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Mechanisms of nitric oxide-mediated inhibition of EMT in cancer

Inhibition of the metastasis-inducer Snail and induction of the metastasis-suppressor RKIP

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Abbreviations: CDDP, cisplatin; DHMEQ, dehydroxymethylepoxyquinomicin; ERK, extracellular signal-regulated kinase; LNCaP, lymph node carcinoma of prostate; MAPK, mitogen-activated protein kinases; MEK, MAP kinase kinase; NFκB, nuclear factorκB; NIK, NFkappa-B-inducing kinase; TAK1, transforming growth factor-beta-activated kinase 1

The role of nitric oxide (NO) in cancer has been controversial and is based on the levels of NO and the responsiveness of the tumor type. It remains unclear whether NO can inhibit the epithelial to mesenchymal transition (EMT) in cancer cells. EMT induction is mediated, in part, by the constitutive activation of the metastasis-inducer transcription factor, Snail and EMT can be inhibited by the metastasis-suppressors Raf-1 kinase inhibitor protein (RKIP) and E-cadherin. Snail is transcriptionally regulated by NFκB and in turn, Snail represses RKIP transcription. Hence, we hypothesized that high levels of NO, that inhibit NFκB activity, may also inhibit Snail, induce RKIP and leading to inhibition of EMT. We show that treatment of human prostate metastatic cell lines with the NO donor, DETANONOate, inhibits EMT and reverses both the mesenchymal phenotype and the cell invasive properties. Further, treatment with DETANONOate inhibits Snail expression and DNA-binding activity in parallel with the upregulation of RKIP and E-cadherin protein levels. The pivotal roles of Snail inhibition and RKIP induction in DETANONOate-mediated inhibition of EMT were corroborated by both Snail silencing by siRNA and by ectopic expression of RKIP. The in vitro findings were validated in vivo in mice bearing PC-3 xenografts treated with DETANONOate. The present findings show, for the first time, the novel role of high, yet, subtoxic concentrations of NO in the inhibition of EMT. Thus, NO donors may exert therapeutic activities in the reversal of EMT and metastasis.

Introduction

Progress has been achieved in the molecular understanding of nitric oxide (NO) in a number of human diseases and its therapeutic applications. However, the effects of NO in cancer development and progression remain controversial, with reports in the literature as a potential anti- or pro-neoplastic agent. The biphasic role of NO in oncology seems to be dependent on its working microenvironment, including the responsiveness of the tumor type, the redox state of the reaction, as well as the final intracellular concentration and the duration of intracellular exposure to nitric oxide.[1-3] As such, abundant evidence in the literature suggests that low NO concentrations may act in tumor cells as enhancers of their survival, proliferation and growth by protecting them from apoptosis, whereas high NO concentrations exhibit cytotoxic effects by promoting DNA damage, protein dysfunctions, gene mutations and tumor cell death, all of which contribute to tumor regression.[4]

Controversy also exists on the role of NO in the regulation of stages participating in the metastatic process, with reports debating on its anti- vs. pro-metastatic activity. Metastasis initiates with the acquisition of invasive and migratory properties by the tumor cells, a process known as epithelial to mesenchymal transition (EMT). Low NO concentrations have been reported to induce tumor cell migration and invasion through the expression of angiogenic and lymphangiogenic factors, whereas, high concentrations of NO inhibit angiogenesis and metastasis.[1,4]

Nitric oxide donors mimic continuous production of endogenous NO in a wide range of time intervals (seconds to days). Thus, multiple biological and (pro- vs. anti-) metastatic responses

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are elicited from NO donors depending on the half-life, concentration and the type of tumor cells exposed to the compound. As such, exogenous administration of NO by NO donors in various tumor models has been reported to result in elevated MMP[5] and CXCR4,[6] expressions, as well as decreased tumor cell adhesion to extracellular matrix components,[7,8] all of which lead to induction of metastasis. On the other side, NO-releasing agents have been shown to suppress the metastatic tumor cell behavior in various in vitro and in vivo tumor models by decreasing angiogenesis and, therefore, tumor cell invasion.[9-13] The anti-invasiveness and anti-metastatic properties of NO have been attributed either to inhibition of angiogenic factors such as β-FGF and TGFβ1,[9-11] or induction of TIMP-2 production and inhibition of p38.[13] Alternatively, NO has been proposed to interfere with the store-operated calcium mobilization through regulation of the NO/cGMP-mediated mechanism,[10] or it can prevent the excessive formation of oxygen radicals and peroxynitrate ($ONOO^-$), which cause cell damage and facilitate tumor metastasis.[12] However, less is known whether and by which mechanisms NO interferes directly in the molecular regulation of EMT.

EMT is known to be the target of a number of constitutively active survival pathways in tumor cells including the NFκB pathway.[14-16] NFκB induces EMT via regulation of the expression of multiple matrix proteases, adhesion molecules and both angiogenic and invasive factors.[14,17-21] In contrast, inhibition of NFκB by various means suppresses EMT and inhibits tumor cell metastasis.[18-22] Recently, NFκB was identified as a transcriptional activator of an additional EMT-inducer gene product, namely, Snail. Snail belongs to the Snail family of transcription factors and is thought to be a hallmark for the initiation of EMT during development and cancer metastasis, directly via transcriptional repression of the metastasis suppressor gene *E-cadherin* and indirectly by contributing to upregulation of mesenchymal gene products.[23,24] Besides E-cadherin, Snail was recently shown to repress the transcription of another tumor suppressor gene product, namely Raf-1 kinase inhibitor protein (RKIP).[25] RKIP is a member of the phosphatidylethanolamine-binding proteins (PEBP) family and among its main functions RKIP inhibits both the NFκB and MAPK signaling pathways. RKIP mediates its inhibitory activity on NFκB and MAPK through physical association with Raf-1 and TAK/NIK and IKK kinases, respectively, leading to inhibition of their activities as kinases.[26,27] The level of RKIP expression is diminished in many primary cancers and is almost absent in several metastatic tumors.[28-30] RKIP overexpression has been shown to inhibit metastasis in experimental cancer models, including prostate cancer, thus RKIP is also known as a metastasis suppressor gene product.[28-31] Snail and RKIP expression levels are inversely correlated in prostate cancer cell lines and patient's samples.[25] Snail and RKIP have also shown opposite effects in the regulation of tumor cell resistance to apoptotic stimuli.[32]

Preliminary findings by us demonstrated that treatment of the EMT-positive human prostate cancer cell lines PC-3 and DU145 with the NO donor, DETANONOate, inhibited their EMT phenotype (Baritaki et al. AACR 101st AACR Annual Meeting 2010, Abstract #: 1466). We hypothesized that DETANONOate-induced inhibition of NFκB[33] may inhibit downstream the metastasis-inducer transcription factor Snail which in turn derepresses the expression of the metastasis-suppressor gene product, RKIP. Since both Snail and RKIP have been shown to regulate the EMT phenotype,[22-24,28-31] the DETANONOate-mediated effects on Snail and RKIP expressions and activities may lead to inhibition of EMT. To test this hypothesis, we examined the following: (1) Does DETANONOate inhibit NFκB signaling in our experimental prostate metastatic cancer cell lines used as experimental models? (2) Does DETANONOate inhibit directly and/or indirectly, the expression and activity of the EMT-inducer, Snail, and whether inhibition of Snail inhibits EMT? (3) Does DETANONOate derepress the activation of the metastasis-suppressor RKIP through the inhibition of Snail and does RKIP overexpression inhibit EMT? and (4) Does treatment of mice bearing PC-3 xenografts with DETANONOate reverse the EMT phenotype and validate the in vitro findings? The present findings concur with the above hypothesis and reveal that DETANONOate treatment, at the concentrations used, inhibits EMT in metastatic prostate cancer lines through interference with the NFκB/Snail/RKIP circuitry.

Results

Inhibition of the EMT phenotype in prostate cancer cell lines by DETANONOate. To examine the role of DETANONOate on the regulation of EMT, we monitored DETANONOate-mediated changes in the expression profiles of gene products that are positively involved in the acquisition of a mesenchymal cell phenotype such as fibronectin and vimentin. Treatment of DU145 and PC-3 cells with 1,000 uM of DETANONOate for 4 and 12 h resulted in both time points in a significant reduction of the high baseline levels of fibronectin and vimentin. Such treatment also restored the expression of the tumor suppressor gene product E-cadherin as assessed by western blot analysis (**Fig. 1A**). Further, tumor cells treatment with DETANONOate for 24 h did not show any significant reversal of the mesenchymal cell phenotype indicating that DETANONOate mediates its effect in a relatively short time window (data not shown). In addition, the invasive properties of the above treated tumor cells were significantly attenuated (> five-fold) after cell treatment with DETANONOate in concentrations greater than 500 uM, as assessed by an in vitro invasion assay. In contrast, lower than 500 uM DETANONOate concentrations didn't result in significant inhibition of invasion (**Fig. 1B**). Cell treatment with the proteasome inhibitor NPI-0052 was used as a positive control.[22] Both cell lines are characterized by constitutive activation of the NFκB signaling pathway. Treatment of DU-145 cells with DETANONOate, used at concentrations of 500 and 1,000 uM, inhibited NFκB DNA-binding activity (**Fig. 1C**). The role of DETANONOate-induced inhibition of NFκB and its relationship to EMT was corroborated by the use of the NFκB inhibitor, DHMEQ. Cell treatment with DHMEQ inhibited EMT and mimicked the effect of DETANONOate (**Sup. Fig. 1**). Overall, the above findings demonstrate that high levels of

DETANONOate (500–1,000 uM) inhibit the EMT phenotype by interfering with the expression of NFκB regulated gene products that are involved in the regulation of EMT.

DETANONOate inhibits the expression of the EMT-inducer Snail and induces the expression of the metastasis suppressor RKIP. The above findings demonstrated that DETANONOate inhibits the EMT phenotype in metastatic prostate carcinoma cell lines. The EMT-inducer Snail is among the genes that are transcriptionally regulated by NFκB and plays a critical role in the acquisition of the mesenchymal cell phenotype and the induction of EMT.[34] In addition, the metastasis suppressor gene product, RKIP, has also been recently shown to be involved in the regulation of EMT.[22] Hence, we hypothesized that DETANONOate-induced inhibition of EMT may be due, in part, to DETANONOate ability to regulate the expression of Snail and RKIP levels. This hypothesis was tested using a variety of experimental designs as described below.

DETANONOate downregulates the expression of the EMT-inducer, Snail. Treatment of the EMT-positive DU145 and PC-3 cells with 1,000 uM of DETANONOate resulted in significant reduction of Snail mRNA and protein levels. Inhibition of Snail mRNA was monitored in PC-3 cells after 2 h of treatment with the peak inhibition observed at 4 and 8 h post-treatment (**Fig. 2A** and upper part). Snail protein levels were found significantly inhibited at 4 h and 12 h post DETANONOate treatment (**Fig. 2A** and lower part), concomitant with the inhibition of the mesenchymal markers observed at the same time points (**Fig. 1A**).

In addition to the inhibitory effect of DETANONOate on the expression of Snail, we further examined whether DETANONOate can also impact on the activity of Snail as a transcription factor. Analysis of Snail DNA-binding activity in PC-3 cells by EMSA revealed that DETANONOate inhibits significantly the DNA-binding activity of Snail as early as 30 min post DETANONOate treatment. The inhibition followed a concentration-dependent pattern as shown at 4 h post-treatment with 500 and 1,000 uM DETANONOate (**Fig. 2B**). Considering the zinc-finger structure of Snail,[35] we hypothesized that the inhibition of its DNA-binding activity by DETANONOate might be due, in part, in a DETANONOate-mediated S-nitrosylation of the Snail protein as we have previously reported for other zinc-finger transcription factors such as YY1.[36] Co-immunoprecipitation assay using an SNO-Cys polyclonal antibody detected a significant level of S-nitrosylated Snail protein in DETANONOate-treated cells (**Fig. 2C**).

The direct correlation of DETANONOate-induced inhibition of Snail and the inhibition of EMT was tested by silencing Snail in DU145 cells by small interference RNA (siRNA). Snail silencing mimicked DETANONOate in terms of inhibiting the mesenchymal markers vimentin and fibronectin and upregulating the epithelial gene product E-cadherin (**Fig. 2D**). These findings demonstrate that DETANONOate inhibits Snail expression and activity. In addition, the findings implicate that Snail inhibition by DETANONOate participates in the inhibition of EMT by DETANONOate.

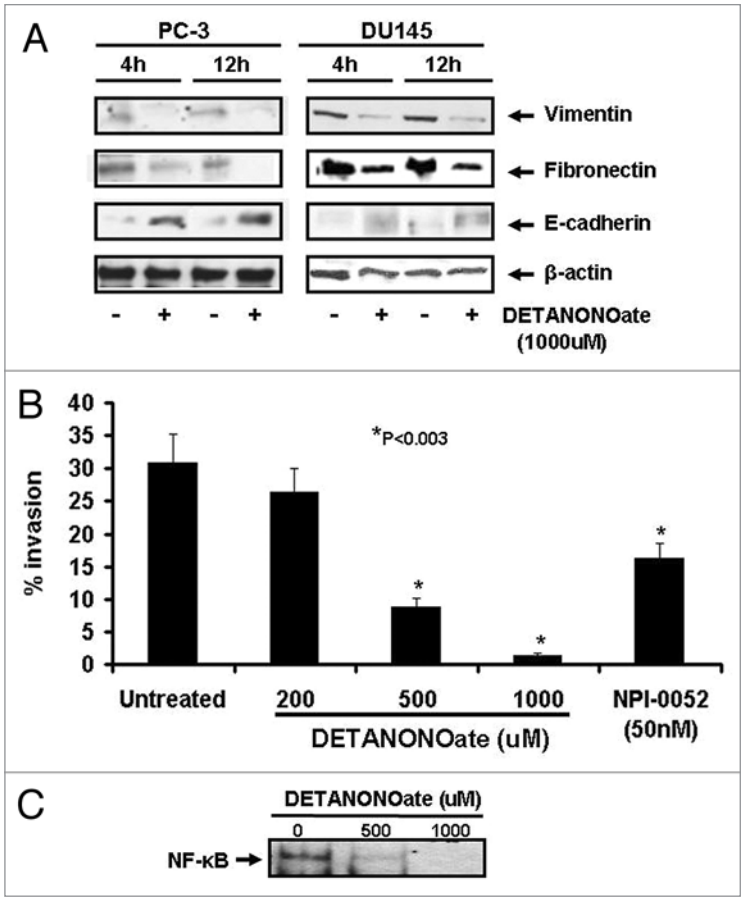

Figure 1. DETANONOate inhibits NFκB and reverses the mesenchymal and invasive cell phenotypes. (A) Reversal of the mesenchymal cell phenotype by DETANONOate. Protein lysates derived from DU-145 and PC-3 cells before and after treatment with 1,000 μM DETANONOate for 4 and 12 h were subjected to western blot analysis for the determination of E-cadherin, vimentin and fibronectin expression. Actin was used as an internal control for loading. (B) DETANONOate inhibits the invasive properties of DU145 cells. 2.5 x 10^4 DU-145 cells were seeded in the upper chamber of the in vitro invasion assay system described in methods, while RPMI 1640 containing 10% FBS was added in the lower chamber. The cells were treated or left untreated with 200, 500 or 1,000 μM DETANONOate and incubated for 22 h. Invaded cells were counted under the microscope after matrigel membrane fixation and staining with crystal violet. Data are expressed as the percent invasion through the matrigel matrix and membrane relative to the migration through the control insert membrane. $p < 0.03$ treated vs. untreated cells (Mann-Whitney U test). (C) Inhibition of NFκB DNA binding activity by DETANONOate. DU145 cells were treated with 500 or 1,000 uM DETANONOate for 4 h and the derived nuclear extracts were subjected to EMSA. β-actin levels were used as loading control.

DETANONOate induces the expression of the metastasis-suppressor RKIP. Time kinetic analysis of tumor cell treatment with 1,000 uM DETANONOate showed upregulation of RKIP mRNA at less than 2 h post treatment (**Fig. 3A**). For protein expression, there was induction at 4 h which increased at 12 h (**Fig. 3B**). The role of RKIP as a metastasis suppressor gene product in several tumors is well established.[8] RKIP transcription

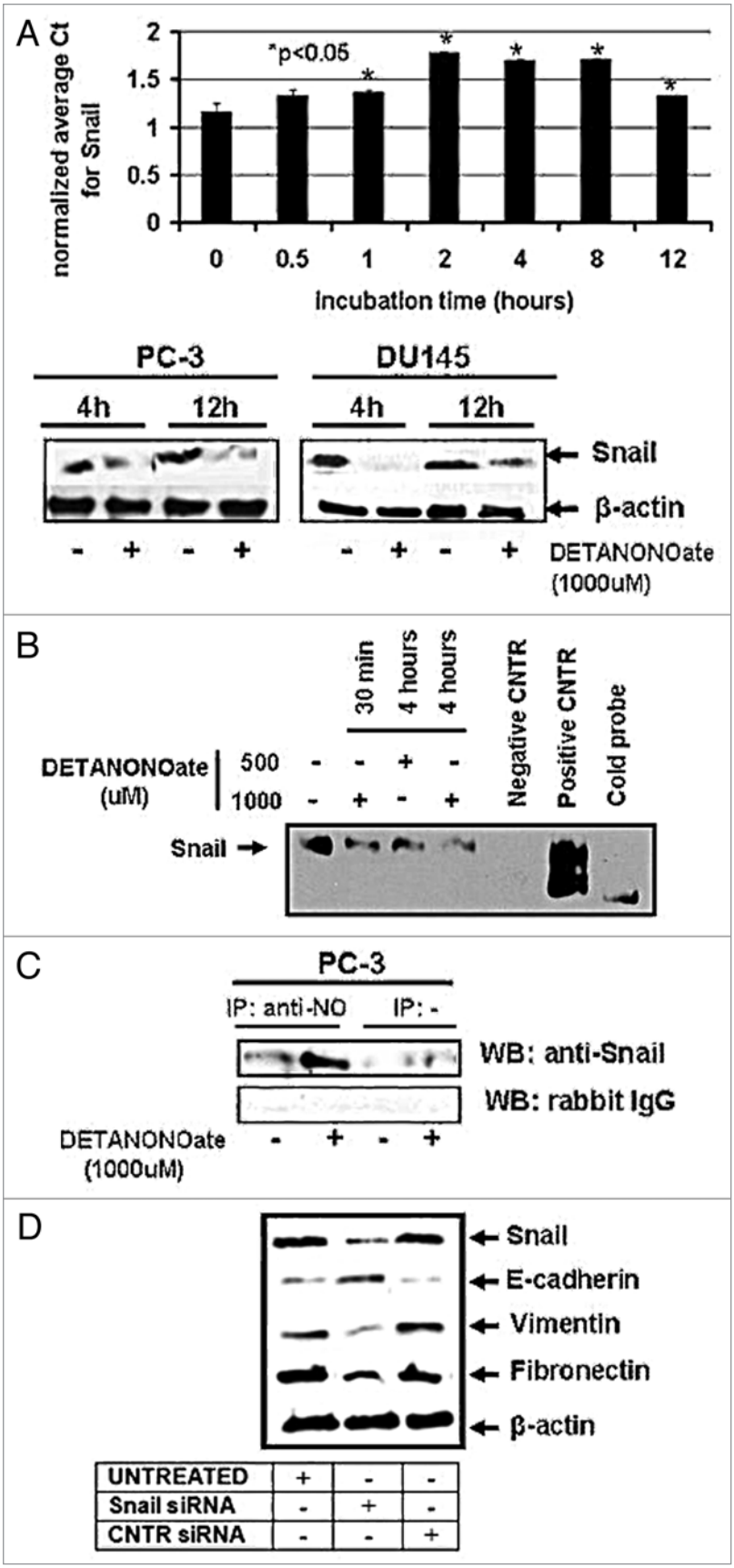

Figure 2. DETANONOate inhibits Snail expression and activity. (A) DETANONOate inhibits Snail expression. Time kinetic analysis of Snail transcripts (upper part) and protein levels (lower part) after cell treatment with 1,000 uM DETANONOate, as assessed by real-time PCR and western blot analyses, respectively. The inhibition of Snail transcript levels are shown as increase in the normalized cycle threshold (Ct) values of the amplified product as function of time. The Ct values of GAPDH assessed in the same samples were used as normalizing controls. Data are expressed as the average normalized Ct values ± STDEV from one experiment performed in triplicates. *p < 0.05: treated vs. untreated cells. (B) Inhibition of Snail DNA binding activity by DETANONOate. DU145 cells were incubated in serum-free medium in the presence of absence of DETANONOate for 30 min or 4 h. The Snail's DNA-binding activity was evaluated by EMSA using a biotin-labeled oligonucleotide Snail probe. (C) DETANONOate induces S-nitrosylation of Snail protein. Immunoprecipitation of S-nitrosylated Snail after DETANONOate treatment (1,000 uM, 4 h). Total cell lysates were used in an immunoprecipitation assay using protein A beads as described in materials and methods. S-nitrosylated proteins were precipitated with an anti-S-nitroso-cysteine (SNO-Cys) antibody and the membranes were immunoblotted with Snail polyclonal antibody. Rabbit IgG was used as a negative control. (D) Direct role of Snail suppression in EMT inhibition. Total cell lysates were harvested from DU 145 cells after suppression of Snail for 72 h using Snail siRNA and subjected to western blot analysis for determination of the protein expression of the indicated gene products. A random nucleotide sequence (CNTR siRNA) was used as a control in the transfection assays. Actin was served as an internal control.

is directly repressed by Snail;[25] thus, we hypothesized that DETANONOate-mediated inhibition of Snail expression could result downstream in RKIP induction. Snail involvement in RKIP regulation was confirmed in DU145 cells transfected either with a wild-type RKIP-Luc reporter or an RKIP-Luc reporter carrying mutations in E-boxes where Snail is bound.[25] Tumor cells transfected with the RKIP reporter carrying mutations for Snail-binding showed enhanced basal promoter activity compared to cells transfected with the wild type RKIP reporter. Co-transfection with Snail siRNA increased the reporter activity in cells transfected with the wild type RKIP reporter whereas there was no change in cells transfected with the mutant RKIP reporter (**Fig. 3C**). The direct role of RKIP induction by DETANONOate in the inhibition of EMT was further examined by ectopic expression of RKIP in DU145 cells using a CMV expression vector. Cells overexpressing RKIP (CMV-HA-RKIP tranfected cells) showed decreased Snail expression, as well as suppressed expression of vimentin and fibronectin and increased levels of E-cadherin (**Fig. 3D**). The direct role of RKIP induction in DETANONOate-mediated inhibition of EMT was further corroborated by reversing the inhibitory

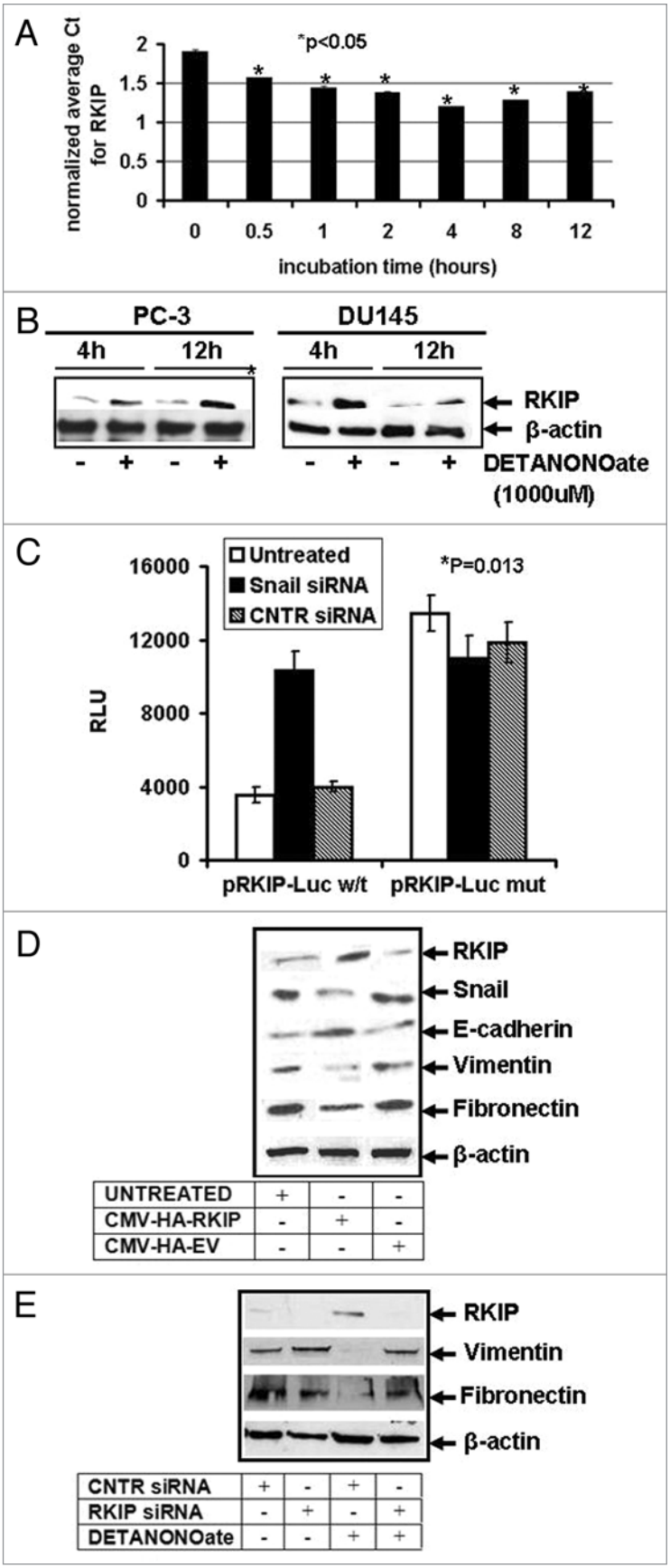

Figure 3. DETANONOate upregulates RKIP expression. (A) DETANONOate upregulates RKIP transcript levels in PC-3 cells as assessed by real-time PCR. The induction of RKIP transcript levels are shown as decrease in the normalized cycle threshold (Ct) values of the amplified product as a function of time. The Ct values of GAPDH assessed in the same samples were used as normalizing controls. Data are expressed as the average normalized Ct values ± STDEV from one experiment performed in triplicates. *p < 0.05: treated vs. untreated cells. (B) Induction of RKIP protein expression by DETANONOate. Protein lysates from DU145 and PC-3 cells were harvested at 4 and 12 h post-treatment with DETANONOate and subjected to western blot analysis for RKIP protein determination. (C) Regulation of RKIP promoter activity by Snail. DU145 cells were co-transfected with Snail siRNA or the relative control siRNA and the pRKIP-Luc wild type (pRKIP-Luc w/t), or the pRKIP-Luc mutant (pRKIP-Luc mut) promoter construct which has mutated 3 out of 5 E-boxes. *p = 0.013: transfected vs. transfected and treated with Snail siRNA cells. (D) Direct role of RKIP in EMT inhibition. Total cell lysates were harvested from DU145 cells after overexpression of RKIP for 48 h using a CMV-HA-RKIP vector and subjected to western blot analysis for determination of the protein expression of the indicated gene products. A CMV empty vector (CMV-HA-EV) was used as a negative transfection control. (E) Silencing of RKIP by siRNA reverses the DETANONOate-mediated inhibition of the EMT phenotype in DU145 cells. Cells were pre-treated with RKIP siRNA or control siRNA for 70 h and then were exposed to DETANONOate (1,000 uM) for additional 12 h. A random nucleotide sequence (CNTR siRNA) was used as a negative silencing control. The expressions of RKIP, vimentin and fibronectin were assessed by western blot analysis. Actin was served as an internal control in all protein assays.

effect of DETANONOate on EMT through silencing of RKIP. DU145 cells pre-treated with RKIP siRNA for 70 h and then exposed to DETANONOate (1,000 uM) for additional 12 h showed similar to baseline (cells not treated with DETANONOate) expressions of vimentin and fibronectin; however, these expressions were significantly upregulated compared to those derived from DETANONOate and control siRNA treated cells (**Fig. 3E**). The reversal of the epithelial phenotype induced by DETANONOate after RKIP silencing was confirmed by microscopic comparison of the morphology of the cells treated with DETANONOate in absence or presence of RKIP siRNA (data not shown).

Overall, the above findings demonstrate that DETANONOate induces the expression of the metastasis-suppressor gene product, RKIP and this induction has a critical and direct role on DETANONOate-mediated inhibition of EMT.

DETANONOate inhibits the EMT phenotype in vivo in mice bearing PC-3 xenografts. To further validate in vivo our in vitro findings on DETANONOate-mediated inhibition of the EMT phenotype, we used athymic nude mice bearing PC-3 xenografts that were treated with DETANONOate or saline every second day for 12 days. At the completion of treatment, tumor biopsies were obtained and analyzed ex vivo by immunohistochemistry for RKIP, Snail, E-cadherin, vimentin and fibronectin expressions (**Fig. 4**). The findings revealed that, similar to PC-3 cells

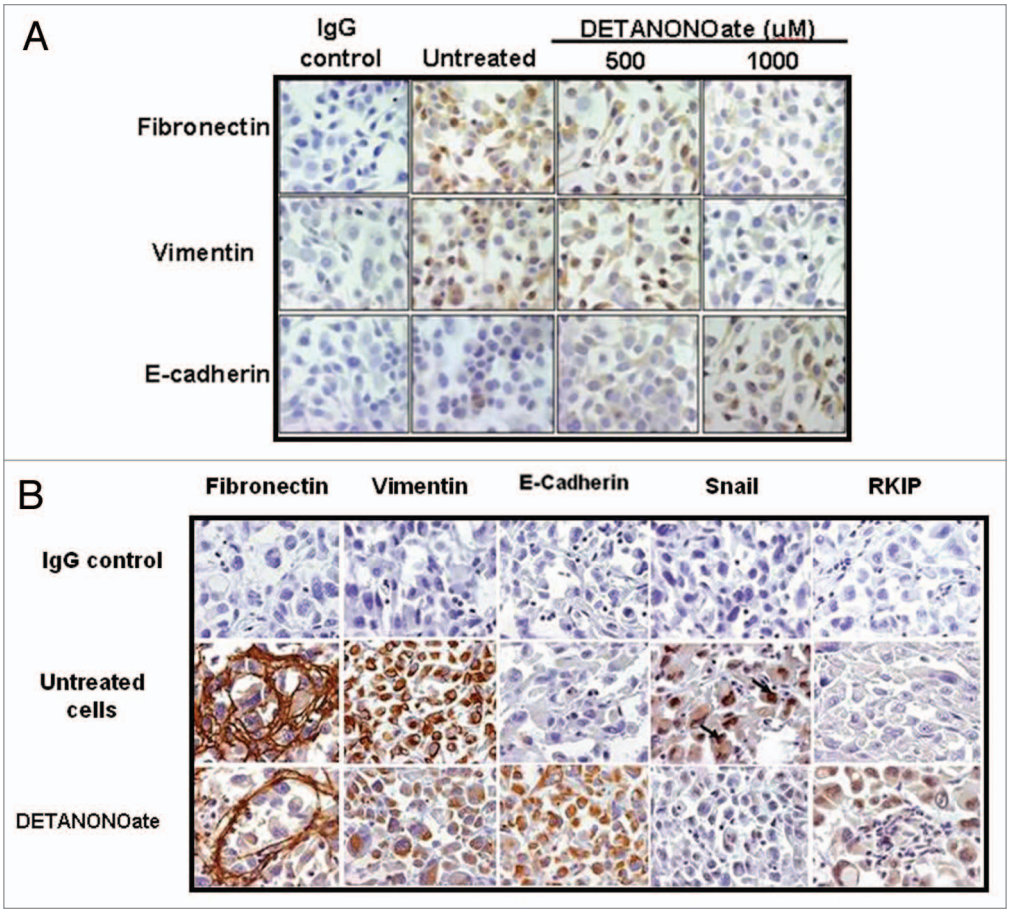

Figure 4. Validation of the NO-mediated reversal of mesenchymal cell phenotype in mice bearing PC-3 xenografts. (A) Immunohistochemical analysis of fibronectin, vimentin and E-cadherin expression in PC-3 cells following treatment with 500 and 1,000 uM of DETANONOate. Rabbit IgG control was used as a negative control. (B) Representative immunohistochemical staining for RKIP, Snail, Fibronectin, and E-cadherin expression in biopsies derived from mice bearing PC-3 tumors. As above, rabbit IgG control was used as negative control.

treated in vitro with DETANONOate (**Fig. 4A**), the biopsies derived from DETANONOate-treated mice had a significant reduction in the expressions of Snail, vimentin and fibronectin, while the expressions of RKIP and E-cadherin were elevated when compared to saline-treated mice (**Fig. 4B**). In addition, treatment of mice bearing PC-3 tumors with DETANONOate alone had no effect in tumor size whereas the combinational treatment with DETANONOate and CDDP reduced significantly the tumor size (Huerta Yepez, et al. AACR 100th AACR Annual Meeting 2009, Abstract #:4542). These findings constitute an in vivo validation of our in vitro observations on the reversal of the mesenchymal metastatic phenotype of PC-3 cells by DETANONONOate.

Discussion

Evidence is presented which demonstrates, for the first time, that high levels of the NO donor DETANONOate inhibit the EMT phenotype in metastatic tumor cell lines used as models. The underlying mechanism of DETANONOate-mediated inhibition of EMT was, in large part, due to the inhibition of the EMT-inducer Snail and induction of the metastasis-suppressor RKIP. Several lines of evidence support the direct role each of Snail and RKIP-modified expressions by DETANONOate in the inhibition of EMT. Consequently, the inhibitory effects of DETANONOate on EMT and cell invasive properties were shown to be the result of the increase in the levels of the metastasis-suppressor factors, RKIP and E-cadherin, and inhibition of the expression of the metastasis-inducer factors, Snail, vimentin and fibronectin. The DETANONOate-induced inhibition of the EMT phenotype shown in vitro was validated in mice bearing tumor xenografts treated with DETANONOate. These findings suggest a novel potential therapeutic application of NO donors in the inhibition of EMT and metastasis in malignant tumor cells.

The modifications induced by DETANONOate and leading to the dysregulation of the NFκB/Snail/RKIP loop, result in the inhibition of the EMT phenotype (**Fig. 5**). Each of these gene products individually modified by DETANONOate was shown to be directly involved in EMT and several lines of evidence supported their roles in EMT. NFκB has an established inducing role in tumor metastasis and EMT. NFκB regulates the expression of multiple adhesion molecules and matrix proteases as well as the expression of genes involved in angiogenesis and cell invasion.[14,17,18] NFκB deficiency in tumor models results in altered

cell morphology and decreased cell motility and invasive potential.[18-21] We and others have previously shown that high subtoxic concentrations of NO, released by NO donors, can serve as an efficient inhibitor of NFκB activity at multiple levels.[37-40] Our findings that DETANONOate-induced inhibition of NFκB and that NFκB plays a role in the inhibition of EMT are in agreement with our observations showing that inhibition of NFκB by its chemical inhibitor DHMEQ[41] results in the loss of the mesenchymal phenotype of DU145 cells (see **Sup. Fig. 1**). We have reported that treatment of prostate tumor and non-Hodgkin's B cell lymphoma (B-NHL) cell lines with DETANONOate reduced the NFκB DNA-binding activity attributed to S-nitrosylation of the NFκB p50 subunit by NO.[33,42,43] The above observations suggest that NO is a potent NFκB inhibitor and that DETANONOate mediates its anti-metastatic properties, in part, via suppression of NFκB signaling.

Several lines of evidence support the role of the inhibition of Snail by DETANONOate as a pivotal factor in the the inhibition of the EMT phenotype. Treatment of prostate metastatic cells with DETANONOate resulted in inhibition of both the expression and activity of Snail. Silencing of Snail by siRNA in our experimental model resulted in inhibition of mesenchymal gene markers and re-expression of E-cadherin, suggesting that the NO-mediated Snail suppression is directly associated with restoration of an epithelial cell phenotype. We have recently demonstrated that overexpression of the stable Snail form, Snail-6SA, in the non-metastatic prostate cell line LNCaP, results in E-cadherin downregulation and acquisition of the EMT phenotype accompanied by expression of vimentin and fibronectin, gene products previously shown to be indirectly regulated by Snail.[22,23] These findings are in agreement with previous studies showing that, in addition to E-cadherin suppression, ectopic Snail expression downregulates other epithelial markers such as mucin1 and cytokeratin-18,[44] and upregulates and redistributes mesenchymal markers such as vimentin and fibronectin.[24] The above findings corroborate previous reports that indicated the suppression of tumor growth and invasiveness by Snail silencing.[45,46] Thus, our findings suggest that NO can serve as a regulator of EMT by interfering negatively with the expression and the transcriptional activity of Snail.

The direct role of RKIP-induced expression by DETANONOate in the inhibition of the EMT phenotype was corroborated by several lines of evidence. Ectopic expression of RKIP in DU145 cells mimicked cell treatment with DETANONOate in terms of reducing the expression of mesenchymal markers and redistributing E-cadherin levels. In contrast, silencing of RKIP by siRNA resulted in reversal of DETANONOate-mediated inhibition of the mesenchymal phenotype of DU145 cells. In prostate cancer cell lines, Snail expression has been reported to be inversely correlated not only with E-cadherin levels but also with the expression of the metastasis suppressor RKIP via direct transcriptional inhibition.[25] Inhibition of Snail by siRNA in DU145 cells resulted in the enhancement of the wild-type but not the mutant RKIP promoter activity and expression, demonstrating the negative regulation of RKIP by Snail. Treatment of DU145 cells with DETANONOate mimicked Snail silencing in terms of restoring

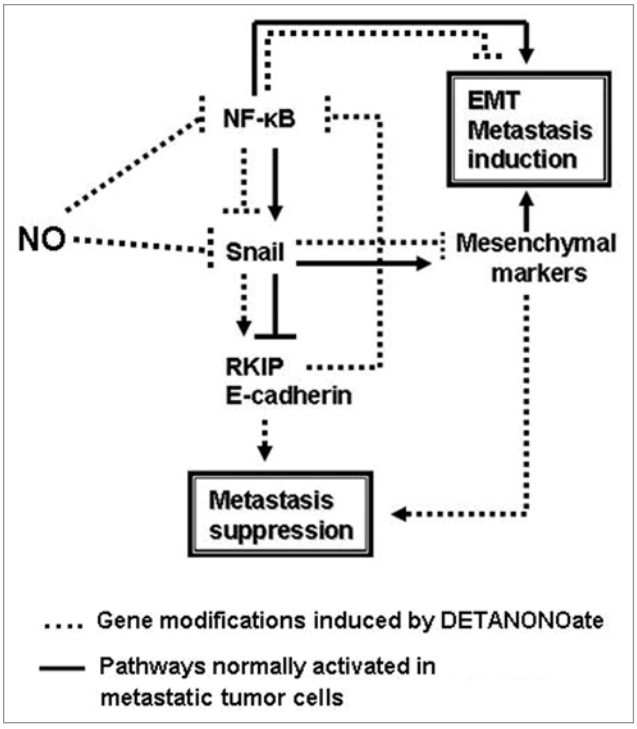

Figure 5. The NFκB/Snail/RKIP crosstalk in DETANONOate-mediated inhibition of EMT. We propose that the NO-mediated inhibition of NFκB activity serves as a modulator of EMT responses not only by itself but also via regulation of downstream EMT-related targets such as Snail. The NO-mediated suppression of Snail expression and activity in turn results in the derepression of its transcriptional targets RKIP and E-cadherin and the inhibition of Snail-regulated mesenchymal markers. RKIP induction attenuates directly a feedback loop of Snail suppression via NFκB inhibition in addition to suppression mediated directly by NO. The reported modifications in the expressions and activities of the above gene products constitute a suggested mechanism by which DETANONOate inhibits the mesenchymal cell phenotype and the migratory and invasive properties of metastatic prostate cells in vitro and in vivo.

RKIP mRNA and protein levels, whereas Snail overexpression in LNCaP cells have been previously shown to result in dramatic RKIP downregulation.[22] The RKIP-mediated inhibition of Snail could be attributed, in part, to the inhibitory role of RKIP on NFκB, which serves as a transcriptional activator of Snail.[34] In addition, the inducing effects of NO on RKIP expression might translate in further inhibition of NFκB and its downstream targets since RKIP is a potent inhibitor of NFκB activation.[27] The induction of RKIP by DETANONOate in our experimental model is supported by findings showing that treatment of normal keratinocytes with the NO donor SNAP results in elevated RKIP mRNA and protein levels and inhibition of proliferation.[47] In vitro and in vivo studies have established RKIP as a metastasis suppressor for prostate and breast cancer, with an inhibitory role on tumor cell intravasation and bone metastasis.[31] Although the underlying mechanism by which RKIP suppresses invasion and metastasis is not completely elucidated, it has been proposed that the anti-metastatic RKIP function in breast tumors is mediated, in part, through interference with a signaling cascade involving MAPK, Myc, LIN28, let-7 and downstream let-7 targets.[48]

Our in vitro findings on the inhibitory role of DETANONOate on RKIP and Snail expressions as well as on the EMT cell phenotype were further validated in vivo in mice bearing PC-3 xenografts. Similar to our in vitro observations, tumor biopsies derived from DETANONOate-treated mice were characterized by the downregulated expression of mesenchymal markers and induction of RKIP and E-cadherin compared with their untreated counterparts. These findings are in agreement with other studies showing the inhibitory effects of NO-releasing agents in metastatic tumor cell behavior both in vitro and in vivo.[9-13]

The challenge regarding NO donors as therapeutic agents is to deliver NO in a sustained and controlled manner. NO donors that spontaneously generate large amounts of NO, independent of iNOS induction, are activated at physiological pH.[49] DETANONOate belongs to the diazeniumdiolates (formerly NONOates) family of NO donors.[50] A range of NONOates has been described with half-lives varying from seconds to hours. At present, NONOates are not used clinically, although they have been tested frequently in different experimental models, including the development, progression and metastasis in cancer.[51] An attractive feature of this class of compounds is that the rate and the selectivity of NO release can be accurately predicted via specific modification of the NONOate structure.[52,53] As such, the predictable nature of NO release by NONOates, especially at concentrations high enough to inhibit tumor progression and metastasis, has introduced them as potential experimental tools for the treatment of certain cancers,[54] although further characterization of these drugs is essential.

Overall, our present findings support the potential antimetastatic properties of DETANONOate used at high levels via inhibition of EMT. We further demonstrate that DETANONOate-mediated modulation of the NFκB/Snail/RKIP circuitry is a novel molecular mechanism by which NO might mediate its anti-EMT function. As such, we identify the NFκB/Snail/RKIP crosstalk as a critical signalling network that interferes not only with tumor cell resistance to apoptotic stimuli[32] but also with initiation of the metastatic cascade (**Fig. 5**). The clinical relevance and significance of inhibiting Snail and restoring RKIP expression by NO releasing agents, alone or in combination with other conventional chemotherapeutics, might correlate with a favorable clinical outcome in terms of cancer progression and spread. Clearly, clinical trials are needed to examine the therapeutic potential of NO donors in the inhibition of EMT and metastasis.

Materials and Methods

Cell lines and reagents. The metastatic bone-derived androgen-independent prostatic adenocarcinoma cell lines DU145 and PC-3 were obtained from the American Type Culture Collection (ATCC). Both cell lines were maintained in media and conditions previously described in references 32 and 33. The NO donor, DETANONOate was purchased from Alexis (Biomol International LP). The NFκB inhibitor DHMEQ was kindly provided by Dr. Umezawa (Keio University, Japan). Stock solutions of DHMEQ and DETANONOate were prepared in DMSO and dH_2O, respectively. Anti-human RKIP, Snail and β-actin antibodies used for western were obtained from Zymed, Abcam and Chemicon, respectively. The anti-human rabbit fibronectin, mouse E-cadherin and goat vimentin antibodies used for western blot analysis and immunohistochemistry were all obtained from Sigma.

Transient transfections. Transient transfections of DU145 cells with the RKIP expression vector CMV-HA-RKIP or the reporter constructs pRKIP-Luc wild type (w/t) and pRKIP-Luc mutant (carrying mutations in three out of the five E-boxes therefore lacking Snail DNA binding) were performed as previously described in references 22, 32 and 55.

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from 1 x 10^6 PC-3 cells before and after treatment with 1,000 μM DETANONOate for the indicated time periods using the RNAasy Mini Kit (Qiagen). The isolated RNA was reversed transcribed to cDNA using the SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed in 20 ul reaction volume using a fluorogenic 5'-nuclease PCR assay (Taqman FAM-labeled fluorogenic probes) in a 7500 Fast Real-Time PCR sequence detection system (all from Applied Biosystems). GAPDH was used as an endogenous control and was detected using the corresponding FAM-labeled fluorogenic probe (Applied Biosystems). Triplicate reactions of each sample were incubated for two minutes at 50°C, denatured for 10 minutes at 95°C and subjected to 40 cycles of annealing at 55°C for 20 seconds, extension at 60°C for 1 minute followed by denaturation at 95°C for 15 seconds. The Ct (cycle threshold) values obtained for RKIP and Snail in each of the tested samples were normalized with the corresponding GAPDH Ct value. The changes in the normalized average Ct values among the tested samples were used as determinants of the initial RKIP or Snail mRNA transcript levels present in the samples.

Application of small interfering RNA (siRNA). The silencing of Snail expression by Snail specific siRNA was performed according to the manufacturer's instructions (Santa Cruz Biotechnology Inc.).[32] Scrabbled siRNA sequence was used as negative silencing control. For the RKIP silencing assays, DU145 cells were pre-treated with RKIP (Santa Cruz Biotechnology Inc.,) or control siRNAs for 70 h followed by 12 h incubation with 1,000 uM DETANONOate. Single treated cells with either RKIP or control siRNAs or DETANONOate were used as comparison controls. Cells were harvested 80–82 h post-transfection and screened for protein expression by western blot.

Western blot analysis. Analysis of RKIP, Snail, vimentin, fibronectin and E-cadherin protein expression was performed in 40 ug protein lysates by western blot analysis as previously described in references 22 and 32. The expression of β-actin was used as an internal control.

In vitro cell invasion assay. Tumor cell invasion studies were performed using 24-well 8 micron Matrigel invasion chambers according to manufacturer's instructions (Becton Dickinson). Cells were starved with basal medium supplemented with 0.1% BSA for 4–5 h prior setting up the assay. RPMI 1640 medium containing 10% serum was used as chemoattractant.

DETANONOate was used at concentrations of 200, 500 and 1,000 uM.

Nuclear extract preparations and electrophoretic mobility shift assay (EMSA). For determination of NFκB and Snail DNA-binding activities, nuclear extracts from DETANONOate-treated and untreated DU145 cells were prepared as previously described in reference 33. Electrophoretic mobility shift assay was performed using the Panomics EMSA Kits with the corresponding biotin-labeled oligonucleotide NFκB or Snail probes (Panomics Inc.).

Immunoprecipitation of S-nitrosylated Snail. The S-nitrosylation of Snail was analysed in PC-3 cells by immunoprecipitation as previously described in reference 33. The immunoprecipitates were pulled down using a rabbit anti-S-nitroso-cysteine (SNO-Cys) polyclonal antibody (Sigma). The immunoprecipitates were resolved on a 12% SDS-PAGE gel and subsequently immunoblotted with a rabbit Snail polyclonal antibody at 1/1,000 dilution (AbCam). Rabbit IgG was used as negative control. The immunostaining was visualized by autoradiography.

In vivo treatment of PC-3-bearing mice with DETANONOate. 1 x 10^6 PC-3 cells were administrated to 8 week-old athymic nude mice by subcutaneous injection as previously described in reference 42 and left to grow for 5 weeks. DETANONOate was administrated intratumoraly under sevofluorane anesthesia at a concentration of 0.4 mg/Kg every second day for 12 days. At the completion of treatment, the mice were euthanized and tumor biopsies were harvested for histological studies.

Immunohistochemistry (IHC). Untreated and treated with 1,000 uM DETANONOate PC-3 cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer (PBS) pH 7.2. Tumor biopsies were fixed by immersing them in the above fixing solution, and then embedded in paraffin. Two micrometer slices were placed on slides and either stained with Mayer's haematoxylin and eosin (H&E) for the histopathological examination, or used for the immunohistochemical techniques. The expressions of RKIP (Santa Cruz, Cat#:73636), Snail (Abcam, Cat#: 17732), Fibronectin (Sigma, Cat#: F3648), Vimentin (Sigma, Cat #: V4630) and E-cadherin (Sigma, Cat#:WH0000999M1) was determined using the corresponding primary antibodies at concentration of 1/500. Immunohistochemistry in biopsies and cells lines was performed as previously described in reference 42. The slides were analyzed under light microscopy (Olympus BX-40).

Immunofluorescence (IF). DU145 cells were grown on glass coverslips and treated with 10 ug DHMEQ for 4 h. Immunofluorescence staining for fibronectin, vimentin and E-cadherin was performed as previously described in reference 22.

Statistical analysis. Significant differences were determined by the Mann-Whitney U and Kruscal Wallis H tests using the SPSS software.

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Note

Supplemental materials can be found at:
www.landesbioscience.com/supplement/BaritakiCC9-24-sup.pdf

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