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Effect of the protein kinase inhibitors, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine H-7 and *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide H-8 on Lewis lung carcinoma tumor progression

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Abstract

The effects of 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine H-7 (a cAMP-dependent protein kinase and protein kinase C inhibitor), *n*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide H-8 (a cAMP- and cGMP-dependent protein kinase inhibitor) and indomethacin (IND, a cyclooxygenase inhibitor) on both the spontaneous metastatic ability of 3LL (Lewis lung carcinoma) tumor cells and anti-tumor host response were studied. The study of tumor progression showed that H-7 and H-8 (2 mg kg^{-1} day^{-1}, i.p., for 8 days) significantly reduced the mean number of metastases (0.8 ± 0.2 and 1.0 ± 0.7, respectively, $P < 0.05$) with respect to the number of lung metastases (4.2 ± 2.1) observed in the control group. In turn, the highest tumor-specific cytotoxicity response (50% increase vs. non-treated target cells) was observed when both animal and tumor cells were treated with H-8. This suggests that the protein kinase inhibitors could inhibit tumor progression toward lung metastases formation by blocking the immunosuppressor mechanism triggered by agents that increase intracellular cAMP.

Keywords: Protein kinase inhibition; H-7; H-8; Cyclooxygenase inhibition; Indomethacin; Cytotoxicity; Metastasis; Lewis lung carcinoma

1. Introduction

Tumor-bearing animals develop generalized immunosuppression, in which prostaglandins secreted by macrophages and tumor cells exert an important role by inhibiting the effector function of several cell types (NK or natural killer cells, LAK or lymphocyte activated killer cells, and Th1 lymphocytes) (Ben-Efraim, 1992). High levels of PGE_2-type prostaglandins have been associated with immunosurveillance suppression and with diminished cytotoxicity toward tumor cells, thereby allowing tumor growth and progression to metastases (Bennett et al., 1985; Fulton and Heppner, 1985). PGE_2 acts through an increase in cyclic AMP (Fulton et al., 1991), which activates protein kinase A. A correlation has been suggested between the immunosuppressor activity of prostaglandins and alterations in effector cell signal transduction. The present study investigated the effects of indomethacin (IND, a cyclooxygenase inhibitor), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine H-7 (an inhibitor of both cAMP-dependent protein kinase and protein kinase C) and *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide H-8 (an inhibitor of both cAMP and cGMP-dependent protein kinase) on the progression of tumors in 3LL (Lewis lung carcinoma) tumor-bearing mice.

2. Material and methods

2.1. Mice and tumor cells

Eight- to ten-week old male syngeneic C57BL/6 mice were purchased from Iffa-Credo (L'Arbresle, France), and housed 10 per cage under standard laboratory conditions. The 3LL tumor cells were maintained under standard tissue culture conditions. Before tumor cell transplant to mice, the cultured cells were assessed by trypan blue exclusion and only single-cell suspensions with a viability

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of over 95% were used in all experiments. The DNA binding dye, Hoechst 33342, was purchased from Sigma (Madrid, Spain).

2.2. Spontaneous metastases

For studies of spontaneous metastases, 100 μl of a 3LL tumor cell suspension (10^6 cells ml^{-1}) was injected into the hindleg of syngeneic mice ($n = 40$). The mice were then randomly divided into four groups of 10 tumor-bearing animals each, and were kept 24 h for the following i.p. treatments: (i) control group (HEPES): a single daily dose (100 μl) of HEPES saline buffer (HEPES 10 mM, NaCl 150 mM); (ii) H-7 group: a single dose of a PKC inhibitor (2 mg kg^{-1} day^{-1}); (iii) H-8 group: a single dose of a protein kinase A inhibitor (2 mg kg^{-1} day^{-1}); (iv) IND group: a single IND dose (2 mg kg^{-1} day^{-1}). The treatment was initiated 1 day after tumor transplant (day 0) for a period of 8 days (days 1–4 and 8–11). Three weeks after tumor cell transplant, the animals were killed, the lungs were removed and blinded observers, using stereoscopic microscopy counted the number of surface lung metastases. Statistical significance with respect to the HEPES group was evaluated by the Mann–Whitney *U*-test. Statistical significance was considered for $P < 0.05$.

2.3. Tumor growth rate

The size of the primary tumor in the hindleg of mice, was measured after tumor cell transplant (day 0)—tumor mass becoming perceptible by day 9—until day 17. Vernier calipers were used to measure the largest (D) and the smallest (d) diameters of the tumor mass. Tumor volume was calculated by the formula: $V = D \times d^2/2$. Statistical significance with respect to the control group (HEPES) was evaluated by the Bonferroni's test.

2.4. Cytotoxicity assays

The effects of the different agents were evaluated on both healthy mice and tumor cells. Cytotoxicity assays were performed to evaluate the susceptibility of tumor cells to be killed by effector cells (splenic lymphocytes and peripheral blood mononuclear cells (PBMC)). The effector cells were obtained from healthy mice previously treated with each pharmacological agent. Animals were treated i.p. with the respective agent (2 mg kg^{-1}) for 4 days and were then immediately killed in order to obtain the effector cells from blood and spleen. The in vitro treatment of 3LL tumor cells consisted of incubation (3 h, 37°C) with H-7 (10^{-5} M), H-8 (10^{-5} M) or IND (10^{-6} M), followed by two further washings of cells with PBS (phosphate buffer saline). In these cases, treatment of cells is indicated in the figures as T + . The controls were tumor cells previously treated with an equivalent volume of HEPES. In this case, treatment is indicated as T − . Effector spleen cells and PBMC were dispersed and depleted of red and dead cells by Ficoll separation (Pharmacia Biosystems, Barcelona, Spain). The blood (approximately 1 ml per mouse) was obtained from the ventricular cavity of heparinized and anesthetized mice. For spleen cell collection, the spleen was removed and perfused with Dulbecco's minimal essential medium. The target cells were labeled by incubation (20 min, 37°C) with Hoechst 33342 (20 μM) in Whitten's medium (NaCl, 5.14 g l^{-1}; $NaHCO_3$, 19 g l^{-1}; KCl 0.36 g l^{-1}; KH_2PO_4, 0.16 g l^{-1}; $MgSO_4 \cdot 7H_2O$, 0.20 g l^{-1}; sodium pyruvate, 0.03 g l^{-1}; lactic acid hemicalcium salt, 0.25 g l^{-1}; glucose 1.0 g l^{-1}; penicillin 0.08 g l^{-1}; streptomycin sulfate, 0.05 g l^{-1}; sodium lactate, 3.26 ml l^{-1} of 70% w/v). Labeled target cells were washed twice in PBS (NaCl, 8.0 g l^{-1}; KCl 0.20 g l^{-1}; $Na_2HPO_4 \cdot 2H_2O$, 1.44 g l^{-1}; KH_2PO_4, 0.2 g l^{-1}) and resuspended at 4×10^5 cells ml^{-1} in Whitten's medium supplemented with 1% foetal calf serum.

Cytotoxicity assays were performed with the standard Hoechst 33342 release assay (Brenan and Parish, 1988). Labeled tumor cells (2×10^4) treated (T +) with IND, H-7, H-8 or treated with HEPES (T −) were mixed with effector cells (splenocytes or PBMC) from IND, H-7 or H-8-treated mice (E +) or from HEPES-treated mice (E −), at a target/effector (T/E) ratio of 1:10 or 1:20 and then the plate was centrifuged at $40 \times g$ for 2 min. Incubation was performed in 0.2 ml of modified Whitten's medium supplemented with 1% foetal calf serum, using 96-well microtiter plates. After 18 h incubation at 37°C, the plate was centrifuged at $850 \times g$ for 5 min and the culture supernatant (150 μl) from each well was transferred to a 96-well microtiter plate. Supernatant fluorescence was determined with a microplate reader (Cytofluor 2350, Millipore Iberica, Madrid, Spain) using the corresponding filters (excitation: 360 nm; emission: 460 nm). The results were expressed as specific release percentage obtained from the following formula: % specific release $= 100 \times (E - S)/(T - S)$, where E is the fluorescence released in the experimental samples, S is the spontaneous fluorescence from the target cells and T is the total fluorescence in the targets. All assays were done in quadruplicate at a T/E ratio of 1:10 and 1:20, with 2×10^4 tumor-labeled target cells. The low-fluorescent modified Whitten's medium supplemented with 1% foetal calf serum without red phenol (0.2 ml per well) was used to carry out all cytotoxic assays.

3. Results

3.1. Spontaneous metastases

The effects of treatment on the superficial lung metastases in 3LL tumor-bearing mice are summarized in Table 1. In the control group treated with HEPES, the metastatic

Table 1
Effect of the treatment with H-7, H-8 and IND on spontaneous lung metastases

Treatments	Incidence		Range	Mean ± S.E.	Median	Values	Statistical significance
	No.	%					
HEPES	8/9	88	0–21	4.2 ± 2.1	2	0,1,1,2,2,3,3,5,21	–
H-7	6/9	66	0–2	0.8 ± 0.2	1	0,0,0,1,1,1,1,2,2	$P < 0.05$
H-8	3/9	33	0–7	1.0 ± 0.7	0	0,0,0,0,0,0,1,1,7	$P < 0.05$
IND	6/9	66	0–4	1.3 ± 0.4	1	0,0,0,1,1,1,2,3,4	n.s.

Mice were inoculated (i.m.) with 3LL tumor cells (10^6) and treated (i.p.) during 8 days with HEPES (100 μl per mouse), H-7 (2 mg kg^{-1}), H-8 (2 mg kg^{-1}) or IND (2 mg kg^{-1}). At 21 days post-transplant, the mice were killed and lung metastases were counted. Statistical significance with respect to the HEPES-treated group (control) was evaluated with the Mann–Whitney *U*-test.

incidence was 88%, with a mean of 4.2 ± 2.1 metastases per lung. Treatment of mice with IND decreased both the incidence of metastases and the mean number of metastases per lung, though the effect was not significant. Treatment with H-7 significantly decreased the number of lung metastases (0.8 ± 0.2, $P < 0.05$); however, the percentage incidence remained high (66%). The mice treated with H-8 showed a significantly diminished ($P < 0.05$) mean value of metastases with respect to the HEPES-treated group (1.0 ± 0.7 vs. 4.2 ± 2.1). Moreover, the median value in H-8-treated mice was zero metastases, and the incidence corresponded to only 33% of the population—i.e., half the incidence obtained in the H-7-treated group.

3.2. Growth rate

Measurements of tumor size were made from day 9 after tumor cell transplant (day +9) until day +18, and tumor volume was calculated measuring the major and minor tumor diameters (Fig. 1). In the mice treated with IND, the animals presented with a significant decrease in tumor volume ($P < 0.005$, Bonferroni's test) with respect to the HEPES-treated group. The remaining groups reached a similar final tumor size at any time of measurement.

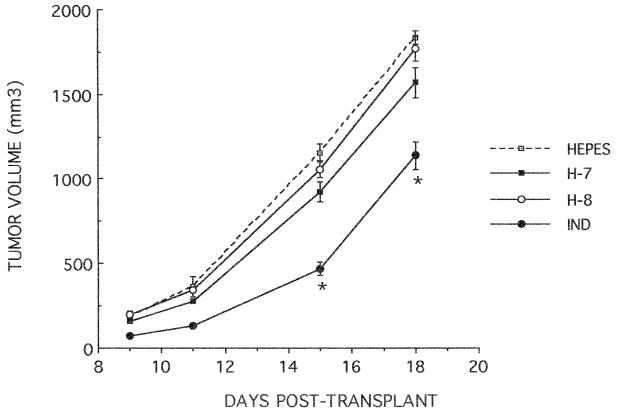

Fig. 1. Tumor growth. Measurements of tumor size were made in mice treated with HEPES (100 μl) or 2 mg kg^{-1} H-7, H-8 or IND from day +9 until day +18 after tumor cell transplant. Vernier calipers were used to measure the largest (*D*) and smallest (*d*) diameters of the hindleg tumor mass. Tumor volume was calculated by the following formula: $V = D \times d^2/2$. Statistical significances ($* P < 0.005$) with respect to the control group (HEPES) were found in the IND-treated group, when analyzed by the Bonferroni's test.

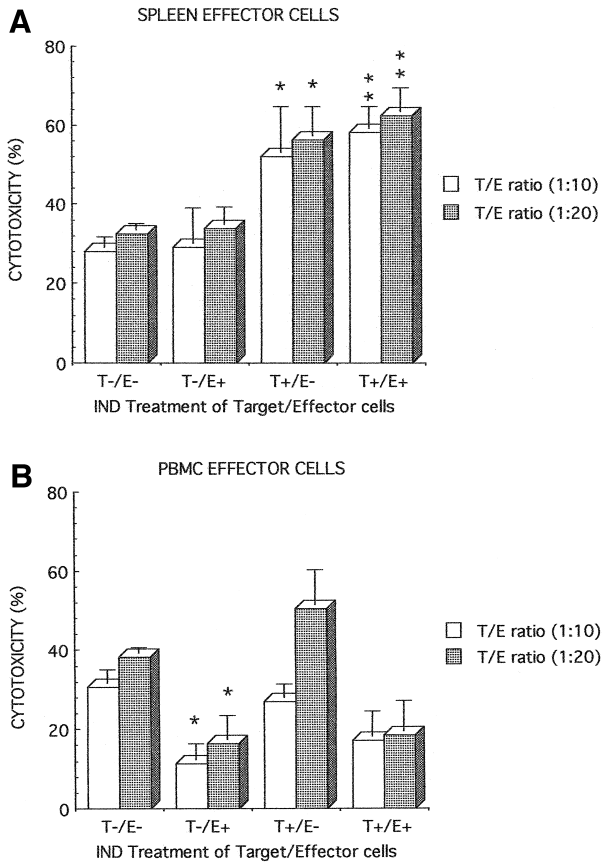

Fig. 2. Effect of IND on cellular cytotoxicity against tumor cells. Percentage cytotoxicity obtained with splenocytes (A) and PBMC (B) from non-tumor bearing animals, after i.p. treatment with a daily dose (4 days) of IND (2 mg kg^{-1}) or 100 μl HEPES (E+ or E−, respectively), against non-treated or IND in vitro-treated (10^{-6} M) tumor cells (T− or T+, respectively). The corresponding tumor cell (T+ or T−) and mice (E+ or E−) treatments are indicated under each column. Cytotoxicity was evaluated using the DNA-binding dye, Hoechst 33342, release assay, and the T/E ratios were 1:10 and 1:20. Statistical significance, calculated with respect to the HEPES-treated group (control, T−/E−), was evaluated by the Student's *t*-test ($* P < 0.05$, $** P < 0.01$).

3.3. Cytotoxic response

We studied the cytotoxic activity of effector cells (splenocytes or PBMC) from healthy treated (E+) or non-treated (E−) mice against 3LL tumor cells respectively treated (T+) or non-treated (T−) with each different pharmacological agent (H-7, H-8 or IND). The results indicated that (Fig. 2A) IND-treated target cells are significantly ($P < 0.05$) more sensitive to the cytotoxic activity of spleen effector cells (T+/E−) than are non-treated tumor cells (T−/E−) at both 1:10 and 1:20 T/E ratios. However, these increases were not significant when PBMC effector cells were used (Fig. 2B). On the other hand, treatment of mice with IND does not significantly modify the cytotoxicity of effector cells (T−/E+) with respect to non-treated mice (T−/E−). However, the incubation of IND-treated tumor cells and effector cells from IND-treated mice (T+/E+) likewise yielded a significantly ($P < 0.01$) higher percentage of cytotoxicity with respect to the controls (T−/E−). In a similar way, the in vitro treatment of 3LL tumor cells with H-8 increased their sensitivity to the cytotoxic activity of effector cells (T+/E−). The results were significant ($P < 0.05$) at 1:10 and 1:20 T/E ratios (Fig. 3A and B)—mainly when splenocytes were used as effector cells. This suggests that an increase in cytotoxic activity might be implicated in a diminished tumor cell capacity to progress toward metastases but the tumor growth inhibition mediated by IND could be associated with inhibitory effects on mechanisms that facilitate tumor growth. Although the percentage of cytotoxicity of splenocytes from H-7-treated mice was higher than that among the controls, neither H-7-treated tumor cells (T+/E−) nor treated mice (T−/E+ or T+/E+) showed significantly increased cytotoxicity (Fig. 4A and B).

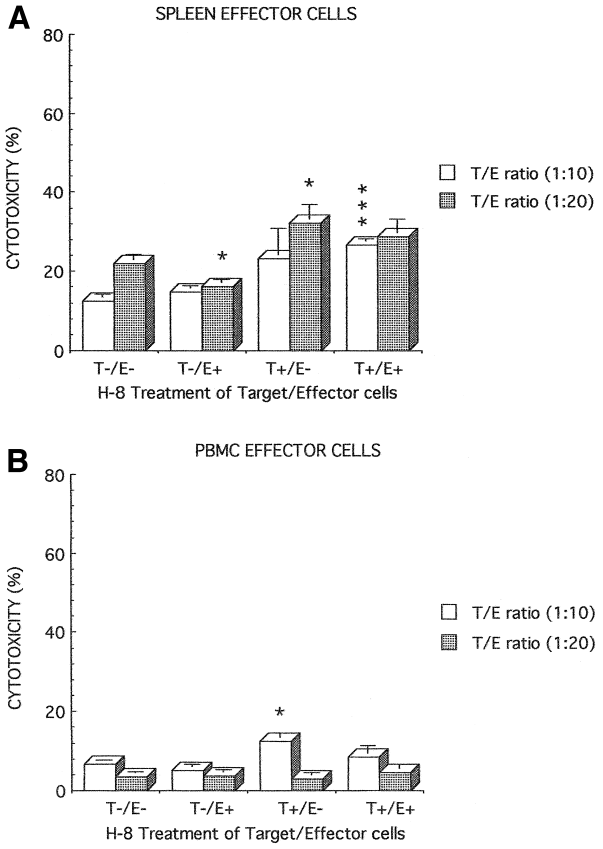

Fig. 3. Effect of H-8 on tumor cells and effector cells from healthy mice. Percentage cytotoxicity obtained with splenocytes (A) and PBMC (B) from non-tumor bearing animals, after i.p. treatment with a daily dose (4 days) of H-8 (2 mg kg^{-1}) or 100 μl HEPES (E+ or E−, respectively), against non-treated or H-8 in vitro-treated (10^{-5} M) tumor cells (T− or T+, respectively). The corresponding tumor cell (T+ or T−) and mice (E+ or E−) treatments are indicated under each column. Cytotoxicity was evaluated using the DNA-binding dye, Hoechst 33342, release assay, and the T/E ratios were 1:10 and 1:20. Statistical significance, calculated with respect to the HEPES-treated group (control, T−/E−), was evaluated by the Student's *t*-test (∗ $P < 0.05$, ∗∗∗ $P < 0.005$).

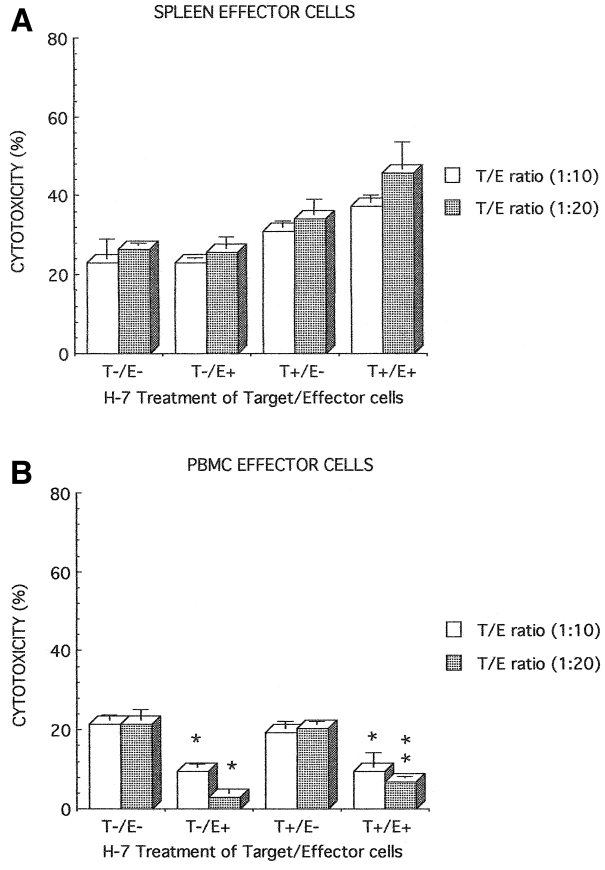

Fig. 4. Effect of H-7 on tumor cells and effector cells from healthy mice. Percentage cytotoxicity obtained with splenocytes (A) and PBMC (B) from non-tumor bearing animals, after i.p. treatment with a daily dose (4 days) of H-7 (2 mg kg^{-1}) or 100 μl HEPES (E+ or E−, respectively), against non-treated or H-7 in vitro-treated (10^{-5} M) tumor cells (T− or T+, respectively). The corresponding tumor cell (T+ or T−) and mice (E+ or E−) treatments are indicated under each column. Cytotoxicity was evaluated using the DNA-binding dye, Hoechst 33342, release assay, and the T/E ratios were 1:10 and 1:20. Statistical significance, calculated with respect to the HEPES-treated group (control, T−/E−), was evaluated by the Student's *t*-test (∗ $P < 0.05$, ∗∗ $P < 0.01$).

4. Discussion

The prevention of metastases is the main objective of anti-tumor therapy, as systemic dissemination is the most common cause of anti-tumor therapy failure (Sugarbaker et al., 1982; Fidler, 1990). Tumor-bearing animals are characterized by a partial or total suppression of immune defenses. This is due in part to an insufficient activity of cytotoxic effector cells against tumor cells as a result of activation of suppressor cells or the release of compounds with inhibitory functions (Watson et al., 1991; Ochoa et al., 1994). In this sense, endogenous prostaglandins (mainly secreted by macrophages and tumor cells) may exert an immunosuppressor effect in tumor-bearing mice (Ben-Efraim, 1992). Although prostaglandins are ubiquitous tissue hormones, exerting pleiotropic effects on cancer cells, their mechanism of action at the molecular and cellular level is not yet clear. Prostaglandins may exert their effect through the activation of adenylate cyclase, resulting in an intracellular increase of cyclic-AMP (Fulton et al., 1991). This induces the activation of protein kinase A, which in turn leads to inhibition of the synthesis of key cytokines involved in the cellular immune response. From a pharmacological point of view, strategies can be designed to interrupt prostaglandin immunosuppressive activity. The present study with 3LL tumor-bearing mice concerned the effect of treatment with a PG synthesis inhibitor (IND) and two cAMP-dependent protein kinase inhibitors (H-7 and H-8), on the spontaneous metastatic potential of 3LL and host cellular immune response against the tumor.

The IND treatment of mice produced a smaller metastatic response than did the control treatment (HEPES), but the decrease was not significant. IND is able to increase the specific cytotoxic ability of the immune system in healthy mice and cause a significant breakdown of tumor growth to yield a smaller tumor mass. These results agree with results of prior experiments both in vitro and in vivo, obtained by us and other groups (Aliño et al., 1989; Shiff et al., 1996; Planchon et al., 1995; Lupulesku, 1996; Connolly et al., 1996). IND has shown anti-tumoral activity in many experimental (Aliño et al., 1989; Fulton, 1987; Fulton and Levi, 1980; Lynch et al., 1978; Plescia et al., 1975; Powles et al., 1973) and human models (Rolland et al., 1980; Al-Saleem et al., 1980; Panje, 1981). Nevertheless, controversial results have been reported with this agent. In some experiments, IND in vivo inhibits tumor genesis (Alexandrov et al., 1996), while in others it actually increases tumor proliferation and induces tumor cells to disseminate and give rise to new metastatic foci (Noguchi et al., 1995). Our previous experimental studies show that IND enhances the anti-tumoral host response (Aliño et al., 1989, 1992), though it could also cause tumor cells to spread (Aliño et al., 1990, 1991, 1992). Thus, pro-metastasizing effects of IND lessen its merit as an anti-tumor agent.

Considering that prostaglandins exert their effect through protein kinase A activation, the selective inhibition of protein kinase A might be expected to disrupt the signal transduction mediated by immunosuppressor prostaglandins, thereby blocking the inhibitory effects at least partially. The results show that the treatment of 3LL tumor-bearing mice with H-7 and H-8 significantly diminished the ability of tumor cells to form lung metastases, though both failed to inhibit primary tumor growth. On the other hand, in vitro treatment of 3LL tumor cells with H-8 makes them more sensitive to the effector cytotoxic cells of healthy mice (splenocytes), in a way similar to the effect of IND. This suggests that H-8 could interfere with tumor progression through the inhibition of prostaglandin signal immunosuppressor pathways. Nevertheless, determination of PKA activity would confirm the effect of these agents at the molecular level. Moreover, we do not know whether this effect can be exclusively attributed to the increased cytotoxic activity in 3LL tumor-bearing mice. In addition, tumor-bearing mice treated with H-7 developed significantly fewer metastases than did the control group, but H-7 proved less effective as an anti-metastatic agent than H-8. Although this effect has been due to the partial inhibitory action on protein kinase A, the cytotoxic response obtained with H-7 failed to reach significantly higher levels than among the controls, and tumor growth did not progress towards a lesser tumor mass volume. Thus, we observed that the protein kinase A inhibitor, H-8, inhibits tumor progression and increases tumor cell sensitivity to cytotoxic effector cells. This treatment is of significant importance in tumor-bearing animals, and opens a new pharmacological pathway for boosting host anti-tumor response while limiting tumor progression.

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