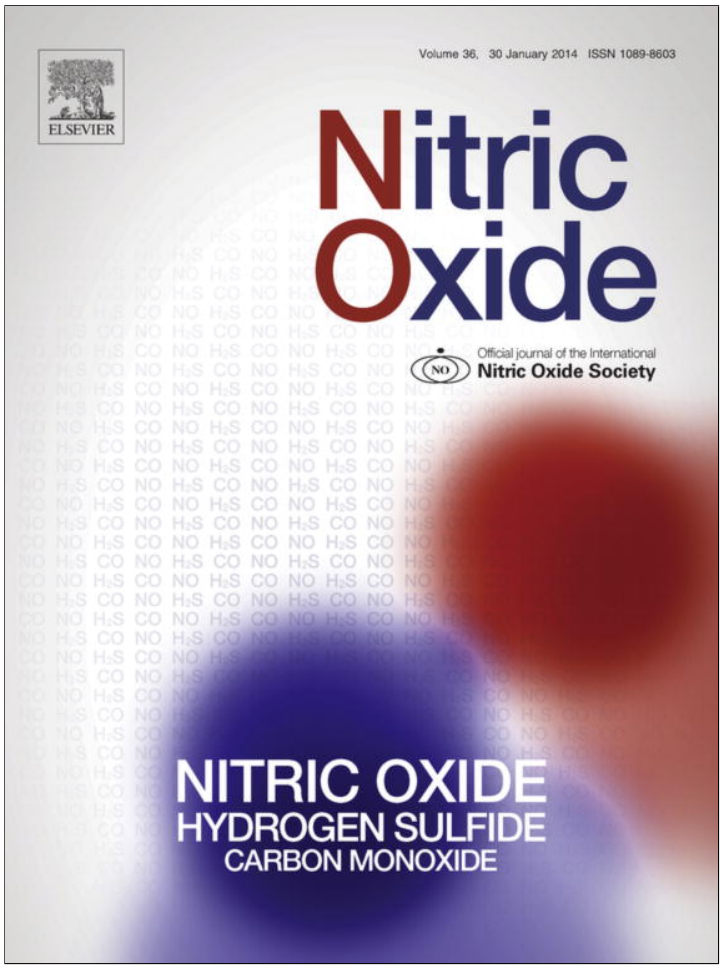

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Inhibition of nitric oxide is a good therapeutic target for bladder tumors that express iNOS

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ABSTRACT

Bladder cancer is the second cause of death for urological tumors in man. When the tumor is nonmuscle invasive, transurethral resection is curative. On the other hand, radical cystectomy is the treatment chosen for patients with invasive tumors, but still under treatment, these patients have high risk of dying, by the development of metastatic disease within 5 years. It is therefore important to identify a new therapeutic target to avoid tumor recurrences and tumor progression. Nitric oxide (NO) is an important biological messenger known to influence several types of cancers. In bladder cancer, production of NO and expression and activity of inducible NO synthase was associated to recurrence and progression. The objective of this work was to analyze if inhibition of nitric oxide production could be considered a therapeutic target for bladder tumors expressing iNOS. Using a bladder cancer murine model with different invasiveness grade we have demonstrated that NO inhibition was able to inhibit growth of bladder tumors expressing iNOS. Furthermore, invasive properties of MB49-I orthotopic growth was inhibited using NO inhibitors. This paper also shows that levels of NO in urine can be correlated with tumor size.

In conclusion, inhibition of NO could be considered as a therapeutic target that prevents tumor growth and progression. Also, urine NO levels may be useful for measuring tumor growth.

Introduction

Bladder cancer (BC) ranks fifth in malignant tumor frequency and second as cause of death from genitourinary cancer in men [1]. Transitional bladder tumors originated from urothelial cells, are the most frequent type of BC, and are classified as non muscle invasive (NMI) when tumors are confined to mucosa (pTa) or to lamina propria (pT1), and as muscle invasive when the detrusor muscle is affected. At the time of diagnosis 80% of tumors are NMI and if the adjacent and distant urothelium is histological normal, transurethral resection is curative. However, recurrences are common in BC, and in many cases progression to muscle invasion may occur. The invasive bladder tumors (pT2–pT4) require more aggressive therapies involving radical cystectomy and/or chemo and radiotherapy [2].

Although many groups have attempted to clarify the biological mechanisms that lead to tumor invasion [3,4], these mechanisms are not yet fully known. Deregulation of extra cellular proteolysis involving plasminogen activator system, matrix metalloproteinase (MMPs) and Cathepsins among others [5,6] are implicated in the invasion process. Recently, our laboratory has developed an orthotopic invasive BC murine model useful to study mechanisms involved in tumor progression. Orthotopic NMI and invasive bladder tumors are generated by electro-cauterization of the bladder wall and subsequent instillation of MB49 and MB49-I bladder cancer cells, respectively. *In vitro*, the MB49-I cell line showed higher invasive properties than MB49 associated with an increase of proteolytic enzymes such as metalloproteinase 9 (MMP-9) and urokinase-type plasminogen activator (uPA) [7]. Both tumor cell lines were able to produce nitric oxide (NO) and express the inducible NO synthase (iNOS) isoform; however, the invasive MB49-I showed higher iNOS expression than the MB49 cell line [8].

NO has been shown to have dichotomous effects on cell proliferation, apoptosis, migration, invasion, angiogenesis and many

Abbreviations: BC, bladder cancer; NMI, nonmuscle invasive; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; NO, nitric oxide; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; L-NAME, N-nitro-L-arginine-methyl-esterHCl; CM, conditioned medium; DAPI, 4′,6-diamino-2-phenylindole.

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other important processes involved in cancer biology. It has shown to be both pro- and anti-tumorigenic, depending on its concentration, time of exposure and tumor microenvironment [9]. The two isoforms that are constitutively expressed (neuronal, nNOS and endothelial, eNOS) produce nano molar NO levels. By the contrary, iNOS isoform, produces higher NO levels, in a micro molar order, in response to inflammatory stimuli [3]. In physiological conditions when the stimuli stop the expression and activity of iNOS decreases. However, if iNOS activity is prolonged in time, carcinogenesis induction, tumor growth and/or tumor progression may occur [10]. Concordantly, it is known that a continuous overexpression of iNOS leads to DNA damage, increased cell proliferation, tumor vascularity and metastatic potential [11].

We previously reported that iNOS, that was not expressed in human normal bladder epithelium, has been found in almost 50% of patients with bladder cancer and, associated with early recurrences [12]. Furthermore, iNOS expression was associated with invasive human BC [8]. Taking into account the relation between invasion and iNOS expression in BC, and the fact that NO could be involved in the activation of MMPs and angiogenesis activities [13,14], it is reasonable to hypothesize that the inhibition of NO should be a good approach in the treatment of patients with bladder tumors expressing iNOS.

In this study, using the MB49/MB49-I murine model, which mimics human disease, we have investigated the role of tumor cell derived iNOS on cell proliferation, migration, proteolytic activity, as well as tumor growth, angiogenesis, and experimental metastasis.

Materials and methods

Cell culture

The murine BC cell line, MB49, the invasive cell line MB49-I and MBT2, were cultured in RPMI-1640 (GIBCO 31800-14). Culture media were supplemented with 2 mM L-glutamine, 80 μg/ml gentamicin and 10% fetal bovine serum FBS as described [15].

Cell viability assay

5×10^4, MB49, MB49-I or MBT2/ml were cultured in 96-well plates with RPMI1640 + 10% FBS. After 24 h incubation, N-nitro-L-arginine-methyl-esterHCl (L-NAME, Sigma) (2 mM) in RPMI supplemented with 2% FBS was added. Cells were cultured for 48 h and cell viability was determined by the MTS assay (Promega, G5421).

Conditioned medium (CM), tumor homogenate preparation and cell lysates

Semiconfluent monolayers growing in 35 mm plates were treated or not with L-NAME (2 mM) for 24 h. Culture medium was replaced by serum-free medium. After 24 h CM was harvested and the numbers of cells in the remaining monolayers were quantified. CM was stored at −80 °C. Tumors or subconfluents monolayers were homogenized in buffer containing 50 mM tris–HCl (pH 8), 100 μg/ml NaCl and 1% Triton. Tissues were homogenized twice for 10 s at 70% power using an Ultra-Turrax® T25. Protein content was determined by the Bradford method.

Western blot assay

Proteins (80 μg) from the different homogenates were electrophoresed on 10% of SDS–PAGE and then transferred to PVDF membranes, as previously [16]. iNOS (1:200) (Abcam 15323), pERK (1:200) (sc-7383), ERK (1:200) (sc-135900), pAKT (1:1000) (sc-7985-R), AKT (1:1000) (sc-8312) primary antibodies were used. Densitometric units of pAKT or pERK were referred to corresponding band of AKT or ERK respectively. Densitometric units of iNOS were referred to the correspondent band of beta-actin (1:20,000) (Sigma A5441). Values were referred as folds of change of control.

MMP and uPA activity

MMP and uPA were determined in CM as previously described [17]. MMP activity was expressed in arbitrary units (AU)/10^6 cells. Caseinolytic uPA activity was referred towards the urokinase standard curve (range 0.1–10 IU/ml). Results are shown in urokinase international units (IU) /10^4 cells.

Cell migration

To analyze the effect of NOS inhibition on cell migration, 1×10^5 cells were seeded on 6-well plates. After 24/48 h, when confluence was reached, a wound with a tip was done in the monolayer and then photographed. Monolayers were then treated or not with L-NAME (2 mM) in RPMI + 2% FBS and after 18 h the same area was photographed. Cell migration was assessed by determining the covered area by the difference between final and initial wound, considering 20 fields per plate.

Nitric oxide (NO) determination

Subconfluent MB49, MB49-I or MBT2 monolayers were treated with or without 2 mM L-NAME. NO was determined in culture supernatant 24 h later using the Griess reagent, as described previously [18].

Immunofluorescence assay

MB49 or MB49-I cells were grown in chamber slides. Both cell lines were treated or not with L-NAME (2 mM) for 24 h. Immunofluorescence was performed as previously described [16] using iNOS (Abcam 15323) (1:200) antibody and rabbit IgG as isotype control. Anti-rabbit-Alexa-488 conjugate (Invitrogen) (1:1000) was used as secondary antibody. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma).

Heterotopic Tumor growth

MB49 or MB49-I cells (2.5×10^5 cells in 0.1 ml) and MBT2 (2×10^6 cells in 0.1 ml) were subcutaneously (s.c) injected into the left flank of syngeneic mice (C57BL/6 J or C3H, respectively) as previously described [18]. Animals were randomly divided into groups that received no treatment (control) or those receiving L-NAME in drinking water (0.5 g/L) (8 mice per group). Tumor growth was registered twice a week by measuring two perpendicular diameters, and the formula $(D \times d)^{1/2}$ (mm) was used to calculate tumor growth, where D is the longer diameter and d is the shorter one. C57BL/6 J mice (approximately 6 weeks old) were obtained from animal facility of the Institute of Oncology Angel H. Roffo (Buenos Aires, Argentina) and C3H mice were obtained from National Academy of Medicine (Buenos Aires, Argentina).

Orthotopic Tumor Growth

Orthotopic MB49 or MB49-I (2.5×10^4 cell/mouse) tumors were developed as described [7] in the bladder of C57BL/6 J female mice. Animals were randomly divided into groups that received no treatment (control) or those receiving L-NAME in drinking water (0.5 g/L) (8 mice per group). Mice were monitored twice weekly for hematuria. Urine was collected at the end of the experiment,

centrifuged and NO was determined using Griess reactive, as described above. Two weeks after tumor cell inoculation mice were sacrificed; bladders were weighed and fixed in 10% formaldehyde, embedded in paraffin and processed for histology. Tumor size was established by determining the weight of the bladders before fixing.

Histology

Conventional H&E for tumor diagnosis and immunohistochemistry for iNOS expression were performed as previously [16]. iNOS (AbCam15323) antibody was used at 1:200 dilution.

Angiogenic Assay

C57BL/6 mice were inoculated intra-dermally (i.d). in both flanks, with 2×10^4 MB49 or MB49-I in RPMI 1640 medium with a drop of Trypan Blue, to identify the inoculation site. The animals were randomly divided into groups that received no treatment (control) or those receiving L-NAME in drinking water (0.5 g/L). Normal skin inoculated only with Trypan Blue was a negative control (8 mice per group). Five days post inoculation mice were sacrificed, the skin was carefully separated from the underlying tissues and the inoculum areas were examined and photographed under magnification (16×). The images were transferred to a computer and the angiogenesis is reported as number of vessels/mm^2 skin.

Experimental metastasis

Male mice were injected in the tail vein with 2.5×10^4 MB49 or MB49-I cells in RPMI 1640 medium. Animals were randomly divided into control and L-NAME group (10 mice per group). Ten days after inoculation mice were sacrificed and examined for superficial lung metastasis. Lungs were removed and fixed in Bouin solution, and the number of surface nodules was determined under stereoscopic microscope at 6.5×.

Ethics statement

Mice were handled in accordance with the international procedure for Care and Use of Laboratory Animals. Protocols were approved by the Institutional Review Board CICUAL, protocol number 2012/02, Institute of Oncology Angel H. Roffo.

Statistical analysis

Three independent experiments were always carried out and only one representative is shown. Results are expressed as the mean and SD or as median and range. Statistically significant values were compared using Student's *t*-test, Kruskal–Wallis or ANOVA and Bonferroni's contrast, using the 3.01 Graph Pad InStat statistical packages. *p* Values lower than 0.05 were considered statistically significant.

Results

L-NAME reduces NO production and iNOS expression

Although MB49-I cells produced more NO that MB49, L-NAME treatment significantly reduced NO levels in both tumor cell lines (Fig. 1A). This decrease in NO levels was not only due to the inhibition of NOS enzymatic activity, but also to a reduction in its expression (Fig. 1B and C). Consistent with these *in vitro* results, we also detected a reduced expression of iNOS in MB49-I tumors growing *in vivo* by L-NAME administration (Fig. 3A). Different from MB49 and MB49-I, MBT2, another bladder cancer cell line didn't express iNOS, and did not produce NO (Fig. 1A and B).

L-NAME inhibits in vitro proliferation of MB49 and MB49-I cancer cell lines

The NOS inhibitor L-NAME significantly inhibited *in vitro* cell viability of MB49 and MB49-I; however, MBT2 cells viability was unmodified (Fig. 2A). Similar results were observed *in vivo*, where oral L-NAME administration was able to inhibit the s.c growth of MB49 and MB49-I tumors without modifying MBT2 tumor growth (Fig. 2B and C). To evaluate if MAPK and PI3 K, involved in proliferation and survival pathways, participate in MB49 and MB49-I growth modulation by NO, the phosphorylation of the two key effector proteins: ERK and AKT, were assayed. L-NAME only decreased ERK phosphorylation in both cell lines, while AKT phosphorylation was not affected (Fig. 2D). L-NAME, also inhibit ERK phosphorylation in MB49 and MB49-I *in vivo* tumors (Fig. 2E).

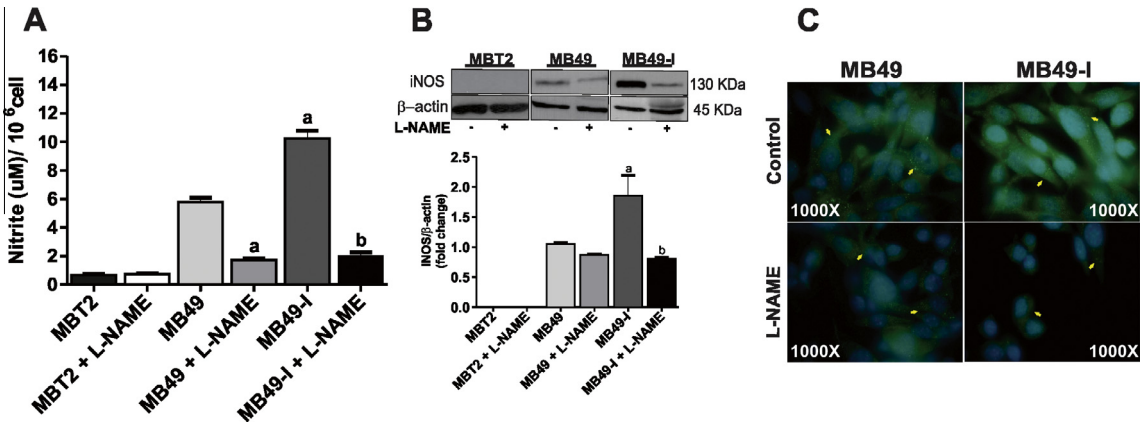

Fig. 1. L-NAME reduced NO production and iNOS expression. (A) NO production was determined in the supernatants of MBT2, MB49 and MB49-I cells treated *in vitro* with L-NAME (2 mM) by Griess reagent. ANOVA: a: $p < 0.001$ vs. MB49; b: $p < 0.001$ vs. MB49-I. (B) Western Blot revealed higher iNOS expression in MB49-I than in MB49 cells. Treatment with L-NAME (2 mM) for 24 h decreased iNOS expression in MB49-I. Densitometric units of iNOS were determinate using analysis software, relativized to beta-actin and referred as a fold of change of control. a: $p < 0.001$ vs. MB49, b: $p < 0.001$ vs. MB49-I. (C) Immunofluorescence staining of MB49 and MB49-I treated with L-NAME (2 mM) for 24 h revealed with anti-iNOS. Cytoplasmic expression (yellow arrow) in MB49-I is greater than in MB49. Cytoplasmic mark is reduced in L-NAME treatment. Magnification 1000× (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

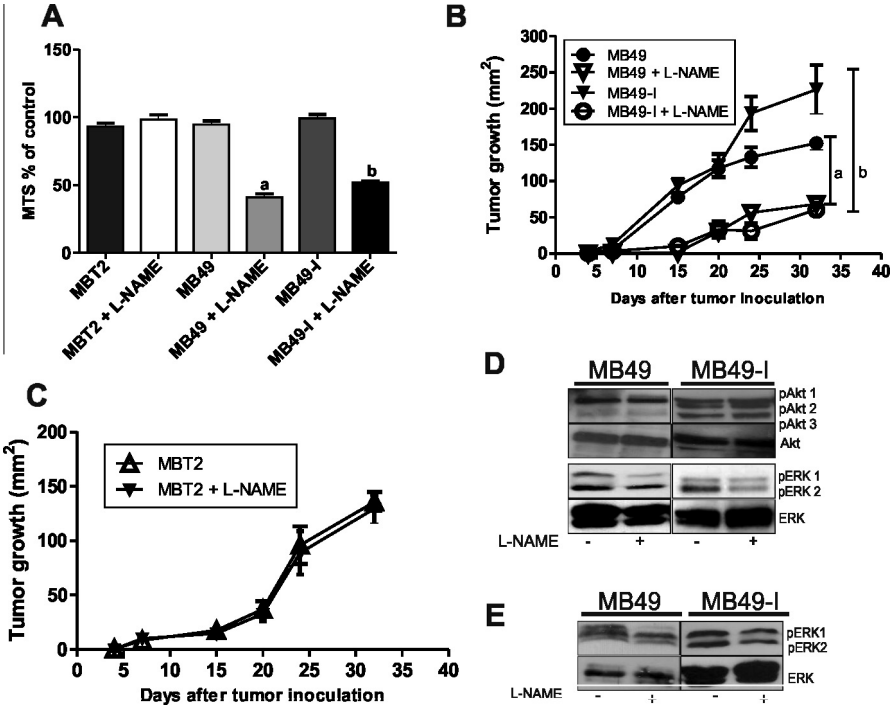

Fig. 2. L-NAME inhibits proliferation *in vitro* of MB49 and MB49-I but not in MBT2 bladder cancer cell lines. (A) MBT2, MB49, and MB49-I cell lines were treated with L-NAME (2 mM) for 48 h. Cell viability was evaluated by a non-radioactive method (MTS). Cell growth was relativized to control. ANOVA: a: $p < 0.001$ vs. MB49; b: $p < 0.001$ vs. MB49-I. (B) Curve of growth of subcutaneous MB49 and MB49-I tumors. Tumors were treated with normal water (Control) or L-NAME (0.5 g/L in drinking water). a: $p < 0.05$ vs. MB49, b: $p < 0.01$ vs. MB49-I (C) Curve of growth of subcutaneous MBT2 tumors. Tumors were treated with normal water (Control) or L-NAME (0.5 g/L in drinking water). (D) Western Blot from MB49 and MB49-I cells homogenates treated with L-NAME (2 mM) to determinate phosphorylation levels of AKT and ERK. (E) Western Blot from MB49 and MB49-I tumors homogenates from control or L-NAME tumor bearing mice to determinate phosphorylation levels of ERK.

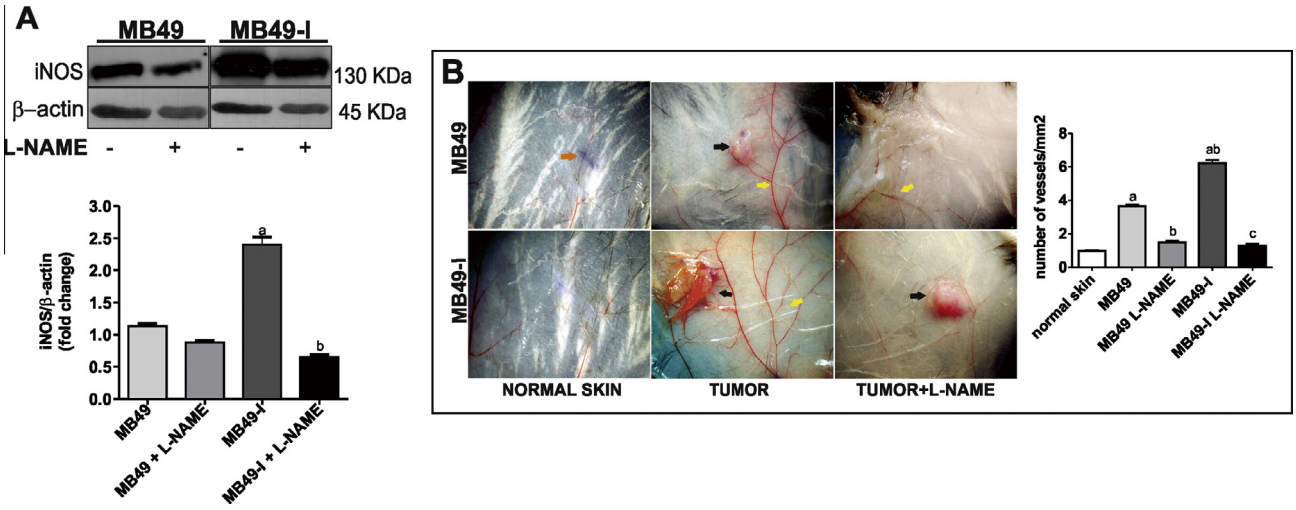

Fig. 3. L-NAME inhibits iNOS expression and angiogenesis *in vivo*. (A) Western Blot revealed higher iNOS expression in MB49-I than in MB49 tumors. Treatment with L-NAME (2 mM) for 24 h decreased iNOS expression in MB49-I. Densitometric units of iNOS were determinate using analysis software, relativized to beta-actin and referred as a fold of change of control. a: $p < 0.001$ vs. MB49, b: $p < 0.001$ vs. MB49-I. (B) Angiogenic assay *in vivo*. Orange arrows indicate inoculation area, yellow arrows vessels and black arrows indicate the tumor (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

L-NAME inhibits proteolytic enzyme activity and invasive migration capacity

It has been previously shown that the invasive BC cell line MB49-I produces higher levels of proteolytic enzymes MMP/9 and uPA than the non-invasive MB49 cell line [7]. Activity of MMP2/9 and uPA was significantly inhibited by L-NAME only in MB49-I cells, while it did not produce any effect in MB49. An *in vitro* wound healing assay was made to analyze the implications of NO in the migratory ability of cancer cell lines. Our results showed that MB49-I had higher migratory capacity than MB49, and that NO inhibition reduced migration only in MB49-I.

Since proteolytic enzymes and migratory activity are associated with tumor progression and metastases, we evaluated the role of NO on *in vivo* experimental lung metastases. Our results showed that oral administration of L-NAME significantly inhibited metastases

generated by MB49-I tumors. Since MB49 originate very few metastatic foci, we could not detect any effect of L-NAME (Table 1).

L-NAME inhibits tumor angiogenesis in vivo

Intra-dermal inoculation of MB49 or MB49-I cells induced an important angiogenic response compared with normal skin; moreover, MB49-I was able to induce the formation of a greater number of blood vessels than MB49 cells. It is noteworthy that tumors generated in these assays showed differences in their size, being MB49-I greater than MB49. Treatment with L-NAME inhibited angiogenesis generated by both cell lines, and also incipient tumor growth (Fig. 3B).

L-NAME inhibits MB49 and MB49-I orthotopic tumor growth

In accordance to what we detected in s.c tumors, oral administration of L-NAME significantly reduced both orthotopic MB49 and MB49-I tumor growth, evaluated as bladders weight (Fig. 4A). Inhibition of tumor growth was confirmed by histopathological studies: only 14% of MB49 and 17% of MB49-I tumor bearing mice treated with L-NAME showed tumor cells, analyzed by H&E staining (Fig. 4B). Just a few tumor foci were detected in bladders from L-NAME tumor bearing mice. Furthermore, while in MB49-I tumors the invasion of muscular layer was demonstrated, in tumors from L-NAME treated mice no muscle invasion was detected (Fig. 4E).

Since MB49 and MB49-I tumors express iNOS (Fig. 3A) and produce NO *in vitro* (Fig. 1A), it is reasonable to assume that increased levels of NO in urine from tumor bearing mice may be found. Therefore, nitrite, a final product of NO, was assayed in urine of these mice. Our results showed that MB49-I tumor bearing mice had significantly higher levels of nitrite than MB49 tumor, and both higher than normal urines. In both cases, nitrite levels in urine decreased significantly by L-NAME treatment (Fig. 4C). A positive correlation between nitrite levels and tumor growth was observed,

Table 1
MMP2/9 activity determined by zimography were signicantly higher in MB49-I than in MB49 cells (a: $p < 0.001$), and inhibits by L-NAME in MB49-I (b: $p < 0.001$). Similarly uPA activity were higher in MB49-I than in MB49 cells (a: $p < 0.001$) and inhibited by L-NAME (b: $p < 0.001$). Migration in wound healing was obtained by differences between initial area and final area. MB49-I were significantly more migratory than MB49 (c: $p < 0.05$). L-NAME inhibits migration of MB49-I (d: $p < 0.001$).

	MMP-9 activity AU/10^6 cel/24h Mean ± SD	MMP-2 activity AU/10^6 cel/24 h Mean ± SD	uPA activity IU/10^4 cel/24 h Mean ± SD	Migration in wound Healing Mean ± SD	Lung metastasis/ mice*
MB49	1.45 ± 0.10	1.02 ± 0.11	1.47 ± 0.02	102 ± 54	5 (1–150)
MB49 + L-NAME	1.50 ± 0.15	1.57 ± 0.15	1.81 ± 0.03	78 ± 35	3 (1–110)
MB49-I	2.16 ± 0.29[a]	1.7 ± 0.3	4.39 ± 0.03[a]	171 ± 23[c]	375 (100–590)[e]
MB49-I + L-NAME	1.02 ± 0.21[b]	0.001 ± 0.010[b]	1.63 ± 0.01[b]	39 ± 10[b]	100 (50–120)[f]

Numbers of lung metastases express as median (range), Kruskal–Wallis. MB49-I induces higher lung metastases than MB49 (e: $p < 0.001$). L-NAME inhibits MB49-I metastases formation (f: $p < 0.001$).
*Ten mice per group.

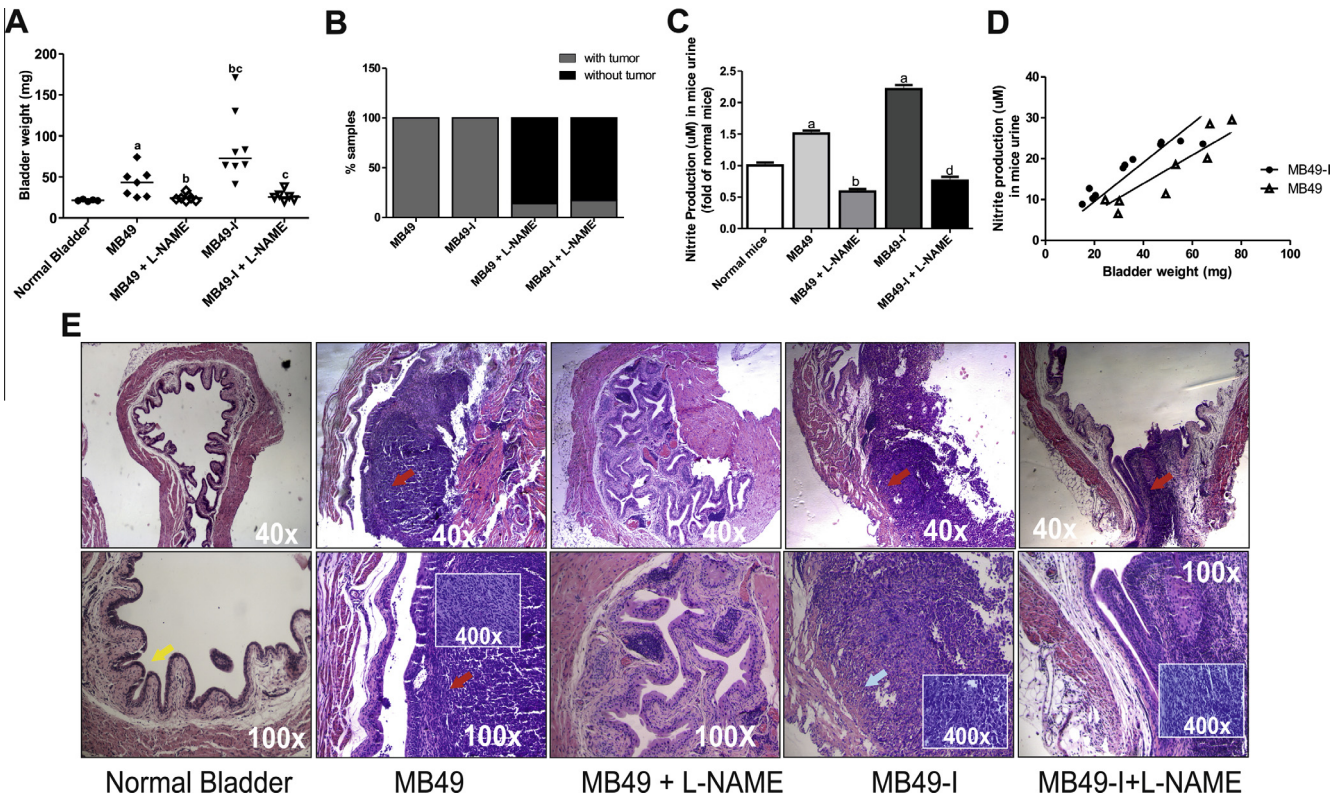

Fig. 4. L-NAME inhibits MB49 and MB49-I orthotopic tumor growth Tumor size is determined as the weight of the bladders. Mann–Whitney: a: $p < 0.05$ vs. normal bladder, b: $p < 0.05$ vs. MB49, c: $p < 0.01$ vs. normal bladder, d: $p < 0.001$ vs. MB49-I. (B) Percentage of mice with/without tumor. (C) NO production was determined in urine of MB49 and MB49-I bearing mice treated or not with L-NAME, by Griess reagent. Mann–Whitney: a: $p < 0.05$ vs. normal mice, b: $p < 0.01$ vs. MB49, c: $p < 0.01$ vs. MB49-I. (D) Lineal Correlation between nitrite production and bladder weight. R^2: 0.82 (MB49-I); 0.78 (MB49). (E) Hematoxilin–Eosine. Yellow arrow indicates normal epithelium, red arrow tumor and blue arrow muscle invasion area. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 4D) indicating that nitrite levels are closely related to tumor size.

Discussion

It has been described that NO affects tumor growth in a bimodal way. These dual effects of NO on cancer arise from its ability to regulate different events such as migration, invasion, angiogenesis that modify tumor growth and metastatic dissemination. It is well established that stimulatory or inhibitory role of NO on the carcinogenic process depends on the quantity produced and the characteristics of the tissue in which the NO is being produced. Thus, in general, low NO levels act as a signal transducer affecting physiological processes such as blood flow regulation and neurotransmission among others. On the contrary, high NO levels have cyto-toxic protective effects against pathogens and certain tumors [19].

iNOS is an inducible NO synthase, responsible for the release of high amounts of NO in a sustained manner for relatively long periods of time, associated with inflammatory processes [3]. The perpetuation of its activity is associated with tumor growth and progression as well as with the metastatic process [20–22]. Previous results from our laboratory are in concordance with these results, since we detected iNOS in cancer tissue from patients with bladder tumors associated with earlier tumor recurrence, invasion and histological grade, while it was not expressed in normal bladder urothelium [8,12]. We have developed a murine model that mimics human NMI and invasive bladder tumors with MB49 and MB49-I, respectively [7]. We showed that MB49 cell line produces lower levels of NO than MB49-I cells, in agreement with their levels of iNOS expression. It was described that transcription factor NF-kB, is an important target for activators or inhibitors of iNOS expression [23]. It have been shown that oxidative stress, LPS, TNF-α, can induced iNOS expression through the activation of NFkB, in different cells. On the other hand, the inhibition was observed at different levels such as direct capture of NF-kB by protein–protein interactions [24,25], inhibition of NF-kB nuclear translocation [26], among others. Through a gen reporter assay we did not observed a modulation of NF-kB with L-NAME, suggesting that this via was not involved in the reduction of the expression of iNOS that we observed. However, we cannot discard that secondary phenomenon can be involved. Since we determined only iNOS expression, we cannot discard that the other two isoforms, nNOS and eNOS, can be expressed in these tumor cell lines.

Using this model, where iNOS/NO pathway is associated with tumor aggressiveness, we evaluated if NO inhibition could be considered as an alternative approach to control tumor progression in bladder tumors expressing iNOS. As expected, our results showed that inhibition of iNOS expression and NO levels by L-NAME inhibited both MB49 and MB49-I cell proliferation, without affecting iNOS negative- MBT2 cells (Figs. 1 and 2). Interestingly, MBT2 cells grow much slower than MB49 and MB49-I in spite of the fact more cells were inoculated, supporting the hypothesis that higher iNOS is involved in tumor progression.

Consistent with our results, it has been shown that NO stimulates the proliferation of MDA-MB-231 and MCF-7 breast cancer cells [27], choriocarcinoma JEG-3 cells [28], ovarian carcinoma HOC-7 cells [29] and Glioma stem cells (GSC) [30]. On the other hand, T24 bladder cancer cells, which do not produce NO, are sensitive to the cytotoxic effects of NO [31]. Thus, according to our results and those mentioned above, we can speculate that NO could function as a growth factor for certain tumor cells.

Since a constitutive phosphorylation of ERK in MB49 and MB49-I was detected we may conclude that MAPK proliferation pathway was involved in both tumor growth. L-NAME was able to down-regulate this pathway, reducing tumor growth (Fig. 2).

Our observations agree with several reports that described that one of the mechanisms that participate in NO-mediated stimulation of cellular proliferation includes MAPK [32].

The metastases formation is a complex process that involves changes in the tumor cells to acquire greater migration and invasion capacity. There are numerous papers showing the promoting role of NO in tumor invasion and metastases, one is the B16-BL6 murine melanoma cells that produced large numbers of lung metastases in NOS2 +/+ than in NOS2−/− mice [33]. Also, nude mice injected with MDA-MB-231 breast cancer cells demonstrated significantly less bone metastases after L-NAME treatment [34]. It was also shown that human colorectal adenocarcinoma cells HRT-18 that express iNOS were more invasive than the non-iNOS expressing HRT-29 cells. If HRT-29 were treated with a NO donor, their invasiveness increased, while with a NO inhibitor, decreased [35].

Proteolytic enzymes such as MMPs and uPA are largely involved in tumor invasion. We previously demonstrated, accordingly with their karyotypes that MB49-I is more invasive and produce higher levels of proteolytic enzymes MMP-9 and uPA than the parental MB49 cell line, as it gains another chromosome 14, which contains genes that codified some proteolytics enzymes, associated with invasion [7,36]. We have now demonstrated that both cell lines also present MMP-2 activity. Both MMP-2/9 and uPA activity were higher in MB49-I than in the MB49 cell line. These activity was significantly inhibited by L-NAME only in invasive MB49-I. Induction of proteolytic enzymes by NO was described in other tumor cells. The treatment of colon adenocarcinoma cells with a NO donor, SNAP, increased MMP-2 and MMP-9 activity reinforcing the idea that NO promotes tumor invasion [37]. The migratory activity was also higher in MB49-I than in MB49 and L-NAME reduced migration only in the first cell line (Table 1). To evaluate metastases formation, we used an i.v inoculation of tumor cells, since in our model spontaneously metastases were only detected in MB49-I orthothopic tumors [7]. Although in i.v inoculation the intravasation process is not involved, it takes into account cell survival and extravasation in target organs. This extravasation is not only associated with cell migration and vessel permeability, also involved protelytic enzymes activities. Taking into account that MMPs and uPA activities were diminished in MB49-I + L-NAME, lower that MB49 + L-NAME, and lung metastases were higher in MB49-I + L-NAME we can not discard that other parameters such as cell migration, vascular permeability, tumor cell survival and growth rate, among others could be involved in metastases formation. In agreement with the *in vitro* results, i.v. inoculation of MB49-I generates higher experimental lung metastases than MB49 cells and L-NAME was able to inhibit MB49-I lung metastases.

The NO is an important mediator in angiogenesis, a primordial event for tumor progression. It was described that NO regulates angiogenesis through a functional switch involving thrombospondin-1 and that L-NAME inhibits angiogenesis blocking the endothelial cell differentiation into vascular tubes [38,39]. Our results show an important angiogenesis in MB49-I and MB49 cells when compared with normal skin. The magnitude of neovascularization correlates with the expression of iNOS, since MB49-I presents more vessels that MB49. Furthermore, NO inhibition reduced vascularization of both tumors and the expression of iNOS *in vivo*. As expected, inhibition of angiogenesis is accompanied by tumor growth reduction (Figs. 3 and 4).

Since orthotopic inoculation is a better approximation to evaluate tumor growth, we assayed the effect of L-NAME in this condition. In the same way we had observed it in *in vitro* and in s.c tumor growth, L-NAME significantly inhibits the orthotopic growth of both tumors (Fig. 4A). Importantly, and consistent with the inhibition of proteolytic enzymes and migration detected

in vitro, muscle invasion caused by MB49-I growing in the bladder, was also repressed through NO inhibition (Fig. 4D).

Previously, we have shown that patients with bladder tumors produced significantly higher urine NO values than the once produced by healthy individuals or by patients successfully treated with non-evidence of disease [40]. Now the orthotopic inoculation of bladder cancer cells in mice allows us to evaluate urinary levels of NO, related to tumor growth. As we had already observed in patients, NO levels were increased in tumor bearing mice compared with normal animals. Taking into account that the tumor is in the bladder, and that nitrite is the first component that it is formed we measure NO in urine as nitrite. In parallel with iNOS tumor expression, MB49-I has higher NO urine levels than MB49 tumor bearing mice. Furthermore, urine NO levels decreased significantly after the treatment with L-NAME (Fig. 4C), and positive correlation between NO levels and tumor size were observed, (Fig. 4D). These results indicate on the one hand that L-NAME inhibits tumor growth and on the other, that NO levels are closely related to tumor size, and could be used to monitor tumor growth.

It was described that specific inhibitors of iNOS such as aminoguanidine or 1400 W were able to inhibited tumor growth of syngeneic or human xenograft [41,42]. On the other hand, no effect was observed in a P22 carcinoma in rats [43]. Nevertheless we observed that 1400 W (5uM) was able to inhibited *in vitro* MB49 and MB49-I proliferation. Also, endovesical administration (5 uM, twice a week) inhibited tumor growth and reduced urine NO levels (data not shown), suggesting that this specific iNOS inhibitor also can be used.

In conclusion, the present results in a murine model and the previous results in patients show that iNOS expression is associated to tumor progression. The inhibition of NO production may be considered as a potential therapy to patients whose tumors express iNOS, since this approach was able to inhibit properties *in vitro* and *in vivo*, related to tumor progression such as proteolytic enzymes activity, migration, viability, angiogenesis, experimental metastases, tumor growth and invasion.

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