Differential modulation of nitric oxide production by curcumin in host macrophages and NK cells

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Abstract Curcumin, the yellow pigment from *Curcuma longa*, has been shown to possess tumoricidal activity. We have earlier reported the induction of apoptosis in AK-5, rat histiocytic cells by curcumin leading to the inhibition of tumor growth in vivo. In this study we have observed differential activation status in host macrophages and NK cells induced by curcumin during the spontaneous regression of subcutaneously transplanted AK-5 tumors. Closer scrutiny of the cytokine profile and nitric oxide (NO) production by immune cells showed an initial down-regulation of Th1 cytokine response and NO production by macrophages, and their upregulation in NK cells, which picked-up upon prolonged treatment with curcumin, culminating in a stronger tumoricidal effect. These studies suggest that the host macrophages and NK cells play an important modulatory role in the remission of AK-5 tumor.

Key words: Nitric oxide; Inducible nitric oxide synthase; Macrophage; NK cell; Tumor regression

1. Introduction

Curcumin, the active component of the rhizome *Curcuma longa* possesses a wide spectrum of therapeutic applications like anti-inflammatory, antioxidant, bactericidal, antihelmintic effects and aids in reducing cholesterol levels [1–4]. This polyphenolic phytochemical is currently accepted as a potent anticancer agent [5,6]. The tumoricidal effect of curcumin has been studied on a wide range of cell lines like mouse sarcoma (S180), human colon cancer cells (HT-29), human kidney cell line 293 and hepatocellular carcinoma (HepG2 cells) [7]. Curcumin also showed antiproliferative activity on human breast tumor MCF-7 cells [8].

Mechanisms of curcumin-mediated tumoricidal action are attributed to the inhibition of protein tyrosine kinase activity, protein kinase activity [9] and arachidonic acid metabolism [7]. Curcumin caused growth arrest and apoptosis of B cell lymphoma by downregulation of egr1, c-myc, bcl-XL, NF-kB and p53 [10]. Curcumin was also found to downregulate proinflammatory cytokines like TNF-α, IL-1β and IL-8 [11]. However its inhibitory effects on inducible nitric oxide synthase (iNOS), an inflammation-induced enzyme producing nitric oxide (NO), may have opposite effects as NO plays an important role in carcinogenesis [11].

Curcumin also prevented tumor growth in animal tumor models [12,13]. AK-5, a rat histiocytoma grows as ascites after i.p. transplantation, killing 100% of the animals, but is rejected spontaneously when transplanted s.c. [14]. The s.c. tumor remission is accompanied by upregulation of Th1 cytokines, involvement of cell-mediated immunity and the interplay of the free radicals [15]. We have earlier shown a delay in tumor rejection after the administration of anticytokine (anti IL-12 and anti IFN-γ) antibodies, mediated through the downregulation of NO production and inhibition of cytotoxicity [16]. NK cells which act as effectors in vivo have been shown to induce apoptosis in AK-5 tumor cells through the production of NO [17]. In the present investigation we show that low doses of curcumin have a differential effect on NK cells (leading to their activation) and on peritoneal macrophages (leading to their inactivation), in terms of NO production and cytotoxic potential. However, prolonged exposure to curcumin induced recovery of this effect, finally leading to a faster remission of the AK-5 tumor.

2. Materials and methods

2.1. Animals and tumors

AK-5 tumor is maintained as ascites by continuous passage of 5×10^6 cells into the peritoneal cavity of a 6 weeks old inbred strain of Wistar rats. Solid AK-5 tumors were obtained after subcutaneous injection of 5×10^6 cells into 4–6 week old rats.

2.2. Curcumin injections

Animals were injected with AK-5 cells subcutaneously. One group of 10 animals received a daily injection of 20 mg of curcumin i.p. until day 20 after tumor transplantation. Tumor growth was measured with a vernier calliper and the size was calculated using the formula $4/3\ \pi\ a^2b/2$ where '*a*' is the shortest diameter and '*b*' is the longest diameter in mm.

2.3. Collection of AK-5 serum

Curcumin treated/untreated tumor bearing animals on different days after tumor transplantation (days 5, 10, 15, 20) were bled through the retro-orbital plexus. The sera were separated by centrifugation and stored at −20°C until further use.

2.4. Preparation of macrophages

Curcumin-treated and untreated AK-5 tumor bearing animals (days 5, 10, 15, 20) were sacrificed and 20 ml of cold PBS containing heparin (10 U/ml) was injected into the peritoneal cavity. The peritoneal lavage was carefully aspirated out and placed at 4°C. The cell suspension was centrifuged for 10 min at $800\times g$ to obtain a white pellet, which was resuspended in IMDM supplemented with FCS (5%) and a mixture of antibiotics (Gibco-BRL). Macrophages were allowed to adhere for 2 h and the floating cells were washed out. Macrophages were cultured in IMDM for 24 h and the supernatants were collected and stored at −70°C for nitrite and cytokine estimations.

2.5. Preparation of NK cells

Splenocytes from normal, immune (AK-5 tumor rejected animals)

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and curcumin-treated animals on days 5, 10, 15 and 20 after tumor transplantation were fractionated on a Ficoll-Hypaque gradient. The lymphocyte fraction was plated in RPMI-FCS for 2 h to allow macrophage adhesion. The non-adherent cells were incubated with mAb 3.2.3 (specific for NKR-P1 receptor, Endogene Inc., USA)-coated Dynal magnetic beads, at a cell:bead ratio of 1:2 for 30 min. The NK cells bound to the beads were separated using Detach-a-bead (Dynal, Oslo, Norway). The purity of the cell preparations was tested by flow cytometry and the preparations containing more than 95% positive cells were used in these experiments.

2.6. Cytokine estimations

Cytokines in the animal sera and culture supernatants were estimated using an enzyme-linked immunofiltration assay (ELIFA, Pierce Chemical Co.). The initial ligand solution (culture supernatant) was bound to nitrocellulose membrane [18] and the membrane was blocked with PBS–BSA solution followed by treatment with appropriate dilutions of anticytokine antibodies. The membrane was washed and treated with horseradish peroxidase-conjugated secondary antibody (1:3000, Boehringer-Mannheim, Germany). The unbound antibody was removed by washing and the signal was produced on the membrane by developing for peroxidase. The standard curves for the cytokines were generated with pure murine cytokines obtained from the National Institute of Biological Standards (Hertfordshire, UK). Recombinant mIL-12 was a kind gift from Dr. U. Gübler, Hoffmann-La Roche.

2.7. NO estimation

The measurement of nitrite in the macrophage–NK cell culture supernatants was an indirect indicator of NO released by the activated cells. Macrophages were cultured for 16 h, whereas NK cells were cocultured with AK-5 cells in the presence of anti AK-5 antibody at an E:T ratio of 50:1 for 4 h. 100 μl of the culture supernatants were mixed with equal volume of Griess reagent and the absorbance was read at 540 nm [19]. Standard curve for nitrite was generated using $NaNO_3$ (Sigma). AK-5 cells did not produce any NO under these conditions.

2.8. Cytotoxicity assays

^{51}Cr-labelled AK-5 cells were cocultured with peritoneal macrophages collected from curcumin treated/untreated AK-5 tumor bearing rats (E:T = 10:1) for 18 h at 37°C. Similarly, a cytotoxicity assay was performed with immune, in vitro and in vivo curcumin-treated NK cells. ^{51}Cr-labelled AK-5 cells were cultured with NK cells in the presence of anti AK-5 antibody (E:T = 50:1) for 4 h. The chromium released into the supernatant was counted and the percentage cytotoxicity was calculated.

2.9. Curcumin treatment in vitro

Peritoneal macrophages from day 15 tumor bearing rats were plated in 24 well plates (1×10^6 cells/well) and different concentrations of curcumin were added (5, 10, 15, 20 μM) in vitro. The cells were incubated for 18 h and the nitrite released in the culture supernatant was measured using Griess reagent. Similarly, NK cells from normal animals were cultured in the presence of different concentrations of curcumin (5, 10, 15, 20 μM). After 18 h treatment, the cells were washed with PBS and used for nitrite determination as described earlier.

2.10. SDS–PAGE and Western blotting

Peritoneal macrophages and splenic NK cells collected from AK-5 tumor (s.c.) transplanted animals (untreated/treated with curcumin in vivo) were washed and boiled in Laemmli's buffer. The proteins were resolved on 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane [20]. The membrane was blocked with 3% PBS–BSA, and incubated overnight at 4°C with anti iNOS monoclonal antibody (Transduction Laboratories, 1:2500). The blots were washed, incubated with antimouse IgG coupled to AP and developed with BCIP/NBT.

3. Results

3.1. Effect of curcumin on AK-5 tumor growth

We have studied the effect of curcumin administered i.p. on the growth of AK-5 tumor transplanted s.c. Curcumin (20 mg/animal) was administered daily up to day 20 after the tumor transplantation. Curcumin-injected animals exhibited larger tumors as compared to the positive controls (Fig. 1). Maximum tumor size was attained by day 15 in both the groups. However the tumors were rejected faster in the curcumin-treated group after day 15 and the tumor size was smaller than the positive control animals by day 20 after the tumor transplantation.

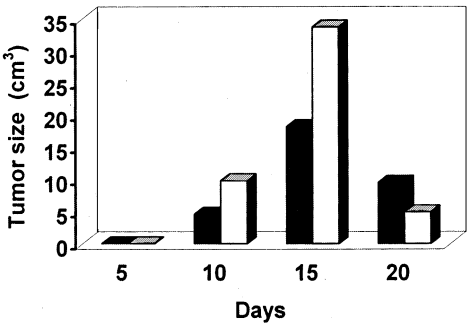

Fig. 1. Effect of curcumin on AK-5 tumor growth on different days. Closed bars represent the control group whereas open bars represent curcumin-treated animals (20 mg/animal/day). Values shown are average of 10 animals per group.

3.2. Effect of curcumin in vivo

We have studied the effect of in vivo administration of curcumin on the cytotoxic activity as well as the production of NO by the peritoneal macrophages and splenic NK cells.

3.2.1. Cytotoxic activity. Macrophages from control and curcumin-treated animals were cocultured with AK-5 tumor cells. Maximum cytotoxicity was achieved with day 15 tumor transplanted macrophages and NK cells (Fig. 2). However, macrophages from curcumin-treated animals possessed lower cytotoxicity as compared to the controls (Fig. 2B). NK cells on the other hand showed higher cytotoxicity against YAC-1 as well as AK-5 cells (ADCC) as compared to the immune NK cells (positive control). Optimal NK cytotoxicity was observed around day 15 (Fig. 2D).

3.2.2. NO production. The NO production pattern correlated well with the cytotoxic activity of macrophages and NK

Table 1
Effect of curcumin on the production of NO by macrophages and NK cells in vitro[a]

Curcumin (μM)	Nitrite concentration	
	Macrophages (μM/10^5 cells)	NK (μg/10^5 cells)
0.0	86.5 ± 4.8	0.42 ± 0.13
2.5	81.2 ± 6.5	0.71 ± 0.09[b]
5.0	67.9 ± 4.5[b]	0.86 ± 0.10[b]
10.0	59.1 ± 2.9[b]	0.91 ± 0.07[b]
20.0	51.9 ± 3.1[b]	0.68 ± 0.11
40.0	29.5 ± 2.7[b]	N.D.
50.0	22.0 ± 2.3[b]	N.D.

[a]Peritoneal macrophages were collected from day 15, AK-5 tumor bearing animals and NK cells were prepared from normal rat splenocytes. 10^5 cells were incubated with curcumin for 18 h. Culture supernatants were tested for nitrite estimations. The values shown are mean ± S.E.M. of two similar experiments. N.D. = not determined.

[b]$P < 0.001$ with respect to untreated controls.

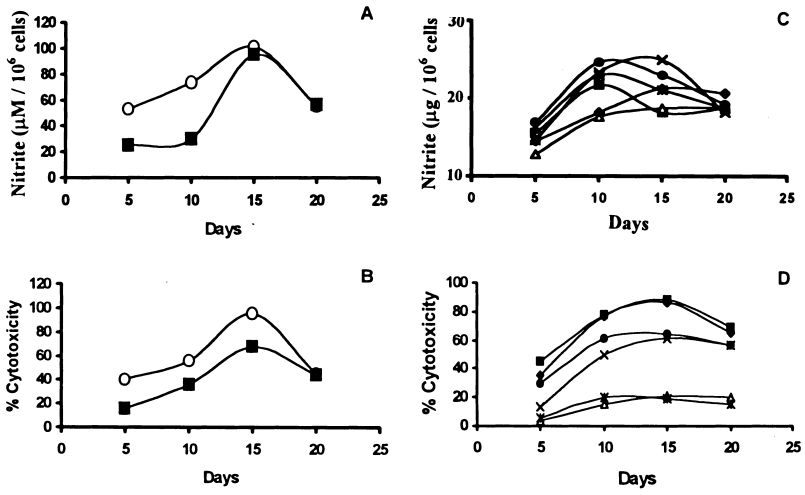

Fig. 2. Effect of curcumin on NO production (A and C) and cytotoxic activity (B and D) of macrophages and NK cells. A, B: Represent macrophages (control ○; curcumin ■) and C and D represent NK cells. C, D: The symbols shown are YAC1+iNK (♦); YAC-1+cur NK (■); AK-5+iNK (▲); AK-5+iNK+αAK-5 (*); AK-5+cur NK (X); AK-5+cur NK+αAK-5 (●). The values shown are representative of three similar experiments.

cells after treatment with curcumin. Nitrite production by curcumin-treated macrophages was lower up to day 15 after tumor transplantation (Fig. 2A). However, curcumin-treated NK cells produced higher quantities of nitrite as compared to the untreated controls, the optimal levels being between days 10 and 15 (Fig. 2C). These observations suggest suppression of macrophage cytotoxicity and NO production by curcumin whereas in NK cells curcumin upregulated production of NO with a corresponding increase in their cytotoxic activity.

3.3. Effect of curcumin in vitro

In order to confirm our in vivo observations, we tested the effect of different concentrations of curcumin on isolated macrophages and NK cells. There was a dose dependent inhibition of NO production by macrophages, whereas NO production by NK cells increased with curcumin concentrations (Table 1), thus confirming our in vivo observations.

3.4. Effect of curcumin on the production of cytokines

We have earlier shown that cytokines like IL-2, IFN-γ [21], IL-12 [22] and TNF-α [23] played a significant role in the activation of host immune cells, which in turn participated in the rejection of AK-5 tumor in syngeneic animals. In the present study we have investigated the cytokine profile in animals after administration of curcumin on different days. The production of IFN-γ, IL-12 and TNF-α increased with tumor growth reaching the peak values between day 15 and 20 in the tumor-transplanted positive control animals (Fig. 3). However, the production of these cytokines in curcumin-injected animals was downregulated on days 5 and 10 after the tumor transplantation. The levels of IFN-γ (Fig. 3A) and IL-12 (Fig. 3C) were elevated significantly on days 15 and 20. The overall cytokine levels remained lower in the curcumin-treated animals as compared to the untreated controls. TNF-α levels on the other hand remained lower throughout the duration of this experiment in curcumin-administered animals (Fig. 3B). We have also studied the release of IL-12 by

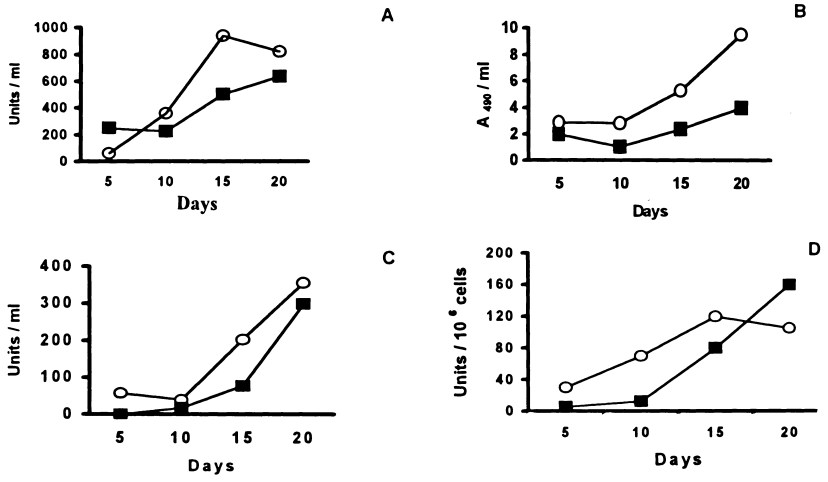

Fig. 3. Effect of curcumin on cytokine levels in AK-5 tumor bearing animals. A: Shows serum IFN-γ. B: TNF-α. C: Serum IL-12. D: Shows the IL-12 levels in the macrophage culture media on different days. Control (○); curcumin-treated (■). The values shown are representative of three similar experiments.

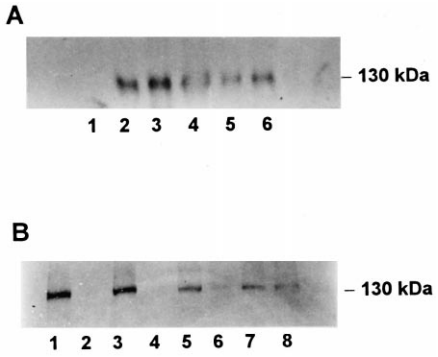

Fig. 4. Effect of curcumin on the iNOS expression by NK cells (A) and macrophages (B). A: Lanes 1, control day 5; 2, curcumin day 5; 3, control day 10; 4, curcumin day 10; 5, control day 15; 6, curcumin day 15. B: Lanes 1, control day 5; 2, curcumin day 5; 3, control day 10; 4, curcumin day 10; 5, control day 15; 6, curcumin day 15; 7, control day 20; 8, curcumin day 20.

peritoneal macrophages in vitro. Macrophages from curcumin-injected animals secreted lower concentrations of IL-12 in the culture medium up to day 15 (Fig. 3D). However, day 20 macrophages produced more of IL-12 as compared to the control cells (Fig. 3D).

3.5. *Expression of iNOS in curcumin-treated macrophages and NK cells*

In order to confirm our earlier results pertaining to NO production after the administration of curcumin in AK-5 tumor bearing animals, we also studied the expression of iNOS protein by Western blotting. There was upregulation of iNOS expression in peritoneal macrophages from s.c. tumor bearing animals (Fig. 4B, lanes 1, 3, 5 and 7). However, curcumin-treated animals did not show any iNOS protein on days 5, 10 and 15 (lanes 2, 4 and 6), whereas on day 20 there was some expression of iNOS observed (lane 8). The iNOS protein was absent in day 5 NK cells, which was upregulated on days 10 and 15 (Fig. 4A, lanes 1, 3, 5). Curcumin treatment induced the expression of iNOS in NK cells on day 5 (lane 2). The level of iNOS expression on days 10 and 15 was not significantly different to the controls (lanes 4 and 6). These results suggest a differential effect of curcumin on iNOS expression by macrophages and NK cells.

4. Discussion

Alternative medicine for treatment of various diseases is getting more popular. Because of limited scientific evidences and lack of mechanistic understanding it is not yet incorporated into the mainstream of medical care. However, alternative medicine is becoming an increasingly attractive approach world-wide and much attention is being given for its systematic study and applications. Curcumin, a common dietary pigment possesses a wide range of therapeutic utilities in traditional Indian medicine [1]. Its role in wound healing, urinary tract disease, liver ailments, hepatitis are well documented, in addition to its use as a cosmetic [1,3]. In addition, curcumin exhibits a variety of pharmacological effects including anti-inflammatory, anticarcinogenic and anti-infectious activities [2,4,5].

In the present investigation, we have tried to elucidate overall immunological effects of curcumin on AK-5 tumor growth and regression in Wistar rats. Curcumin, a known antioxidant, has been shown to inhibit activation of free-radical-activated transcription factors like NF-kB and AP-1 where it is identified as an antioxidant [9]. Also downregulation of iNOS gene expression in activated murine macrophages is demonstrated upon treatment with curcumin [24], yet, curcumin is known to induce tumor cell apoptosis [13]. Thus downregulation of NO production may prevent carcinogenesis on one hand, whereas, overall suppression of free radical production during the physiological process may encourage the development of the tumor. On closer scrutiny, the AK-5-transplanted animals showed an initial downregulation of NO production and reduced cytotoxic capabilities in host macrophages (day 5 and 10) after curcumin treatment which led to the development of larger size tumors (Fig. 1). At the same time however, the NK cells showed progressive upregulation of NO production and cytotoxicity (Fig. 2). The peritoneal macrophages were probably affected by the overall suppression of IFN-γ, TNF-α and IL-12 levels as observed in the sera (Fig. 3). The serum cytokine profile was significantly downregulated during the initial growing phase of the AK-5 tumor suggesting an inhibitory effect of curcumin administration. Curcumin-mediated TNF-α downregulation has also been reported earlier [25]. Kang and coworkers on the other hand, have reported a downregulating effect of curcumin on IL-12 production, thereby aiding in Th1-cytokine-mediated diseases [26]. In the present investigation we have also observed reduced IL-12 production in the sera and in the macrophage supernatants collected from day 5 and 10 curcumin-treated animals as compared to the control animals (Figs. 2 and 3). Curcumin-treated animal macrophages showed very low NO production and no detectable iNOS protein (Fig. 4B, lanes 2, 4). In NK cells however, there was an upregulation of NO production and iNOS protein (Fig. 4A). Surprisingly, the results reversed from day 15 onwards, when a progressive upward trend in the serum cytokine profile, polarizing Th1 type response, as well as macrophage-mediated IL-12 production on days 15 and 20 after curcumin treatment in AK-5 tumor bearing animals was observed. There was also an increase in macrophage-mediated cytotoxicity and NO production (Fig. 2).

Detectable levels of iNOS protein in day 15 and 20 macrophages were observed (Fig. 4B, lanes 6, 8). However, in NK cells, there was a high level of NO production and the presence of iNOS protein from day 5 onwards with no significant changes until day 20. A similar trend was observed by Brouet and his group where curcumin downregulated the production of iNOS proteins in LPS/IFN-treated RAW 264.7 cells after 6 h, whereas detectable levels of iNOS proteins were seen after 18 h [27]. These observations suggest that lower concentrations of curcumin have an inhibitory effect on macrophages (i.e. on days 5 and 10) which is lost after sustained treatment with curcumin over a longer period of time. The ultimate effect being the revival of Th1 cytokine response, production of NO and increased cytotoxicity leading to the rejection of the tumor.

Thus curcumin seems to be an interesting molecule possessing anticancer activity. We have recently shown the generation of reactive oxygen species after the treatment of the tumor cells with curcumin, which in turn induced apoptosis in tumor cells [28]. Thus curcumin, which has been reported to downregulate NO production in vitro, showed a differential effect in vivo, where it induced activation of immune cells leading to

the regression of the tumor. These observations suggest a dual effect of curcumin on tumor cells, one directly by inducing apoptosis and secondly through the activation of immune cells.

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References

[1] Nadkarni, K.M. (1976) in: Indian Materia Medica (Nadkarni, K.M., Ed.), pp. 414–417, Popular Prakashan, Bombay.
[2] Kuttan, R., Bhanumathy, P., Nirmala, K. and George, M.C. (1985) Cancer Lett. 29, 197–202.
[3] Sharma, O.P. (1976) Biochem. Pharmacol. 25, 1811–1812.
[4] Ramprasad, S.P. and Sisir, M. (1956) J. Sci. Ind. Res. India 156, 239–241.
[5] Mehta, K., Pantazis, P., McQueen, T. and Aggarwal, B.B. (1997) Anticancer Drugs 8, 470–481.
[6] Hanif, R., Qiao, L., Shiff, S.J. and Rigas, B. (1997) J. Lab. Clin. Med. 130, 576–584.
[7] Jiang, M.C., Yang-Yen, H.F., Yen, J.J. and Lin, J.K. (1996) Nutr. Cancer 26, 111–120.
[8] Simon, A., Allias, D.P., Duroux, J.L., Basly, J.P., Durand-Fontanier, S. and Delage, C. (1998) Cancer Lett. 129, 111–116.
[9] Chen, H.W. and Huang, H.C. (1998) Br. J. Pharmacol. 124, 1029–1240.
[10] Han, S.S., Chung, S.T., Robertson, D.A., Ranjan, D. and Bondada, S. (1999) Clin. Immunol. 93, 152–161.
[11] Chan, M.M., Huang, H.I., Fenton, M.R. and Fong, D. (1998) Biochem. Pharmacol. 55, 1955–1962.
[12] Kawamori, T., Lubet, R., Steele, V.E., Kelloff, G.J., Kaskey, R.B., Rao, C.V. and Reddy, B.S. (1999) Cancer Res. 59, 597–601.
[13] Khar, A., Ali, A.M., Pardhasaradhi, B.V.V., Begum, Z. and Anjum, R. (1999) FEBS Lett. 445, 165–168.
[14] Khar, A. (1993) Int. J. Oncol. 2, 393–398.
[15] Bhaumik, S. and Khar, A. (1998) Nitric Oxide Biol. Chem. 2, 467–474.
[16] Bhaumik, S. and Khar, A. (1998) Apoptosis 3, 361–368.
[17] Jyothi, M.D. and Khar, A. (1999) Nitric Oxide Biol. Chem. 5, 409–418.
[18] Ijsselmuiden, O.E., Herbrink, P., Meddens, M.J., Tank, B., Stolz, E. and Van Ejik, R.V. (1989) J. Immunol. Methods 119, 35–43.
[19] Green, L.C., Wagner, D.D.A., Glogowski, J., Skepper, P.L., Wishnok, J.S. and Tannenbaum, S.R. (1982) Anal. Biochem. 124, 131–138.
[20] Laemmli, U.K. (1970) Nature 227, 680–685.
[21] Kausalya, S., Varalakshmi, Ch. and Khar, A. (1995) J. Interferon Cytokine Res. 15, 647–654.
[22] Hegde, S.P., Bright, J.J., Kausalya, S. and Khar, A. (1995) Cell. Immunol. 162, 241–247.
[23] Khar, A., Leela Kumari, A. and Ali, A.M. (1993) Int. J. Oncol. 2, 399–402.
[24] Prasain, J.K., Tezuka, Y., Hase, K., Basnet, P., Dong, H., Namba, T. and Kadota, S. (1998) Biol. Pharm. Bull. 21, 371–374.
[25] Chan, M.M. (1995) Biochem. Pharmacol. 49, 1551–1556.
[26] Kang, B.Y., Song, Y.J., Kim, K.M., Choe, Y.K., Hwang, S.Y. and Kim, T.S. (1999) Br. J. Pharmacol. 128, 380–384.
[27] Brouet, I. and Ohshima, H. (1995) Biochem. Biophys. Res. Commun. 206, 533–540.
[28] Bhaumik, S., Anjum, R., Rangaraj, N., Pardhasaradhi, B.V.V. and Khar, A. (1999) FEBS Lett. 456, 311–314.