HCl; 100 μL) then sodium dodecylsulphate (3%; 20 μL) were added to the harvested cells in each well. Absorbance at 550 nm was measured by use of a spectrophotometric microplate reader (Bio-tek, Model EL311s, Winooski, Vermont, USA).

Fluorescence microscopy

Yac-1 cells were incubated in the presence of acriflavine or AG60 and cells stained with acriflavine fluorescence were photographed by use of a Jenamed 2 with blue filter (450 nm) (Zeiss, Jena, Germany). Magnification was × 40.

Data analysis

Statistical comparisons were performed by use of computer programs for pharmacological calculations (Tallarida & Murray 1987). One-way analysis of variance was used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $\alpha = 0{\cdot}05$ or 0·01.

Results

The anti-tumour effect of acriflavine or guanosine, or both, was first evaluated in ICR mice subcutaneously implanted with Ehrlich carcinoma cells (1×10^6 mouse^{-1}). Percent inhibition of the daily growth rate was determined by measuring tumour diameter. The tumour growth rate increased by 0·073 cm^2 per day in tumour-implanted mice. Intramuscular acriflavine treatment at the dose of 15 mg kg^{-1} day^{-1} for 14 days caused $27 \pm 5\%$ inhibition in the tumour growth, as determined by tumour diameter during the 2-week treatment regimen. Thus, acriflavine treatment alone suppressed the tumour growth by 32% relative to control with the tumour growth rate being noted as 0·05 cm^2 day^{-1}. Minimum regression ($12 \pm 6\%$) in tumour growth was noted in the animals treated with guanosine alone at the daily dose of 15 mg kg^{-1} (0·059 cm^2 day^{-1}). Treatment of animals with AG60 at 30 mg kg^{-1} resulted in $61 \pm 4\%$ inhibition of the tumour growth rate during the treatment regimen (day 5 to day 13) in the subcutaneously-xenografted tumour diameter at the inguinal site with the daily growth rate being observed as 0·025 cm^2 day^{-1}. The 1 : 1 (w/w) ratio of acriflavine to guanosine was selected as the optimum on the basis of preliminary experiments and the limit of guanosine solubility.

The Ehrlich tumour excised after 14-day treatment was weighed. The tumour weight in Ehrlich carcinoma cell-implanted ICR mice treated with vehicle was $0{\cdot}89 \pm 0{\cdot}42$ g. Acriflavine or guanosine treatment at 15 mg kg^{-1} for 14 days resulted in decreases in tumour weight of 36 and 34% ($0{\cdot}25 \pm 0{\cdot}07$ g and $0{\cdot}59 \pm 0{\cdot}21$ g), respectively. Treatment of Ehrlich carcinoma-implanted mice with acriflavine and guanosine (1 : 1) concomitantly at the dose of 30 mg kg^{-1} resulted in 96% reduction of tumour weight at 14 days post-treatment ($0{\cdot}04 \pm 0{\cdot}03$ g, significant, $P < 0{\cdot}05$). Excised Ehrlich tumour mass is comparatively depicted in Fig. 1a. Tumour growth and progression was completely suppressed in response to AG60 treatment. Fig. 1b shows histopathological findings of Ehrlich carcinoma cell invasion. Extensive invasion of Ehrlich carcinoma cells into the dermis in ICR mice is apparent. Fig. 1c clearly demonstrates inhibition of Ehrlich carcinoma cell proliferation by AG60 treatment. Necrosis at the borderline between the carcinoma tissue and the dermis was noted. Infiltration of inflammatory cells was observable. Extensive and progressive necrosis was apparent in the centre of the tumour mass.

To establish the optimum dose of AG60 effective in inhibiting tumour growth, the anti-tumour effect was examined at various doses. The daily tumour growth rate measured at the doses employed is listed in Table 1. The rate of tumour growth was inhibited by 32% in animals treated daily with acriflavine at a dose of 15 mg kg^{-1}. Because a dose of 20 mg kg^{-1} or more was lethal, the anti-tumour effects of acriflavine could not be further assessed. Minimal inhibitory effects ($\sim 20\%$ inhibition) were obtained with guanosine alone at the doses employed (15 to 90 mg kg^{-1}). Although histochemical examination revealed partial necrosis in certain areas of the tumour after guanosine treatment, the inhibitory effect of guanosine was compromised by the growth of tumour cells adjacent to normal tissue. Thus, guanosine alone, at doses from 15 to 90 mg kg^{-1} failed to suppress tumour growth effectively. Interestingly, enhanced dose-dependent inhibition of tumour growth rate was observed in the animals treated with AG60. A maximum 65% inhibition was noted at a dose of 30 mg kg^{-1}, AG60.

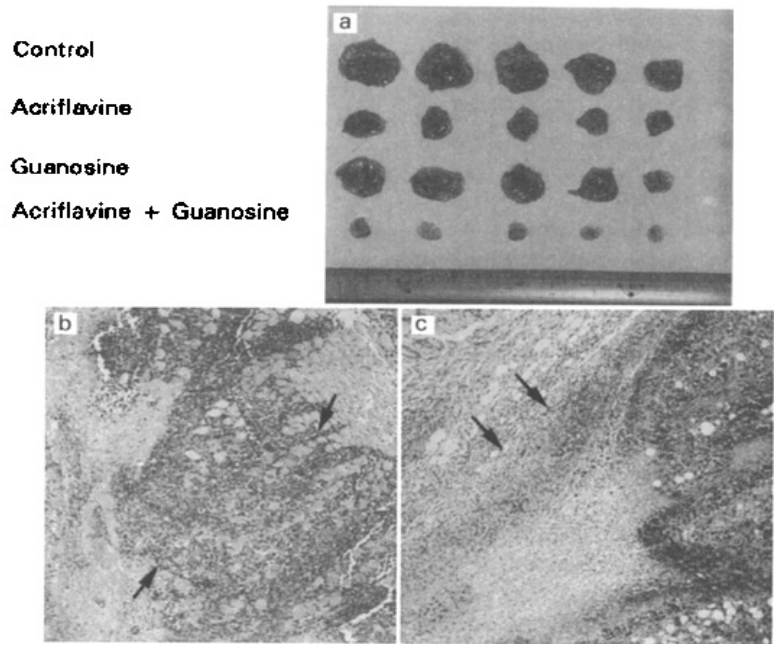

FIG. 1. The effects of acriflavine, guanosine or AG60 on Ehrlich carcinoma formation in ICR mice. a, Gross features of excised Ehrlich carcinoma 14 days after tumour implantation. Tumour mass in the left inguinal area was substantially shrunken after AG60 treatment (acriflavine, 15 mg kg^{-1} day^{-1}; guanosine, 15 mg kg^{-1} day^{-1}; AG60, 30 mg kg^{-1} day^{-1}, i.m., 14 days). b, Representative histopathologic findings of Ehrlich carcinoma cell invasion without drug treatment. (Hematoxylin and eosin staining, × 10), → tumour extension in deep dermal muscle layer. c, Representative section of Ehrlich tumour cells subcutaneously inoculated in the inguinal area with AG60 treatment. Progressive necrosis in the centre of tumour mass is apparent (Hematoxylin and eosin staining, × 10), → infiltration of inflammatory cells.

Table 1. Dose-related effect of acriflavine or guanosine, or both, on the growth of Ehrlich tumour.

Treatment	% Control of tumour growth for dose of (mg kg^{-1})							
	0	10	15	20	30	50	70	90
Acriflavine	100	–	69	–	–	–	–	–
Guanosine	100	–	81	–	–	78	102	70
Acriflavine + guanosine	100	84	–	63	30	–	–	–

Ehrlich tumour was subcutaneously implanted in ICR mice and tumour formation was evaluated at 14 days post-treatment. Values represent mean of relative tumour growth (n = 5).

Table 2. Effect of acriflavine or guanosine, or both, on body weight of Ehrlich tumour-implanted ICR mice.

Treatment	Body weight (g) at day				
	0	3	6	9	12
Control	17·9 ± 0·9	21·5 ± 1·5	24·8 ± 1·5	26·9 ± 1·8	28·3 ± 2·1
Acriflavine	20·0 ± 0·8	23·5 ± 0·7	19·8 ± 0·7*	18·8 ± 1·1*	22·1 ± 1·3*
Guanosine	18·8 ± 0·7	22·9 ± 0·9	26·2 ± 0·9	27·7 ± 1·4	29·9 ± 1·5
Acriflavine + guanosine	21·2 ± 1·7	23·7 ± 2·7	20·3 ± 2·9*	18·7 ± 2·4*	18·2 ± 2·8*

Body weights were measured after acriflavine (15 mg kg^{-1} day^{-1}, i.m.) or guanosine (15 mg kg^{-1} day^{-1}, i.m.) treatment, or both (AG60, 30 mg kg^{-1} day^{-1}, i.m.). The values are mean ± s.d. (n = 5). Data were analysed by one-way analysis of variance followed by the Newman–Keuls test; $*P < 0.01$ compared with control.

The ED50 of AG60 against Ehrlich carcinoma cells was 23 mg kg^{-1} (95% confidence limit, 20·0–27·5 mg kg^{-1}, Table 1). Daily doses of AG60 of 40 mg kg^{-1} or more were, however, lethal. The toxicity of acriflavine or guanosine, or both, was also determined in ICR mice after a single injection of the agents. The LD50 of acriflavine was ~30 mg kg^{-1} (i.p.). The LD50 of AG60 given intramuscularly was ~55 mg kg^{-1} in male or female ICR mice.

Body weight changes were monitored in Ehrlich carcinoma cell-implanted ICR mice. For mice treated with either vehicle or guanosine, daily increases in body weight were 0·87 g or 0·90 g day^{-1}, respectively, resulting in 58% and 59% increases at day 12 after tumour implantation (Table 2). Treatment with either acriflavine or AG60, however, completely suppressed body-weight increases during the treatment, indicating that acriflavine was the agent active in suppressing growth of animals. Preliminary studies support the hypothesis that acriflavine perturbs intracellular glucose transport, as partly evidenced by the significant increases in plasma glucose levels after acriflavine or AG60 treatment (Table 3).

The anticancer effect of AG60 was examined in CDF1 mice bearing Ehrlich ascitic tumour cells (1×10^7). The weight of tumour cells collected from ascitic fluid 11 days after tumour cell implantation was 3·7 g $mouse^{-1}$ (Table 4). Whereas treatment with guanosine alone (15 mg kg^{-1} day^{-1}, 10 days) initiated 24 h after Ehrlich ascitic tumour cell implantation failed to suppress tumour-cell proliferation, acriflavine was effective in suppressing ascitic tumour cell proliferation by ~95% (Table 4). AG60 treatment (30 mg kg^{-1} day^{-1}, 10 days) resulted in substantial (95%) suppression of the growth of Ehrlich ascitic tumour. Although the tumour-cell volume

Table 3. The effect of acriflavine on the plasma glucose levels.

Treatment	Plasma glucose level (mg dL^{-1})	Percent of control value
Control	116 ± 16	100
Acriflavine	162 ± 6*	140
Guanosine	120 ± 8	103
Acriflavine + guanosine	178 ± 35**	153

Acriflavine or guanosine, or both, was administered intramuscularly to Sprague–Dawley rats at a dose of 20 mg kg^{-1} as a single injection. Blood samples were collected 24 h later. The values are mean ± s.d. (n = 5). Data were analysed by one-way analysis of variance then the Newman–Keuls test; $*P < 0.05$, $**P < 0.01$ compared with control.

Table 4. The effect of acriflavine or guanosine, or both, on the proliferation of Ehrlich ascitic tumour cells intraperitoneally implanted in CDF1 mice.

Treatment	Cells in ascitic fluid (g)	Percent of control value
Control	3·75 ± 0·55	100
Acriflavine	0·19 ± 0·02*	5
Guanosine	3·98 ± 0·54	106
Acriflavine + guanosine	0·18 ± 0·03*	5

Acriflavine (15 mg kg^{-1} day^{-1}) or guanosine (15 mg kg^{-1} day^{-1}), or both (AG60, 30 mg kg^{-1} day^{-1}), was administered to the tumour-bearing mice. Intraperitoneal washed fluid was centrifuged at 3000 *g* and the volume of packed cells was measured. The values are mean ± s.d. (n = 5). Data were analysed by one-way analysis of variance then the Newman–Keuls test; $*P < 0.01$ compared with control.

measured after treatment with acriflavine alone was comparable with that after treatment with AG60, microscopic examination revealed that after acriflavine treatment ~25% of the cells were ascitic Ehrlich cells whereas only ~10% of degenerated tumour cells were collected from the ascitic fluid in AG60-treated animals (i.e. no living tumour cells were observed). Inflammatory cells including neutrophils were predominant after AG60 treatment (Fig. 2). Similar results were obtained from animals intraperitoneally implanted with L1210 leukaemia cells (data not shown).

Ehrlich ascitic tumour-bearing animals which survived were further challenged by implantation with 1×10^7 Ehrlich ascitic tumour cells in order to determine further anticancer effects in terms of the survival rate. All of the Ehrlich ascitic tumour cell-bearing mice died between 26 and 33 days after the second implantation of tumour cells, whereas all of the animals treated with AG60 for 14 days survived during the 60-day observation period.

The anti-tumour effect of AG60 (30 mg kg^{-1} day^{-1}, 7 days) was further determined in CDF1 mice intraperitoneally implanted with 1×10^6 P388 leukaemia cells. Fig. 3 shows proliferated P388 leukaemia cells in the intraperitoneum 7 days after tumour cell implantation. Consecutive daily treatment of mice with AG60 for 7 days after P388 cell implantation resulted in inhibition of tumour proliferation.

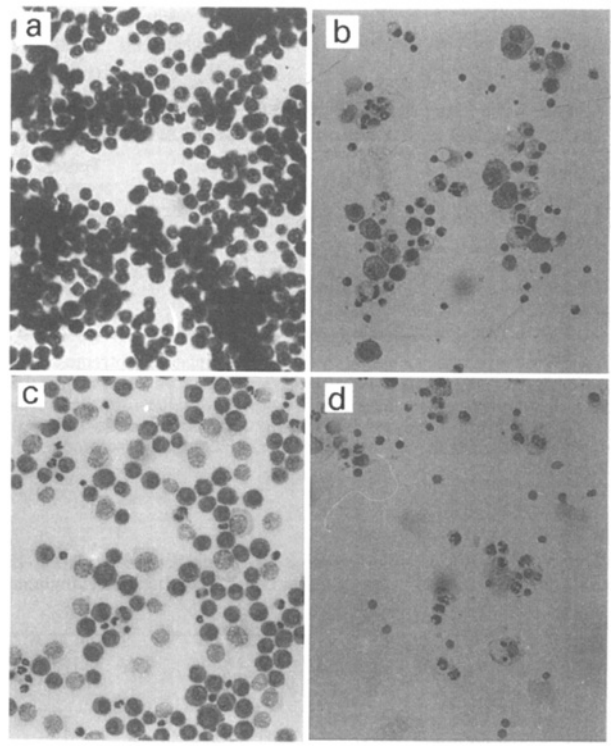

FIG. 2. Microscopic examination of the Ehrlich ascitic tumour cells. Anti-tumour effects of acriflavine or guanosine, or both, were examined in CDF1 mice intraperitoneally implanted with the tumour cells (1×10^7). Touch-prints of the intraperitoneal fluid were assessed 10 days after implantation (× 20). a, Proliferation of the tumour cells is observed without drug treatment. b, Intraperitoneal cells in the animals treated with acriflavine (15 mg kg^{-1} day^{-1}, i.p., 10 days) include 25% tumour cells. c, Guanosine-treated mice (15 mg kg^{-1} day^{-1}, i.p., 10 days) furnished 60% of living tumour cells. d, AG60 treatment (30 mg kg^{-1} day^{-1}, i.p., 10 days) resulted in complete suppression of tumour-cell proliferation.

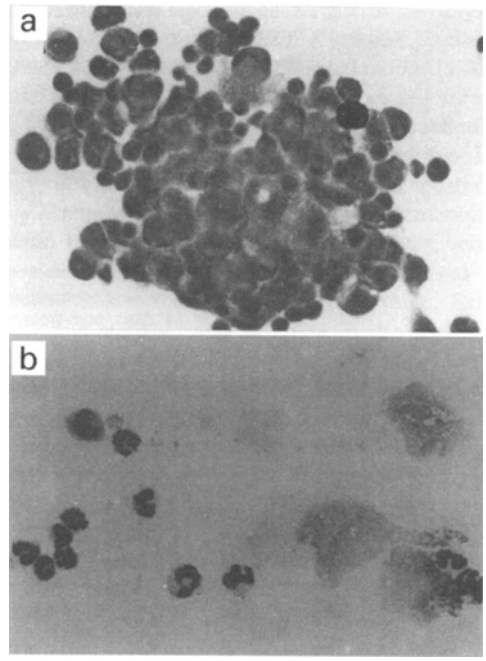

FIG. 3. Anti-tumour effect of AG60 (30 mg kg^{-1} day^{-1}, i.p., 7 days) in CDF1 mice intraperitoneally implanted with P388 leukaemia cells. Leukaemia cells were microscopically examined using touch-prints of the intraperitoneum. a, Intraperitoneally proliferating leukaemia cells without drug treatment (Giemsa staining, × 40). b, Proliferation of leukaemia cells was completely suppressed by AG60. Segmented neutrophils and a few histiocytes are noted (Giemsa staining, × 40).

The anti-tumour effect of AG60 against P388 cells was determined as a function of dose. Although a daily dose of 10 mg kg^{-1} of AG60 failed to reduce intraperitoneal P388 cell growth effectively, moderate suppression of tumour growth was observed in animals treated with 20 mg kg^{-1} of AG60. P388 tumour cell growth was completely suppressed by doses of 30 mg kg^{-1} or more.

Yac-1 cells, which are floating cells in culture, were used to examine the time-dependent morphological changes of tumour cells in the presence of AG60. The IC50 values of acriflavine, guanosine and AG60 for Yac-1 cells were $2{\cdot}11 \pm 0{\cdot}28$, > 100 and $1{\cdot}89 \pm 0{\cdot}24$ μg mL^{-1}, respectively. Fig. 4 shows the results from fluorescent microscopic analysis of Yac-1 cells cultured in the presence of AG60 and depicts the high binding affinity of acriflavine with plasma and nuclear membranes. Certain areas in the nucleoplasm were also stained with acriflavine. Similar morphological changes of the cells were observed in the presence of acriflavine. Guanosine alone failed to affect cell viability. Time-dependent changes in morphology such as bleb formations, cytoplasmic swellings and ballooning of Yac-1 cells by AG60 or acriflavine might represent energy depletion of the cells, followed by osmotic lysis due to cationic influx (Fig. 5).

Discussion

This study demonstrates for the first time that the anti-tumour effect of acriflavine is increased by concomitant treatment with guanosine. There are three possible mechanistic explanations of the enhanced tumour specificity of AG60.

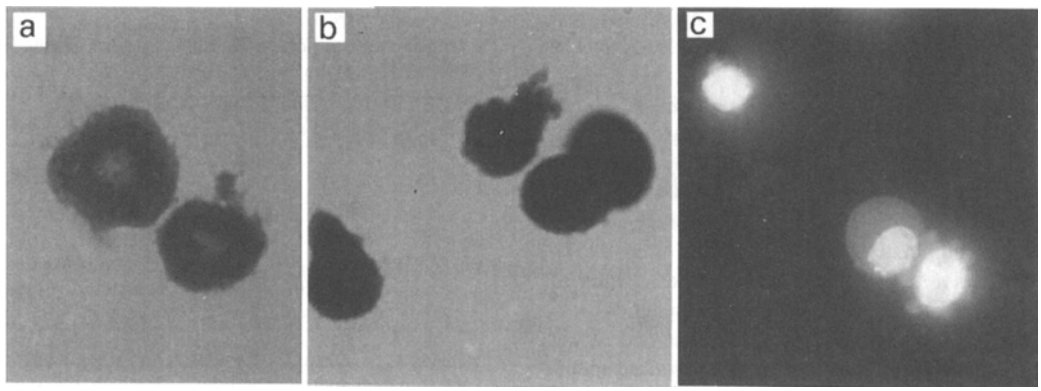

FIG. 4. Fluorescent microscopic analysis of Yac-1 cells cultured in the presence of AG60 (× 100). a, Yac-1 cells cultured in the absence of drug were stained with trypan blue. b, Yac-1 cells were incubated in the presence of 0·5 μg mL^{-1} AG60 for 24 h and stained with trypan blue. Multiple blebs and initiation of ballooning of the cytoplasmic membrane were noted. c, Fluorescent microscopic examination shows the high binding affinity of acriflavine with plasma and nuclear membranes, and morphological changes of the cells. Time-related morphological changes such as bleb formations, swelling and ballooning of Yac-1 cells are apparent 24 h after incubation in the presence of AG60 (50 μg mL^{-1}). Similar results were obtained with 25 μg mL^{-1} acriflavine.

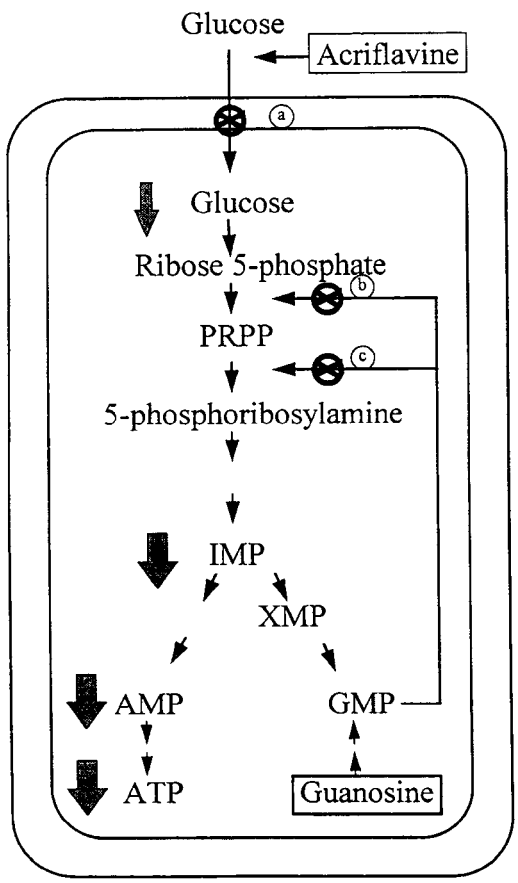

FIG. 5. The potential blocking of glucose utilization by acriflavine and guanosine nucleotides; PRPP is ribose phosphate pyrophosphate. a, Blocking of glucose transport by acriflavine; b, inhibition of ribose phosphate pyrophosphokinase; c, inhibition of glutamine-ribose phosphate pyrophosphate amidotransferase.

Firstly, it is highly likely that anti-tumour effects of acriflavine result from the non-specific blocking of the functions of plasma membrane-bound proteins, which would result in impairment of nutrient transport. In addition, excessive guanosine administered can be utilized as a precursor of guanine nucleotides in tumour cells, which would be involved in the blocking of ATP production through feedback enzyme inhibition and in signal transduction pathways (i.e. DNA and RNA). For example, guanosine is easily degraded by purine nucleoside phosphorylase into guanine and ribose-1-phosphate, and subsequently guanosine 5′-monophosphate is produced from guanosine or guanine, or both, by the catalysis of hypoxanthine guanine phosphoribosyl transferase. Excessive guanylates produced from guanine inhibit ATP production by a feedback mechanism. Thus, sequential blocking of energy production by both acriflavine and guanosine will lead to rapid depletion both of ATP and probably of oxygen utilization. This potential mechanism of action is supported in part by the morphological changes of Yac-1 cells in the presence of AG60 and by the increases in plasma glucose levels with concomitant cessation of body weight increases during treatment of animals with acriflavine or AG60. Enhanced cytotoxicity was further supported by the intense fluorescent acriflavine staining of the cells in the presence of guanosine, probably as a result of the disruption of plasma membrane and staining of intracellular organelles with acriflavine.

Secondly, phosphodiester bonds in DNA or RNA might also be the site of acriflavine binding through intercalation (Bose et al 1966), a hypothesis supported by the complete inhibition of nucleolar RNA synthesis and the partial inhibition of other RNA and DNA synthesis in *Trypanosoma rhodesiense*.

Thirdly, the tumour-cell surface might be altered by acriflavine. The induction of changes in tumour-cell-surface antigens by acriflavine has been reported and seems to be mediated through the promotion of lectin-mediated cellular agglutination (Chakraborty et al 1984; Chakraborty & Bose 1987). Studies support the hypothesis that increased aggregation of tumour cells is mannose lectin-mediated. A similar phenomenon was observed in experiments performed with castanospermine, suggesting that increased aggregation in the cells treated with cytotoxic agents might depend on mannose lectin-mediated cell-to-cell adhesion. Acridines without amino groups and derivatives with two heteroatoms in the middle ring would be ineffective. The compounds with free amino groups appeared to prevent RNA and protein synthesis (Scholtissek & Becht

1966). Agglutination testing supported the hypothesis that modulation of the plasma membrane by acridines leads to cell-to-cell interaction.

Among the possible mechanisms postulated above, energy depletion is highly likely to be associated with the synergistic enhancement of acriflavine anti-tumour effects by guanosine. The acriflavine binding sites in the plasma membrane would include polymeric molecules of phosphate, sulphate, or carboxyl groups, or combinations of these (Roth et al 1967). Inhibition of the active site was overcome by the addition of triton X-100 micelles or phospholipid (e.g. phosphatidyl serine) vesicles, which supports the hypothesis that one of the active binding sites for acriflavine is the phospholipid membrane. Acriflavine has been used to stain DNA, RNA and acid mucopolysaccharide. The observation that most of these compounds perturb the lipid bilayer and consequently inhibit enzyme activity non-specifically (Canellakis & Chen 1979), as supported by the multiple sites of interaction of the acridine derivatives with protein kinase C, and that detergent micelles or phospholipid overcome the inhibition of protein kinase C, supports the hypothesis that acriflavine preferentially partitions into the bilayer. It is likely that the amino groups present in acridine interact with two phosphate groups present in phospholipid by ionic bonding (Hannun & Bell 1988). Because protein kinase C is probably important in tumour promotion and cell regulation, increases in acriflavine perturbation of protein kinase C activity through modulation of cell-membrane integrity might also be associated with the anti-tumour effects of AG60. Structure-function studies have shown that other acridines lacking the amino groups and 1-aminoanthracene failed to inhibit protein kinase C activity, indicating that nitrogen in the ring structure and the amino substituent are necessary for the effects of the compounds; this is further supported by the higher potency of the compounds in the inhibition of protein kinase C (Hannun & Bell 1988).

This study clearly demonstrates that whereas guanosine alone is minimally cytotoxic to tumour cells it enhances the anti-tumour effects of acriflavine against a variety of cancer cells and is relatively non-toxic. It has also been shown that combinational therapy of acriflavine with other agents is synergistically effective in eradicating certain viruses (Mathe et al 1994). This combination of acriflavine and guanosine reported in this study appears to be more effective than other reported combined therapy (Santelli & Valeriote 1978; Iigo & Hoshi 1984; Iigo et al 1987). Enhancement of the anti-tumour activity of acriflavine by combination with guanosine will provide a preclinical basis for clinical applications and the usefulness of this combination against malignancy. The lack of serious adverse effects of these agents, such as the bone marrow depression and gastrointestinal cell damage observable with conventional cancer chemotherapy, should further prompt clinical application of this preclinical pharmacological observation.

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