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Inhibition of angiogenesis, tumour growth and metastasis by the NO-releasing vasodilators, isosorbide mononitrate and dinitrate

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1 The effect of the nitric oxide (NO)-producing nitrovasodilators isosorbide mononitrate (ISMN) and isosorbide dinitrate (ISDN) were assessed on (a) the *in vivo* model of angiogenesis of the chick chorioallantoic membrane (CAM) and (b) on the growth and metastatic properties of the Lewis Lung carcinoma (LLC) in mice.

2 Isosorbide 5-mononitrate (ISMN) and isosorbide dinitrate (ISDN), inhibited angiogenesis in the CAM dose-dependently. ISMN was more potent in inhibiting this process. Both compounds were capable of completely reversing the angiogenic effect of α-thrombin. These effects of ISMN and ISDN on angiogenesis were comparable to those previously observed with sodium nitroprusside which generates NO non-enzymatically.

3 Mice, implanted intramuscularly with LLC, received daily i.p. injections of ISMN for 14 days resulting in a significant decrease in the size of the primary tumour and a reduction in the number and size of metastatic foci in the lungs. ISDN had a similar but less pronounced effect than that observed with ISMN.

4 Addition of ISMN or ISDN to cultures of bovine, rabbit and human endothelial cells and to cultures of LLC cells had no effect on their growth characteristics.

5 These results indicate that ISMN and ISDN inhibit angiogenesis and tumour growth and metastasis in an animal tumour model. The possibility should therefore be considered that these nitrovasodilators which are widely used therapeutically and have well characterized pharmacological profiles, may also possess antitumour properties in the clinic.

Keywords: Isosorbide 5-mononitrate; isosorbide dinitrate; nitric oxide; vasodilators; tumour growth; metastasis; angiogenesis

Introduction

In the last decade nitric oxide (NO) has been shown to be an omnipresent intercellular messenger with a remarkably wide repertoire of biologicial actions. Its role as a major regulator in the nervous, immune and cardiovascular systems as well as in pathophysiological states (septic shock, hypertension, stroke and neurodegenerative diseases) is increasingly appreciated (Bredt & Snyder, 1994; Gross, 1995). Recently, it was shown that in the *in vivo* chick chorioallantoic membrane (CAM), the spontaneous generator of NO sodium nitroprusside (SNP) (Ignarro *et al.*, 1981) and the NO precursor L-arginine (Palmer *et al.*, 1988) inhibited, while the NO-synthase inhibitors N^G-monomethyl-L-arginine (L-NMMA) and N^G-nitro-L-arginine methylester (L-NAME) (Moncada *et al.*, 1991), stimulated new vessel formation (angiogenesis) (Pipili-Synetos *et al.*, 1994). These observations suggested that NO may be an endogenous suppressor of angiogenesis.

Angiogenesis, in the mature human, is usually limited to the reproductive cycle and the wound healing process. This type of angiogenesis proceeds in an orderly and highly regulated manner and is self-limiting (Folkman & Singh, 1992; Maragoudakis, 1993). All other forms of angiogenesis in the adult organism are pathological with the primary example of the growth and metastasis of solid tumours where the development of a vascular network is a prerequisite for both processes to occur. Angiogenesis has therefore been recognised as a potential target for controlling tumour growth and metastasis, leading to a search for inhibitors of angiogenesis as an alternative less toxic therapeutic intervention (Maione & Sharpe, 1991).

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Based on the above, the objective of the present study was to examine whether NO, being an inhibitor of angiogenesis, might also inhibit the growth of primary tumours and the development of metastasis in mice implanted with Lewis Lung carcinoma (LLC). For this purpose the long acting vasodilators (and widely used antianginal drugs) isosorbide dinitrate (ISDN) and isosorbide mononitrate (ISMN) which act through NO release (Feelisch & Noack, 1987) were used and their antiangiogenic, antitumour and antimetastatic ability assessed.

Methods

The *in vivo* CAM angiogenesis model, initially described by Folkman (1985) and modified as previously reported (Maragoudakis *et al.*, 1988) was used. Briefly, fresh fertilized eggs were incubated for 4 days at 37°C when a window was opened on the egg shell exposing the CAM. The window was covered with sterile cellophane tape and the eggs were returned to the incubator until day 9 when the test materials were applied. The test materials or vehicle and 0.5 μCi [U-^{14}C]-labelled proline, were placed on sterile plastic discs and were allowed to dry under sterile conditions. The control discs (containing vehicle and radiolabelled proline) were placed on the CAM 1 cm away from the disc containing the test material. A sterile solution of cortisone acetate (249 nmol/disc) was routinely incorporated in all discs in order to prevent an inflammatory response. The loaded and dried discs were inverted and placed on the CAM, the windows were covered and the eggs incubated until day 11 when assessment of angiogenesis took place.

Biochemical evaluation of angiogenesis

Collagenous proteins represent 80% of the total basement membrane proteins formed by the CAM during the chick embryo development (Maragoudakis *et al.*, 1988). The extent of their biosynthesis has been shown to correlate well with new vessel formation. This biosynthesis reaches a maximum between days 8 and 11 and coincides with maximal angiogenesis in the CAM as shown by morphological evaluation of vascular density. Furthermore, at day 10, collagenous protein biosynthesis is 11 fold higher than that of day 15 when angiogenesis has reached a plateau. Biochemical evaluation of newly formed vessels was performed by determining the extent of collagenous protein biosynthesis in the CAM lying directly under the discs. Briefly, the area under the disc was cut off, placed in an appropriate buffer and protein biosynthesis was stopped. Non-protein bound radioactivity was removed by washing with trichloroacetic acid. Discs containing radioactivity were resuspended and subjected to collagenase digestion. The resulting radiolabelled tripeptides corresponding to basement membrane collagen and other collagenous material synthesized by the CAM from [U-^{14}C]-proline, were counted and expressed as c.p.m. mg^{-1} protein.

Morphological evaluation of angiogenesis

For morphological evaluation, eggs were treated as above in the absence of radiolabelled proline. At day 11 the eggs were flooded with 10% buffered formalin, the plastic discs were removed and the eggs were kept at 37°C until dissection. A large area around the discs was cut off and placed on a glass slide and the vascular density index (expressed as number of blood vessels) was measured by the method of Harris-Hooker *et al.* (1983). Harris-Hooker evaluation underestimates by approximately 10% (compared to the biochemical evaluation of angiogenesis) the changes in the vascular network. This is an expected limitation of this method as some vessels are probably collapsed and do not show up under the stereoscope.

Determination of NO release from the CAM in vitro

The CAM from day 9 embryos was used for the determination of NO release *in vitro*. CAM from 20 eggs was dissected into 4 pieces each into a Petri dish containing Hanks balanced salt solution (HBSS) pH 7.4. Thirty six (36) of these pieces were then divided between three beakers (control and two treatment groups) containing 10 ml of HBSS alone (wet weight of tissue 0.83 g), or the appropriate amounts of ISMN (wet weight of tissue 0.83 g) or ISDN (wet weight of tissue 0.94 g) dissolved in HBSS and were maintained at room temperature. Samples were taken 5 min after the introduction of the tissue into the HBSS, according to the following protocol: 100 µl from each sample were added to polypropelene vials containing the reaction mixture which consisted of H_2O_2 (500 µM), luminol (30 µM) and an appropriate amount of HBSS to make up a total volume of 500 µl. The vial was then stirred vigorously and the emission was recorded in a Berthold Autolumat LB953 luminometer. Chemiluminescence peaks were converted to nmol of NO by fitting them on to a standard curve constructed with increasing concentrations of pure NO as previously described (Delikonstantinos *et al.*, 1995). Results are expressed as nmol g^{-1} of wet weight of tissue.

Lung Lewis carcinoma and endothelial cell proliferation assay

Lewis lung carcinoma was maintained by sequential transplantation in C57BL mice. In preparation for the proliferation assay, tumours were allowed to grow in mice for 14 days when they were excised and cells were obtained as previously described (Kline & Platonova, 1980). The cells were suspended in RPMI containing 10% foetal bovine serum and antibiotics and were seeded in 1 ml aliquots containing 5,000 cells into 12-well plates.

Bovine brain capillary endothelial cells, human umbilical vein endothelial cells, bovine cortex endothelial cells and bovine aortic endothelial cells were obtained as previously described (Gospodarowicz *et al.*, 1986). They were then suspended in DMEM containing 10% newborn calf serum and antibiotics and they were seeded in 1 ml aliquots containing 5,000 cells into 12-well dishes.

All cells received bFGF (2.5 ng ml^{-1}) every other day plus either buffer (controls) or the indicated concentrations of ISMN or ISDN. Cells were counted after 6 days with a Coulter particle counter. Values are expressed as % of control and are the means of duplicate determinations which varied by $<10\%$ of the mean.

Animal studies

Male C57BL mice, 6–8 weeks old, (obtained from the Experimental Laboratory of Theagenion Cancer Institute) were acclimatized and caged in groups of four or less. All mice were fed on animal chow *ad libitum*.

Animals with tumours ranging between 0.8–1.5 cm^3 were killed and the skin of the right hind leg overlaying the tumour was cleaned with betadine. Tumours were then excised under sterile conditions and a suspension in normal saline was subsequently obtained by mincing the tumour with scissors and passing it through a series of sequentially smaller hypodermic needles of 22–30 gauge. The final concentration was adjusted to 2×10^7 cells ml^{-1} and the suspension was kept on ice. After the site was cleaned with ethanol, the right hind leg was inoculated subcutaneously with 2×10^6 cells in 0.1 ml. The animals were allowed to rest for 48 h and were subsequently divided into the indicated number of groups which received daily intraperitoneal injections (0.2 ml) of either treatment (ISMN or ISDN dissolved in sterile normal saline containing 2.5% DMSO) or vehicle (2.5% DMSO in sterile normal saline).

After 14 days the animals were killed and tumour size was measured with a microvernier using the formula $(a\times b\times c)/2$ cm^3 (where a = length, b = width and c = depth at the site of inoculation). The primary tumours along with the lungs were then excised and fixed in buffered formalin.

Histological examination of the lung specimens

The fixed lung specimens were embedded in paraffin according to standard histological procedures and four sections were made through each specimen. They were then stained with haematoxylin-eosin. Evaluation of metastasis in the fixed and stained lungs was performed by microscopic examination of the number of tumour foci in each animal. Mean number of foci was calculated by dividing the sum of the number of metastases for each animal within a group, by the number of animals in the group.

Materials

The following drugs were used: collagenase type VII from *Clostridium histolyticum*, cortisone acetate, bFGF (Sigma Chemical Co., Poole Dorset). L-[U-^{14}C]-proline (specific activity 273 mCi mmol^{-1}) was obtained from New England Nuclear (Boston, MA). α-Thrombin was a gift from Dr J. W. Fenton and had the following characteristics: specific activity 3287 units mg^{-1} protein, active site concentration 5.3×10^{-5} M (Fenton *et al.*, 1991). Isosorbide 5-mononitrate (ISMN, containing 20% lactose) and isosorbide dinitrate (ISDN, containing 60% lactose) were a gift from ELPEN (Athens, Greece) and were dissolved in 2.5–10% DMSO. Tissue culture media, antibiotics, newborn and bovine calf serum were obtained from ICN Flow Labs (U.K.). Plastic discs used were 13 mm round tissue culture coverslips from Nunc Inc. (Naperville, IL, U.S.A.).

Fresh fertilized eggs were obtained locally (Ioannina, Greece) and kept at 10°C before incubation at 37°C.

Calculation and statistics

For each egg, collagenous protein biosynthesis under the disc containing the test material or vehicle, was calculated as c.p.m. mg^{-1} protein. Collagenous protein biosynthesis, or number of vessels, under the disc containing the test material, was then expressed as % of that under the control disc. The results were analyzed by Student's paired or unpaired *t* test and *n* signifies the number of eggs or animals for each treatment. The graphics and statistics were performed using the Slide-WritePlus for Windows computer programme.

Results

Effect of ISMN and ISDN on angiogenesis in the CAM in vivo

Isosorbide mononitrate (ISMN) from 25–210 nmol/disc caused a dose-dependent inhibition in basal (unstimulated) angiogenesis as shown by a decrease in collagenous protein biosynthesis (Figure 1). This inhibition ranged between 3.7±7.0%–37.4±9.1% of control (n=7–10). Isosorbide dinitrate (ISDN) from 25–210 nmol/disc also caused an inhibition in basal angiogenesis ranging between 11.7±3.1%–22.3±2.6% of control (n=10–14) (Figure 1). It can be seen that ISMN was more potent in inhibiting angiogenesis than ISDN. In these experiments morphological evaluation performed by the method of Harris-Hooker *et al.* (1983) showed that the vascular densities under the discs containing 210 nmol of ISMN or ISDN were reduced by 22.5±3.3% (n=7, P<0.001) and 21.6±4.8% (n=5, P<0.001) respectively. Sodium nitroprusside (SNP) which acts through NO formation, has been shown to reverse completely the angiogenic effect of α-thrombin (Pipili-Synetos *et al.*, 1994). Similarly both ISMN and ISDN (at 210 nmol/disc) were capable of completely reversing the angiogenesis promoting effect of α-thrombin (6.7 nmol/disc) (Figure 2a, 2b). Under these conditions the combination of α-thrombin with ISMN or ISDN caused a 15.1±8.1% and 11.9±6.2% (n=9–10) decrease in collagenous protein biosynthesis compared to a 62.4±13% (n=10) increase in this parameter caused by α-thrombin alone.

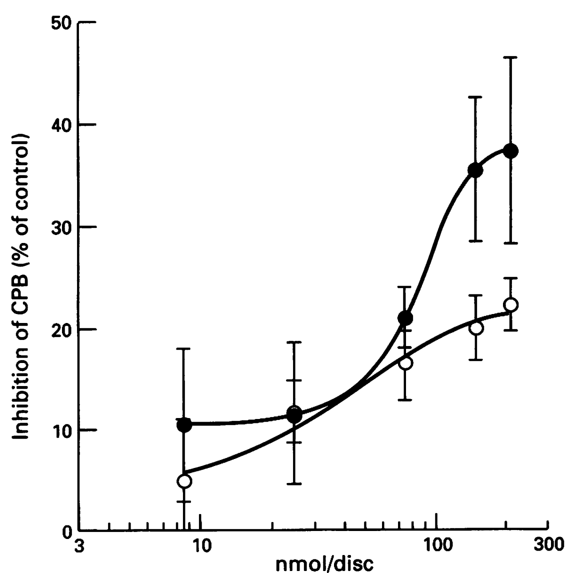

Figure 1 Effect of isosorbide mononitrate (●) and dinitrate (○) on angiogenesis in the chick chorioallantoic membrane *in vivo*, expressed as collagenous protein biosynethesis (CPB). Results are expressed as mean±s.e mean % of control. The number of observations *n* is indicated in the text.

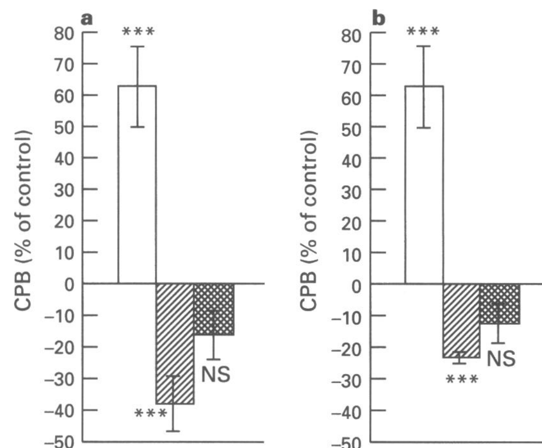

Figure 2 Reversal of the angiogenic effects (expressed as collagenous protein biosynthesis, CPB) of (a) α-thrombin (THR) (6.7 nmol/disc) by 210 nmol/disc isosorbide mononitrate (ISMN) (a) or 210 nmol/disc isosorbide dinitrate (ISDN) (b) in the chick chorioallantoic membrane *in vivo*. In (a) THR, open column; ISMN, hatched column; ISMN+THR stippled column; in (b) THR, open column; ISDN, hatched column; ISDN+THR, cross-hatched column. Results are expressed as mean±s.e mean % of control and are compared by paired *t* test. Asterisks denote statistical significance of the calculated percentage difference between control and test. ***P<0.01. The number of observations *n* is indicated in the text.

Effect of ISMN and ISDN on NO release from the CAM in vitro

The CAM *in vitro* released NO in the absence of any stimulus. This release was 253 nmol g^{-1} of wet weight of tissue. In the presence of 5×10^{-6} M ISMN or ISDN, NO release was 302 and 410 nmol g^{-1} tissue respectively. These amounts indicate that almost the entire amount of the vasodilators was converted to NO within the first 5 min of incubation of the tissue. These observations were obtained from a total of 20 eggs.

Effect of ISMN and ISDN on endothelial cell cultures

ISMN or ISDN, at concentrations ranging from 0.78–50 μM, had no significant effect on the proliferation of b-FGF-stimulated endothelial cells from bovine brain capillaries, human umbilical vein, bovine adrenal cortex capillaries and bovine aorta (Figure 3a, 3b).

Effect of ISMN and ISDN on Lung Lewis carcinoma primary tumours and pulmonary metastasis

ISMN injected daily at 200 μg/animal caused a significant reduction in the size of primary LLC in mice (Figure 4). Fourteen days after implantation, the size of the primary tumours implanted on the right hindleg was 3.3±0.31 cm^3 (n=20) for the control group compared to 1.4±0.14 cm^3 (n=16, P<0.01) for the group which received 200 μg ISMN daily. Increasing the amount of the daily injection to 600 μg/animal did not cause a further reduction in the size of the tumours (2.7±0.33 cm^3, n=11). Under these conditions the effect of ISMN, although still statistically significant (P<0.05), was less pronounced.

The lungs of the animals which received 200 μg were examined for the presence of metastatic foci. Metastatic foci were counted in each individual animal from a control group of 8 and from a group of 10 which received 200 μg ISMN (Figure 5). It was found that ISMN reduced the number of metastases in the lungs to 0.70±0.25 foci/animal compared to 2.25±0.42 foci/animal in the control group and this reduction was stastically significant (P<0.01).

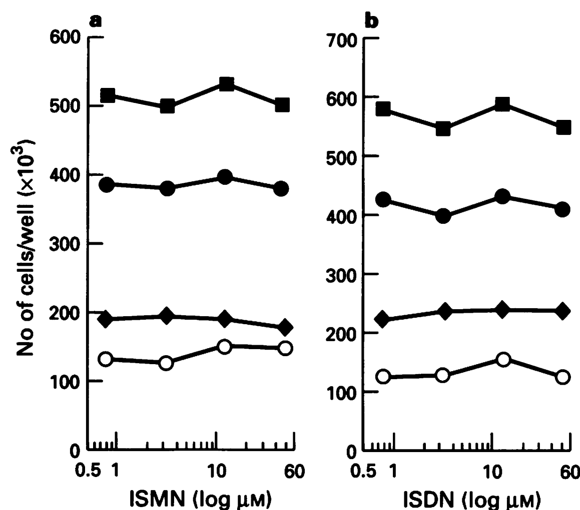

Figure 3 Effect of 0.5–50 μM isosorbide mononitrate (ISMN) (a) or isosorbide dinitrate (ISDN) (b) on the proliferation rate of bFGF (2.5 ng ml^{-1})-stimulated endothelial cells from bovine brain (●), aorta (■) and adrenal cortex (◆) and from human umbilical vein (○). Values are expressed as % of control and are the means of duplicate determinations which varied by < 10% of the mean.

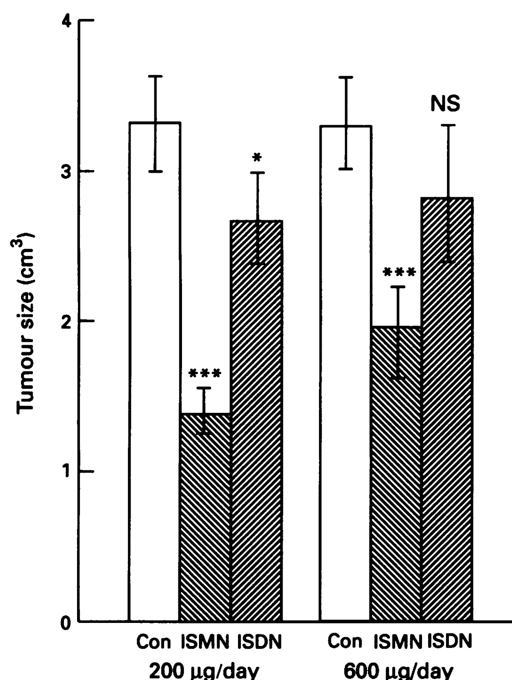

Figure 4 Effect of 200 μg and 600 μg of isosorbide mononitrate (ISMN) or isosorbide dinitrate (ISDN) injected daily (for 14 days) into mice inoculated with 2×10^6 cells of LLC, on tumour size (measured with a microvernier using the formula $(a \times b \times c)/2\,cm^3$, where a = length, b = width and c = depth at the site of inoculation). Results are expressed as mean ± s.e cm^3 and are compared to the controls (con) by unpaired *t* test. Asterisks denote statistical significance between control and test. ***$P < 0.01$, *$P < 0.05$. The number of animals *n* is indicated in the text.

ISDN (200 μg/animal) had a similar but smaller effect on both the size of the primary tumour as well as on the number of metastatic foci in the lungs (Figure 4). The size of the primary tumour was reduced to 1.95 ± 0.27 cm^3 ($n = 16$) compared to 3.3 ± 0.31 cm^3 ($n = 20$) and this reduction was statistically significant ($P < 0.01$). Daily injection of 600 μg/animal had no significant effect on the size of the primary tumour 2.8 ± 0.49 cm^3, $n = 9$, Figure 4). The number of metastatic foci in the lungs of the animals receiving 200 μg daily was reduced to 1.75 ± 0.46 foci/animal, $n = 8$, compared to 2.25 ± 0.42 foci/animal ($n = 8$) found in the control group

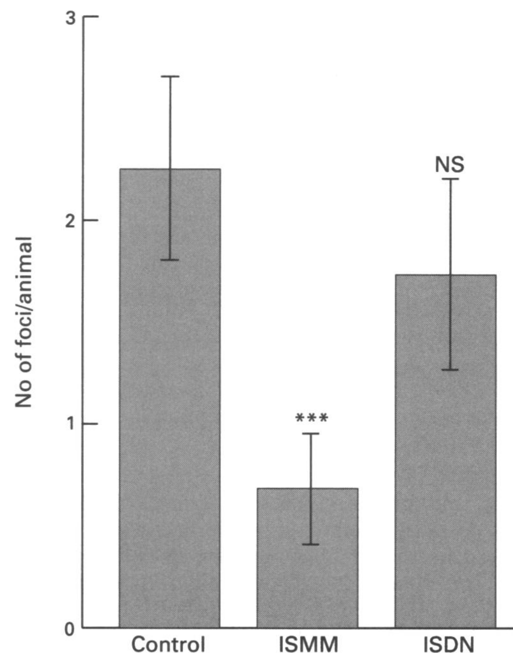

Figure 5 Effect of 200 μg of isosorbide mononitrate (ISMN) or isosorbide dinitrate (ISDN) injected daily (for 14 days) into mice inoculated with 2×10^6 cells of LLC, on the development of pulmonary metastatic foci. Mean ± s.e number of foci was calculated by dividing the sum of the number of metastases for each animal within a group, by the number of animals in the group; tests are compared to the controls by unpaired *t* test. Asterisks denote statistical significance between control and test. ***$P < 0.01$. The number of animals *n* is indicated in the text.

(Figure 5). This reduction was not statistically significant. It can be seen that the effects of ISDN on both the size of the primary tumour as well as on the pulmonary metastasis were less pronounced than those of ISMN.

When LLC cells were grown in culture, neither ISMN nor ISDN (from 0.5–50 μM) had any effect on the proliferation of these cells (data not shown).

Discussion

In the present study it was shown that the NO-generating anti-anginal vasodilators, ISMN and ISDN (Harrison & Bates, 1993) were capable of inhibiting angiogenesis in the CAM *in vivo* (in agreement with previous work from this laboratory showing that NO is an endogenous antiangiogenic mediator, Pipili-Synetos *et al.*, 1994), and tumour growth and metastasis in mice implanted with LLC. This latter effect is in agreement with recent findings where the NO synthase inhibitor N^G-nitro-L-arginine ethyl ester (L-NAME) potentiated pulmonary metastasis of B16 melanoma or Lung Lewis carcinoma cells injected into mice via the tail vein (Yamamoto *et al.*, 1994) and suggests a role for the endogenously produced NO in the metastatic process. The CAM *in vivo*, is a system which may not be disturbed between the time of the application of the test substance and the time of assessment of angiogenesis. In addition, as these time points are 48 h apart, determination of NO at the end time point could not be taken as a direct effect of the vasodilator applied on the disc. However, since (a) NO release from the CAM *in vitro* increases in the presence of ISMN and ISDN and (b) in most other systems the biological effects of organic nitrates are directly linked to their ability to activate soluble guanylate cyclase through NO release (Feelisch & Noack, 1987; Harrison & Bates, 1993), it is likely that these compounds exert their antiangiogenic effect through NO formation. In view of the fact that both tumour growth and metastasis depend on new vessel formation, the antitumour and antimetastatic effect of both agents may be a consequence of their antiangiogenic properties. Furthermore, neither compound af-

fected the proliferation of LLC cells in culture, an observation which indicates that the antitumour effect of ISMN/ISDN is not a consequence of a direct action on tumour growth.

The antiangiogenic effect of both agents was modest under basal conditions (37% and 23% respectively). However, they were capable of completely reversing the α-thrombin-induced 60% increase to 15% decrease in angiogenesis. Under stimulated conditions therefore these compounds confer a strong, inhibition which in the present study amounts to a net effect of 75%. The precise mechanism underlying this effect has not been clarified in the present study. However, the fact that neither compound had any effect on the growth of vascular endothelial cells from four different anatomical sites, indicates that their inhibitory effect on angiogenesis is not due to inhibition of endothelial cell proliferation. Yang *et al.* (1994) and Ziche *et al.* (1994) recently reported that SNP and nitroglycerin inhibited and stimulated endothelial cell proliferation, respectively. The reason for the discrepancy between our data and the data of these authors, although not entirely clear, could be attributed to the fact that in the present study, (a) bFGF-stimulated endothelial cells were used (b) different NO-releasing compounds were studied and (c) different endothelial cells were used. Since new vessel formation normally involves stimulation by various growth factors, including bFGF (Folkman & Singh, 1992), the protocol used here seems pertinent, if endothelial cell proliferation is to be associated with angiogenesis. Furthermore the members of the vasodilator family exhibit a considerable variation in their biological activity which is mainly due to the multiple pathways responsible for their biotransformation in different tissues (Harrison & Bates, 1993). ISMN was more effective than ISDN in terms of both antiangiogenic and antitumour-antimetastatic ability. Although both nitrates contain nitrogen in an oxidation state of five and thus require enzymatic reduction to release NO (Harrison & Bates, 1993), ISDN is more potent in terms of activating guanylate cyclase and releasing NO, compared to ISMN (Feelisch & Noack, 1987). On the other hand, ISMN possesses a longer half-life than ISDN (5 h compared to 48 min, Martindale, the Extra Pharmacopeia, 1982; 1989) in the circulation, since it is not subjected to hepatic inactivation. Since both the experimental systems used here were *in vivo* systems, the longer half-life appears to be the crucial factor as far as the effectiveness of the two compounds was concerned.

The observed anti-tumour effects were considerably reduced when the amount of drug injected daily was increased from 200 to 600 μg/animal. This could be due to the fact that the effect of ISMN or ISDN on tumour growth consists of two components. (a) The antiangiogenic effect, causing deprivation of the nutrients necessary for the tumour to grow and (b) the vasodilator effect, facilitating the transport of nutrients and thus causing the opposite effect. In addition, vasodilatation of a parent vessel is believed to be an early event in angiogenesis, causing the endothelial cells to stretch and thus become responsive to bFGF and other growth factors (Folkman & Singh, 1992). The net effect will therefore depend on the balance between the two opposing actions and will be determined by the relative potency of ISMN or ISDN as inhibitors of angiogenesis and vasodilators respectively. An alternative explanation could be that 600 μg might be sufficiently high to cause tolerance. A common problem associated with the long term use of nitrovasodilators is the development of rapid tolerance (Abrams, 1980). This however is normally the result of frequent administration of the drugs and even then recovery can be accomplished by overnight periods of no therapy. The experimental protocol used here involved a single daily injection of ISMN or ISDN with sufficient periods of rest (24 h). Therefore, although haemodynamic parameters were not determined, the occurrence of tolerance was not considered as a viable alternative explanation.

For a tumour to grow beyond a few mm^3 and metastasize, it must switch to an angiogenic phenotype (Folkman & Singh, 1992). This can be achieved by a shift in the balance between inducers and inhibitors of angiogenesis which may be formed either by the tumour itself or by cells recruited by the tumour. The fact that increasing the availability of NO through NO-releasing compounds inhibits angiogenesis in the CAM as well as tumour growth and metastasis, suggests that NO may be one of the endogenous angiogenic suppressors which is down-regulated when neovascularization is initiated. The evidence provided in the present study, linking the inhibitory effect of NO on tumour growth and metastasis, to inhibition of angiogenesis, is indirect. In order to be able to establish a meaningful relationship between number of vessels/tumour area in treated and untreated animals and attribute it to a direct antiangiogenic effect of the nitrovasodilators, a positive control using a known equipotent antitumour agent devoid of any antiangiogenic activity should be included. Such experiments form the basis of further studies and will be carried out in the immediate future.

The possible mechanisms for the antiangiogenic effect of NO, with the exclusion of endothelial cell proliferation, may involve interactions with vascular elements other than the endothelial cells. A likely target for NO might be the blood platelet since both platelet aggregation and adhesion are inhibited by NO (Radomski & Moncada, 1991). Angiogenesis and tumour growth and metastasis are processes which are believed to involve platelet activation (Tsopanoglou *et al.*, 1993; Rickles & Edwards, 1983; Karpatkin *et al.*, 1988; Gasic *et al.*, 1968; 1973; Pearlstein *et al.*, 1984; Nierodzik *et al.*, 1991; 1992; Honn *et al.*, 1992). Activated platelets may adhere to the vascular endothelium, increase cell permeability and initiate proliferative phenomena through the release of growth factors (Page, 1988) which are well characterized angiogenic molecules (Folkman & Singh, 1992). Antibody-induced thrombocytopaenia has been shown to reduce markedly metastases in experimental tumour models including LLC (Gasic *et al.*, 1968; Pearlstein *et al.*, 1984). However, the effect of antiplatelet agents such as aspirin and prostacyclin, on metastasis has been controversial or non-reproducible (Gasic *et al.*, 1973; Honn *et al.*, 1981; Karpatkin *et al.*, 1988). Interestingly, in all these studies, the antiplatelet agents used had little or no effect on platelet adhesion in contrast to NO which inhibits this process. Karpatkin *et al.* (1988) were able to show that agents inhibiting the interaction between platelets and adhesive proteins such as factor VIII and fibronectin, were able to inhibit pulmonary metastases in mice induced by three different tumour cell lines. Furthermore, Brooks *et al.* (1994) have been able to establish a strong connection between the expression of the vascular integrin $a_v\beta_3$ (the endothelial cell receptor for factor VIII and fibronectin) and angiogenesis in the CAM. It is therefore likely that the effects of NO in angiogenesis and tumour growth and metastasis are at least partly mediated via inhibition of platelet adhesion (a) to the vascular wall and/or (b) to tumour cells creating aggregates which then interact with the vascular wall. The fact that NO-releasing compounds completely reverse the angiogenic effect of thrombin further supports the above hypothesis since thrombin, a major platelet activator, has been shown to enhance platelet-tumour adhesion (Nierodzik *et al.*, 1991; 1992) and metastasis in tumour animal models.

It is noteworthy that both NO and thrombin exhibit a different profile regarding their effect in neovascularization when they are applied in the rabbit corneal assay of angiogenesis compared to their effects in the CAM. In the corneal assay, thrombin alone is unable to stimulate angiogenesis (Knighton *et al.*, 1982) whereas SNP potentiates new vessel formation induced by either prostaglandin E_1 or substance P (Ziche *et al.*, 1994). The angiogenic effect of thrombin is in fact restored when it is combined with platelets and stimulates their release. The rabbit cornea is a tissue devoid of blood vessels whereas the CAM is a vascularised one. This structural difference may have several implications on the pathways mediating angiogenesis in the two systems, the most important being the involvement of platelets.

In conclusion, the NO-releasing compounds ISMN and ISDN inhibited angiogenesis in the CAM and tumour growth and pulmonary metastasis in mice implanted with LLC. The effect of these compounds is independent of endothelial cell

growth and does not involve direct inhibition of tumour growth. The underlying mechanism for these effects may involve platelet activation and this is currently under investigation. In view of the fact that both ISMN and ISDN have been widely used with well characterized pharmacological properties and free of the unwanted effects of the commonly used antiproliferative antitumour agents, they should be investigated as potential antitumour drugs in the clinic.

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