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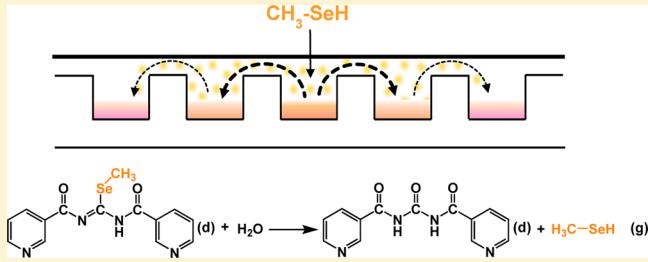
## Cytotoxic and Proapoptotic Activities of Imidoselenocarbamate Derivatives Are Dependent on the Release of Methylselenol

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 Supporting Information

**ABSTRACT:** In the search for new molecules with potential antiangiogenic activity, we found that several imidoselenocarbamate derivatives effectively suppressed the expression of vascular endothelial growth factor (VEGF) induced by hypoxia in NCI-H157 tumor cells. Mechanistic studies indicated that these compounds inhibited STAT3 phosphorylation triggered by hypoxia, suggesting that inhibition of STAT3 function may play a role in VEGF inhibition. Moreover, these molecules showed interesting proapoptotic and antiproliferative effects. Both the presence of selenium, but not sulfur, and the nature of the radical substituents were important for activity. Interestingly, under hypoxic conditions, several methyl imidoselenocarbamate derivatives released methylselenol, a highly reactive and cytotoxic gas, which was responsible for their biological activities. The kinetics of the release of methylselenol by these molecules was highly dependent on the nature of the substituent radicals and correlated with their early proapoptotic activity. Our results support the notion that pharmacological activities reported for methyl imidoselenocarbamate derivatives are dependent on the release of methylselenol. Given the well-known antitumor activities of this compound, imidoselenocarbamate derivatives represent a promising approach to develop new drugs that release methylselenol in a controlled way.



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### INTRODUCTION

Angiogenesis, the process by which the existing vascular network expands to form new blood vessels, is a tightly regulated process that is essential during growth, wound healing, and development, as well as cancer progression. The main regulator of physiological and pathological angiogenesis is the vascular endothelial growth factor (VEGF), which is required for tumor growth, invasion, and metastasis in many animal models.<sup>1</sup> VEGF expression is highly stimulated through hypoxia-inducible factor 1 (HIF-1), a transcription factor that consists of two subunits, HIF-1 $\alpha$  and HIF-1 $\beta$ . Under hypoxia, HIF-1 $\alpha$  accumulates, translocates to the nucleus, and dimerizes with the constitutively expressed HIF-1 $\beta$ .<sup>2</sup> There, the heterodimeric HIF-1 factor binds to hypoxia-response elements (HREs) located within the promoter of a battery of genes related to the adaptative response to hypoxia.<sup>3</sup>

Anti-VEGF therapies are being actively investigated as potential anticancer treatments, either as alternatives or as adjuncts to conventional chemo- or radiation therapy. Among the strategies used, there are neutralizing monoclonal antibodies against VEGF or its receptor, small molecule tyrosine kinase inhibitors of VEGF receptors, and soluble VEGF receptors that act as decoy receptors for VEGF.<sup>2</sup> An important problem with these strategies, however, is that they inhibit both tumor and physiological angiogenesis, leading to relevant therapeutic secondary effects that limit their clinical usefulness. There is a

need to develop new inhibitors that target tumor angiogenesis more specifically.

Hypoxia is a hallmark of solid tumors that has been associated with tumor progression, metastasis, and poor patient survival.<sup>3</sup> In this work, we searched for new molecules with the ability to inhibit the production of VEGF induced by hypoxia. For this purpose, we tested the biological activity of a library of structurally diverse compounds synthesized by the Department of Organic and Pharmaceutical Chemistry at the University of Navarra, including selenium (Se)-containing molecules. Se is a well-known antioxidant at physiological levels obtained by normal nutritional intake, while supranutritional Se supplementation has been proposed to reduce the risk of cancers of several organs.<sup>4</sup> Several hypotheses have been advanced to account for the anticarcinogenic effects of Se, but the underlying mechanisms remain to be elucidated.<sup>4,5</sup> Ip and co-workers were the first to link Se metabolism with the chemopreventive and antitumor activity of different Se compounds.<sup>6</sup> They showed that a monomethyl Se metabolite, such as methylselenol, may be the active Se in vivo against chemically induced mammary carcinogenesis, and the chemopreventive efficacy of a given Se compound may depend on their metabolic conversion rate to that active form.<sup>7,8</sup>

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Nowadays, precursors of methylselenol are the Se compounds that show great promise in cancer prevention and therapy.<sup>4,9,10</sup>

In this work, we found that several imidoselenocarbamate (isoselenourea) derivatives inhibited VEGF expression and induced apoptosis at low micromolar concentrations in H157 cells. The presence of Se was essential for their biological properties, since substitution of Se by sulfur led to severe loss of activity. Interestingly, we found that methyl imidoselenocarbamate derivatives released methylselenol at different rates, and the kinetics or liberation correlated with their early proapoptotic activity. Imidoselenocarbamates have been previously shown to possess a broad range of biological activities, including antiproliferative, antitumoral,<sup>11,12</sup> and antileishmanial activities.<sup>13</sup> Our data support the notion that methylselenol released by these compounds is responsible for the reported activities of imidoselenocarbamate derivatives. As far as we know, this is the first report of a series of molecules that release methylselenol in a controlled way.

## EXPERIMENTAL PROCEDURES

**Reagents and Antibodies.** Imidoselenocarbamate and imidothiocarbamate derivatives were synthesized, purified, and characterized as reported,<sup>12</sup> dissolved in anhydrous dimethyl sulfoxide (DMSO), and added to the medium at the indicated concentrations. In all cases, the concentration of solvent in culture medium did not exceed 0.5% (v/v). Apigenin, epigallocatechin-3-gallate (EGCG), wortmannin, seleno-L-methionine (MSE), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB or Ellman's reagent), dimethyl diselenide, and sodium borohydride ( $\text{NaBH}_4$ ) were purchased from Sigma (St. Louis, MO). L-Methionine  $\gamma$ -lyase (METase) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The antibodies against Akt, phospho-Akt (Ser 473), phospho-ERK1/2 (Thr202/Tyr204), signal transducer and activator of transcription 3 (STAT3), and phospho-STAT3 (Tyr705) were from Cell Signaling Technology (Boston, MA). Anti-VEGF antibody (MAB293) was from R&D (Minneapolis, MN). HIF-1 $\alpha$  antibodies were purchased from BD Biosciences (Erembodegem, Belgium), and  $\beta$ -actin antibody was purchased from Sigma.

**Cell Culture.** The lung cancer squamous cell carcinoma NCI-H157 cell line was purchased from Promocell SL (Barcelona, Spain) and cultured in RPMI 1640 medium with Ultraglutamine 1 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Life Tec. Gibco Invitrogen Corporation, Grand Island, NY), 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Tec. Gibco Invitrogen Corporation) (complete medium). Cells were grown in a humidified atmosphere containing 5% CO<sub>2</sub> at 37 °C. Microenvironmental hypoxic conditions (1% O<sub>2</sub>) were achieved in an airtight humidified chamber infused with a mixture of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub> (Air Liquide, Madrid, Spain). The chamber was monitored with an O<sub>2</sub> sensor (Dräger Pac 5000, Dräger, Lübeck, Germany), and the temperature was maintained at 37 °C.

**Western Blotting.** For Western blot analysis,  $6 \times 10^5$  H157 cells were seeded on 60 mm culture dishes in complete medium for 40 h. Cells were exposed to medium without serum containing 25 mM HEPES for 2 h and then pretreated with the indicated concentrations of each compound for 30 min, followed by exposure to hypoxia for 6 h. Total cellular extracts were obtained by homogenizing the cells in Triton ice-cold buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 0.1 mM DTT, and 1  $\mu\text{g}/\text{mL}$  aprotinin. The protein concentration of the samples was determined by BCA (bicinchoninic acid assay). Samples were treated with Laemmli sample buffer, separated by SDS-PAGE under reducing conditions, and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell Bioscience, Dassel, Germany). Membranes were blocked with 10% nonfat milk in TBS containing 0.1% Tween-20 for 1 h at room temperature and then incubated overnight at 4 °C with the primary antibody at the suitable dilution. Blots were incubated with a HRP-conjugated secondary

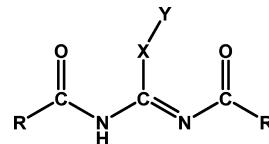
antibody for 1 h and developed with Lumi-Light Plus Western blotting substrate (Roche, Mannheim, Germany). Blot images were scanned with a GS-800 calibrated densitometer and analyzed using Quantity One software (Bio-Rad, Hercules, CA).

**Screening Assay To Measure the Effect of Compounds on VEGF Production in H157 Cells.** H157 cells ( $4 \times 10^4$  per well) were plated in 96-well plates (TPP, St. Louis, MO) and cultured until confluence was reached. Medium was then removed, and cells were washed and incubated with different concentrations of each compound in 200  $\mu\text{L}$  of serum-depleted medium under normoxia or hypoxia for 24 h. Triplicates were used for each experimental condition. Supernatants (100  $\mu\text{L}$ ) were added to a 96-well biotest microfiltration apparatus (Bio-Rad) with a multichannel pipet, filtered under vacuum through a Protran nitrocellulose membrane (Whatman, Dassel, Germany), and washed twice with 100  $\mu\text{L}$  of TBS. The membrane was then blocked with 10% nonfat milk in TBS containing 0.1% Tween-20 for 1 h, and VEGF was immunodetected with MAB293 anti-VEGF antibody (R&D) and quantified as before. Dose-response curves were obtained by nonlinear regression of the original data using GraphPad Prism (GraphPad Software, La Jolla, CA). VEGF secretion into the culture medium was also measured by Western blot under nonreducing conditions with the same antibody as described.<sup>14</sup> Briefly, cells were grown in P-60 plates in complete medium, washed twice with PBS, and incubated in medium without serum under normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 24 h. Supernatants were then collected and concentrated with a centrifugal filter device (Millipore, Bedford, MA), and the amount of VEGF was measured by Western blot under nonreducing conditions.

**Cell Viability and Cell Proliferation Assays.** For cell viability assays,  $4 \times 10^4$  H157 cells per well were seeded in 96-well plates (TPP) in complete medium and incubated for 24 h before experimental treatments. Then, cells were washed twice with PBS and treated in triplicate with compounds in medium without serum containing 25 mM HEPES and L-glutamine (GIBCO, Invitrogen, Carlsbad, CA) and further incubated in normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 24 h. After this time, medium was removed, and viable cells were measured using the neutral red assay.<sup>14</sup> The neutral red assay was performed by incubating the cells for 90 min at 37 °C with 200  $\mu\text{L}$  of fresh media, supplemented with 40 mM HEPES to avoid neutral red precipitation, and 50  $\mu\text{L}$  of a solution prepared by diluting 1 mg/mL neutral red (Sigma Chemical) with an equal volume of 1.8% NaCl solution. Then, cells were washed twice with PBS and lysed with 100  $\mu\text{L}$  of 50% NaH<sub>2</sub>PO<sub>4</sub>-ethanol. The absorbance of the extracted dye was read at 540 nm in a Multiskan Ex plate reader (Thermo Fisher Scientific, Vantaa, Finland). Proliferation assays were performed as described.<sup>15</sup> Briefly, H157 (3000 cells/well) were plated and then treated with compounds prepared in complete medium containing 25 mM HEPES for 72 h in normoxic conditions. Then, medium was removed, and the number of cells was quantified.

**Apoptosis Assay and Flow Cytometry.** Annexin V labeling was carried out using flow cytometry analysis according to the manufacturer's protocol (R&D). Cells ( $5 \times 10^5$ ) were seeded on 60 mm culture dishes in complete medium and incubated for 40 h. The medium was then removed, and cells were incubated with different concentrations of each compound in 2 mL of serum-depleted medium for 5 h. Cells were washed twice with 2 mL of PBS, trypsinized, collected by centrifugation, and resuspended in 100  $\mu\text{L}$  of ice-cold phosphate-buffered saline (PBS). Then, 100  $\mu\text{L}$  of a solution with annexin V-FITC and propidium iodide-PE was then added, and samples were incubated at room temperature, avoiding light exposure. After 15 min, 400  $\mu\text{L}$  of a binding solution was added, and the emitted fluorescence was quantified by flow cytometry within 1 h in a FACScan (BD PharMingen, San Diego, CA).

**Kinetics of Methylselenol Release.** The kinetics of release of free thiols and selenols was determined by the Ellman's Test by using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as an indicator.<sup>16</sup> DTNB reacts fast and quantitatively with thiols and selenols to give a bright yellow derivative (TNB<sup>+</sup>). Reactions were performed in 100 mM phosphate buffer, 1 mM EDTA (pH 8). The buffer solution was deaerated by bubbling nitrogen through the solution at room

Table 1. Biological Activity of Imidoselenocarbamate Derivatives<sup>a</sup>

compd	X	Y	R	VEGF		cell viability LD <sub>50</sub> ± SD (μM)		cell proliferation	
				IC <sub>50</sub> ± SD (μM)		hypoxia	normoxia	GI <sub>50</sub> ± SD (μM)	
1a	Se	methyl	phenyl	2.4 ± 0.2		4 ± 1	5.8 ± 0.1	2.1 ± 0.1	
1b	S	methyl	phenyl	>20		>20	>20	>20	
1c	Se	ethyl	phenyl	6 ± 1		2.5 ± 0.3	3 ± 2	5.1 ± 0.2	
1d	Se	isopropyl	phenyl	7 ± 1		3.4 ± 0.3	7 ± 1	18 ± 3	
1e	Se	methyl	3,5-dimethoxyphenyl	11 ± 1		4.9 ± 0.3	11 ± 1	1.0 ± 0.1	
1f	Se	isopropyl	3,5-dimethoxyphenyl	12 ± 1		31 ± 4	12 ± 1	8.2 ± 0.3	
1g	Se	methyl	4-chlorophenyl	>20		>20	>20	2.7 ± 0.1	
1h	Se	isopropyl	4-chlorophenyl	23 ± 1		11 ± 2	13 ± 3	5.9 ± 0.8	
1i	Se	methyl	4-nitrophenyl	>20		>20	>20	>20	
1j	Se	methyl	4-trifluoromethylphenyl	3.3 ± 0.2		1.4 ± 0.1	2.3 ± 0.3	1.3 ± 0.1	
1k	Se	methyl	4-cyanophenyl	3.1 ± 0.3		4.4 ± 0.8	4.6 ± 0.7	1.2 ± 0.1	
1l	Se	methyl	4-tert-butylphenyl	15 ± 3		8.8 ± 0.2	17 ± 2	2.8 ± 0.8	
1m	Se	methyl	4-methylphenyl	1.2 ± 0.5		2.2 ± 0.2	1.6 ± 0.2	1.6 ± 0.1	
1n	Se	methyl	methyl	3.8 ± 0.6		4.7 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	
1o	S	methyl	4-tert-butylphenyl	19 ± 12		>100	81 ± 23	52 ± 9	
1p	S	methyl	4-chlorophenyl	>20		>20	>20	>20	
1q	S	methyl	3-pyridyl	21 ± 2		>100	>100	>100	
1r	Se	methyl	3-pyridyl	1.5 ± 0.2		5.7 ± 0.2	7.6 ± 0.1	1.2 ± 0.1	
1s	S	methyl	2-chloro-3-pyridyl	15 ± 5		81 ± 10	76 ± 20	84 ± 7	
1t	Se	methyl	2-chloro-3-pyridyl	3.3 ± 0.6		5 ± 1	1.5 ± 0.2	0.8 ± 0.1	
apigenin				5.9 ± 0.1		>100	>100	35 ± 4	
EGCG				12 ± 2		>100	>100	12 ± 1	

<sup>a</sup>Inhibition of hypoxia-induced VEGF expression, cell viability, and cell proliferation were measured as described in the Experimental Procedures. All experiments were independently performed at least three times. Note that cell viability and VEGF expression were measured in cells cultured in medium without serum. Apigenin and EGCG were used as reference compounds.

temperature, and then, 100 μM DTNB (from a 10 mM stock solution in ethanol) was added to the buffer. The reaction was started by adding 5 μL of each compound, prepared at 10 mM in anhydrous DMSO, to 200 μL of this solution. The release of free selenols or thiols was followed at room temperature in a 96-multiwell plate by measuring the absorbance of the samples at 405 nm in a multiscan EX plate reader (Thermo) at the indicated times, using *N*-acetylcysteine (10–100 μM) as a standard. Spontaneous DTNB hydrolysis is slow but detectable at pH 8, so a DTNB blank was incubated under the same conditions, and its value was subtracted from each measurement. The same experiments were also performed in phenol red-free RPMI 1640 medium (Biowhittaker). As a negative control in these assays, we used a seleno compound that does not release selenols: 2-(4-(trifluoromethyl)benzoyselenyl)acetic acid.<sup>17</sup>

**Head Space Gas Chromatography Mass Spectrometry (HS-GC-MS) Analysis.** HS-GC-MS was performed as described.<sup>18</sup> Dimethyldiselenide and methylselenol were used as standards. Dimethyldiselenide was prepared in methanol at 10 mM. Methylselenol was prepared in situ in vials for HS-GC-MS analysis: 2.4 μL of dimethyldiselenide was dissolved in 1 mL of ethanol, then 20 mg of NaBH<sub>4</sub> was added, and the vial was capped using a rubber septum with a crimp cap. Compound 1n was prepared by dissolving 15 mg of the compound in 5% DMSO/H<sub>2</sub>O. The vial was capped as before, and the sample was kept at ambient temperature for 24 h before the analysis.

Chromatographic analyses were carried out on an Agilent 6890 gas chromatograph coupled to a 5973N quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). A HP-5MS capillary column (30 m × 0.25 mm i.d.) coated with a 0.25 μm film of stationary phase (5% phenyl methylsiloxane) was used. The flow rate of the high purity helium carrier gas was 1 mL/min. The split/split less inlet was operated in split mode, and the split ratio was 50:1. The injector was

held at 250 °C, and the transfer line to detector was held at 300 °C. The GC oven temperature was 60 °C isothermal for 1 min, 50 °C × min<sup>-1</sup> to 140 °C, then 1 min isothermal. The mass spectrometer was operated in electron impact mode (EI, 70 eV). The selected ion monitoring (SIM) conditions were established selecting the most abundant fragment ions of standards and the relative intensity of each one, according the characteristic Se isotope patterns of compounds containing one or two Se isotopes.<sup>18</sup>

## RESULTS

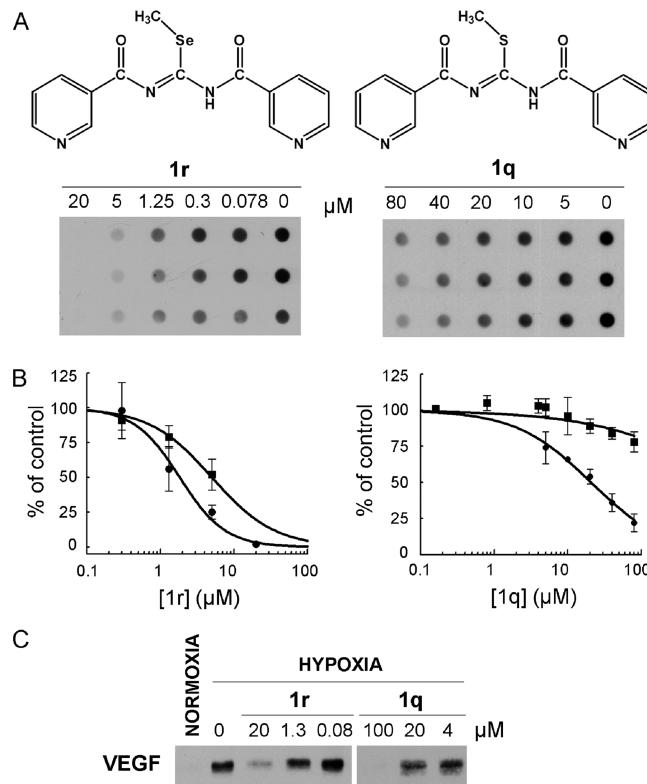
**Imidoselenocarbamate Derivatives Inhibit Hypoxia-Induced VEGF Expression in H157 Cells.** We used a screening assay to measure the effect of a large number of newly synthesized compounds on the secretion of VEGF induced by hypoxia by lung tumor cells. This screening was carried out with H157 cells in 96-well plates that underwent hypoxia (1% O<sub>2</sub>) for 24 h. Supernatants were collected and filtered through a nitrocellulose membrane using a dot-blot apparatus, and VEGF was immunodetected with a suitable antibody.<sup>14</sup> The viability of cells, cultured under hypoxia or normoxia, and the effect of each compound on cell proliferation were simultaneously measured in 96-well plates. Using this scheme, we tested the activity of 180 structurally diverse compounds. From these, several imidoselenocarbamate (iso-selenourea) derivatives showed the highest biological activity and were further evaluated.

Table 1 shows the biological activity of 15 imidoselenocarbamate derivatives and five imidothiocarbamate analogues

included in this study for comparison purposes. Apigenin and EGCG, two well-known polyphenols that inhibit HIF-1 $\alpha$  and VEGF expression induced by hypoxia,<sup>19,20</sup> were included in the screening as reference compounds. The analysis of the activity of five pairs of sulfur and Se analogues (**1q–1r**, **1s–1t**, **1b–1a**, **1o–1l**, and **1g–1p**) indicated that the presence of Se was essential for biological activity. In several cases (as in **1s–1t**), the Se derivative was 50–100 times more cytotoxic and antiproliferative than the corresponding sulfur analogue. Moreover, all Se compounds were more active than sulfur analogues in inhibiting hypoxia induced-VEGF expression. The effect of changing the “Y” alkyl chain length bound to the Se atom was also analyzed (**1a–c–d**, **1e–f**, and **1g–h**), and the order of activity was found to be methyl > ethyl > isopropyl. This difference was more clearly seen in the inhibition of the proliferation of H157 cells. The screening was also designed to detect molecules capable to selectively kill tumor cells under hypoxia. These compounds would potentially be very interesting for the treatment of solid tumors. However, none of the tested derivatives proved to be much more cytotoxic in hypoxic than in normoxic conditions.

**Compound 1r Inhibited VEGF Expression Induced by Hypoxia Irrespective of HIF-1 $\alpha$  Levels.** The most interesting compound found was **1r**, which inhibited VEGF ( $IC_{50} = 1.5 \mu M$ ) and cell proliferation ( $GI_{50} = 1.2 \mu M$ ) at low micromolar concentrations (Figure 1). This compound was selected for further analysis, along with the corresponding sulfur analogue **1q** ( $IC_{50} = 21 \mu M$ ,  $GI_{50} > 100 \mu M$ ), which was included for comparison purposes. The regulation of VEGF in response to hypoxia is thought to be mediated primarily through the transcription factor HIF. Under hypoxic conditions, HIF-1 $\alpha$  peaked at 4–6 h in H157 cells and then slowly decreased.<sup>14</sup> For this reason, we measured the effect of **1q** and **1r** on HIF-1 $\alpha$  expression in response to hypoxia. Apigenin, a flavonoid that decreased hypoxia-induced HIF-1 $\alpha$  protein levels,<sup>18</sup> was used as a positive control. As shown in Figure 2A, compounds **1q** and **1r** did not affect HIF-1 $\alpha$  expression. Recently, we have found that several flavonoids impair VEGF transcription by an alternative mechanism that did not depend on HIF expression.<sup>14</sup> We showed that these flavonoids suppressed hypoxia-induced STAT3 tyrosine phosphorylation, and this activity correlated with their potency as VEGF inhibitors. We tested whether **1r** impaired STAT3 activation mediated by hypoxia. As shown in Figure 2B, **1r** showed a dose-response inhibition of STAT3 phosphorylation following exposure to hypoxia. To test if this inhibition may account for the observed reduction in VEGF expression, we tested the effect of tyrphostin AG490 and PP2 on HIF-1 $\alpha$  and VEGF protein expression. Tyrphostin AG490 is an efficient tyrosine kinase inhibitor of JAK/STAT activation, and PP2 is a selective inhibitor for src-family tyrosine kinases, which have been implicated in STAT3 activation by hypoxia. We found that tyrphostin, but not PP2, inhibited STAT3 phosphorylation and VEGF expression induced by hypoxia without modifying HIF-1 $\alpha$  levels (Figure 2C).

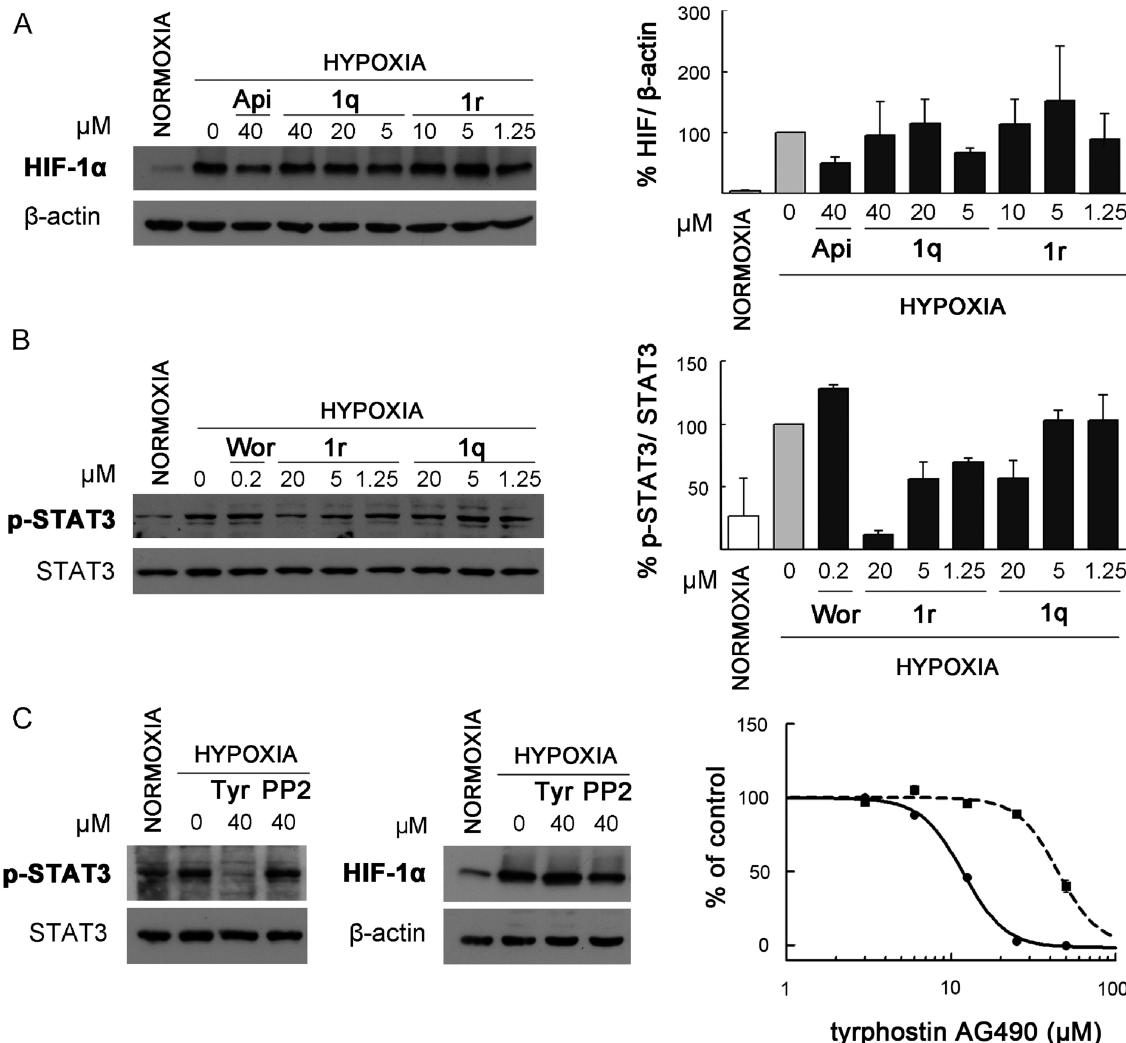
**Imidoselenocarbamate Derivatives Released Methylselenol under Hypoxic Conditions.** While performing the screening for new VEGF inhibitors in 96-well plates under hypoxia, we realized that the presence of high concentrations of certain methyl imidoselenocarbamate derivatives in a well decreased cell viability in the neighbor wells of the same multiwell dish. To study this effect, cells were seeded in 96-well plates, and **1r** was added at 40  $\mu M$  to three consecutive wells



**Figure 1.** Effect of **1r** and **1q** on hypoxia-induced VEGF expression. (A) Effect of **1r** and **1q** on the production of VEGF by H157 cells as measured by dot blot. H157 cells were seeded in 96-well plates, treated in triplicate with different concentrations of **1r** or **1q**, and incubated for 24 h under hypoxia in medium without serum. Then, 100  $\mu L$  of each supernatant was filtered through a nitrocellulose membrane in a dot blot apparatus. The membrane was then blocked, and VEGF was immunodetected with MAB293 anti-VEGF antibody. (B) Dose-response curves for the inhibition of hypoxia-induced VEGF expression (●) and cell viability (■) in H157 cells by **1r** and **1q**. (C) Effect of **1r** and **1q** on hypoxia-induced VEGF expression as measured by Western blot. H157 cells were incubated under normoxia (21%  $O_2$ ) or hypoxia (1%  $O_2$ ) for 24 h in medium without serum. Then, supernatants were collected and concentrated with a centrifugal filter device, and VEGF was detected by Western blot.

(Figure 3A). Plates were cultured under hypoxia or normoxia, and cell viability was measured on the whole plate after 24 h of incubation. As shown in Figure 3B, when plates were incubated under hypoxic conditions, there was a direct relationship between the distance of each well to the treatment and cellular viability. This result suggested that a volatile and cytotoxic compound was released, which diffused to the adjacent wells under hypoxic conditions. To ascertain if this liberation was due to metabolic transformation of the compound within the cells, the experiment was repeated by adding the compound to three wells containing only culture medium. Under these conditions, gas liberation occurred to the same extent and only under hypoxic conditions (data not shown). The fact that methylselenol was released irrespective of the presence of the cells led us to the conclusion that metabolic cell transformation of the compounds within the cells was not necessary.

Analyzing the chemical structure of **1r**, it seemed possible that the cytotoxic compound released might indeed be methylselenol. Methylselenol is a gas that can be produced in vitro by incubating MSE with bacterial methioninase (METase).<sup>21</sup> So, we repeated the above experiment adding



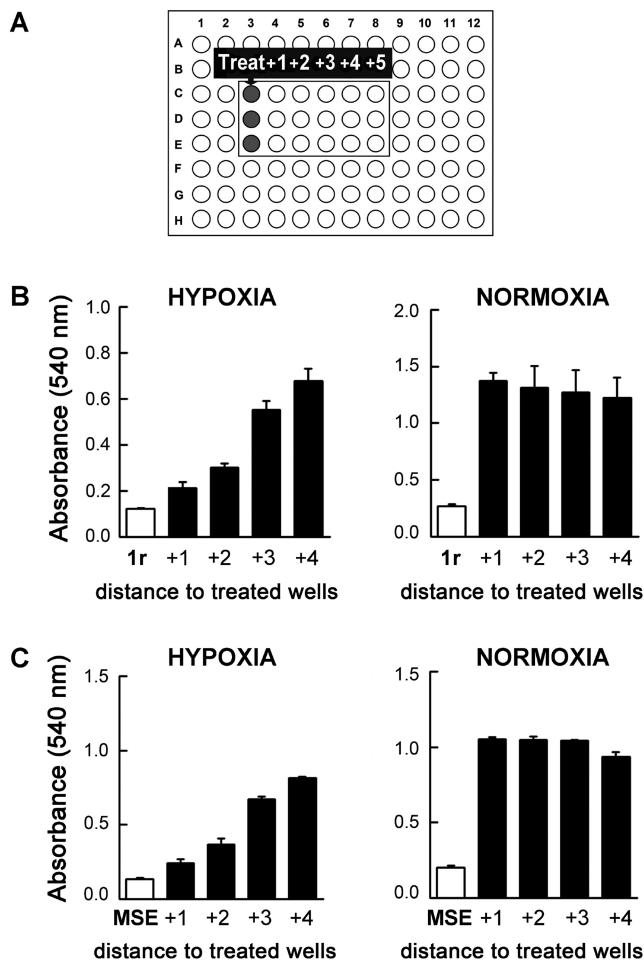
**Figure 2.** Effect of **1r** and **1q** on HIF-1 $\alpha$  expression and STAT3 activation induced by hypoxia in H157 cells. Subconfluent H157 cells were pretreated with the indicated concentrations of compounds for 30 min before they were incubated under hypoxia for 6 h. Total cell extracts were analyzed by immunoblotting with antibodies against the indicated proteins. (A) Effect of **1q** and **1r** on HIF-1 $\alpha$  expression. (B) Effect of **1q** and **1r** on STAT-3 phosphorylation. Mean ratios from densitometric analyses of HIF/ $\beta$ -actin and pSTAT3(Tyr<sup>705</sup>)/STAT3 were obtained from three independent experiments. (C) Effect of tyrphostin (AG490) and PP2 on HIF-1 $\alpha$  expression and STAT phosphorylation. Dose-response curves for tyrphostin AG490 on the inhibition of hypoxia-induced VEGF expression (●) and cell viability (■). Api, apigenin; Wor, wortmannin; Tyr, tyrphostin AG490; and PP2, Src family kinase inhibitor.

40  $\mu$ M selenomethionine and methioninase to the cells. Notably, this treatment produced the same effects on H157 cells under normoxia and hypoxia than those found with 40  $\mu$ M **1r** (Figure 3C), suggesting that the gas released by **1r** was in fact methylselenol. On the basis of these results, we proposed that methylselenol was released by hydrolysis following the reaction shown in Figure 4A.

Using the same bioassay, we tested whether other methyl imidoselenocarbamate derivatives were able to release methylselenol. As shown in Figure 4B, compounds **1n**, **1t**, and to a lesser extent **1a**, clearly decreased cell viability in adjacent wells. It was possible that some of the inactive products also released methylselenol but in reduced quantities that would make them undetectable. Therefore, we repeated the experiment adding a higher concentration of each compound in the wells (200  $\mu$ M). Under these conditions, it was found that compounds **1m** and **1a** also released methylselenol under hypoxia (data not shown). Compound **1i** showed only marginal activity. We were not able to detect liberation of methylselenol from this compound,

reinforcing the notion that methylselenol was responsible for the biological activity of methyl imidoselenocarbamate derivatives.

We directly confirmed that a volatile selenol was released by a quite simple experiment described in Figure 5A. In this experiment, it was clearly shown that the small tube containing **1n** released a gas that reacted with Ellman's reagent, changing the color of the large tube. Moreover, we measured the kinetics of the release of methylselenol from imidoselenocarbamate derivatives using Ellman's reagent. Imidothiocarbamate derivatives **1s** and **1q** were also tested for comparison purposes. In these assays, compounds were added directly to a solution containing DTNB (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, and 100  $\mu$ M DTNB, pH 8), and the rate of thiol or selenol release was measured spectrophotometrically at 405 nm. Both selenol and thiol compounds reacted with Ellman's reagent, although methylselenol release by imidoselenocarbamate derivatives was considerably faster than thiol release by imidothiocarbamate derivatives (Figure 5B). Moreover, the rate of



**Figure 3.** Compound 1r releases methylselenol, which diffuses to the adjacent wells under hypoxia. H157 cells were seeded in 96-well plates in complete medium and incubated for 24 h before experimental treatments. Then, cells were washed twice with PBS and incubated in medium without serum under normoxia (21% O<sub>2</sub>) or hypoxia (1% O<sub>2</sub>) for 24 h. (A) Plate layout diagram for the experiment. Treatments (in triplicate) were only added to the black wells, while cell viability was measured on the whole plate using the neutral red assay. (B) Effect of 40 μM 1r on the viability of adjacent wells under hypoxic or under normoxic conditions. Columns show the mean and standard deviations obtained from the indicated wells. (C) Effects of methylselenol on cell viability under hypoxic and normoxic conditions. Cells were cultured for 24 h under hypoxic or normoxic conditions, and cell viability was measured as above. Methylselenol was enzymatically generated by incubating 0.01 U/mL recombinant methioninase (METase) with 40 μM their substrate MSE.

methylselenol generation was notably influenced by R substituent. The rate of liberation of methylselenol was found to decrease in the order: methyl > 3-pyridil > 2-chloro-3-pyridil (**1n** > **1r** > **1t**).

Table 2 shows the kinetic constants and the half-life of the compounds analyzed. Kinetic release of methylselenol was determined in two different media: buffer phosphate [100 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, and 100 μM DTNB (pH 8)] and phenol red-free RPMI 1640 medium containing 100 μM DTNB. The rate of release of methylselenol in RPMI medium occurred about 10 times faster than in the case of phosphate buffer, probably as a consequence of the pH rise found in culture media containing bicarbonate (such as RPMI) when studies are performed at room atmosphere.<sup>22</sup> In addition, the

half-lives of Se derivatives (**1n**, **1r**, and **1t**) were considerably shorter than those of the corresponding thiol analogues (**1s** and **1q**).

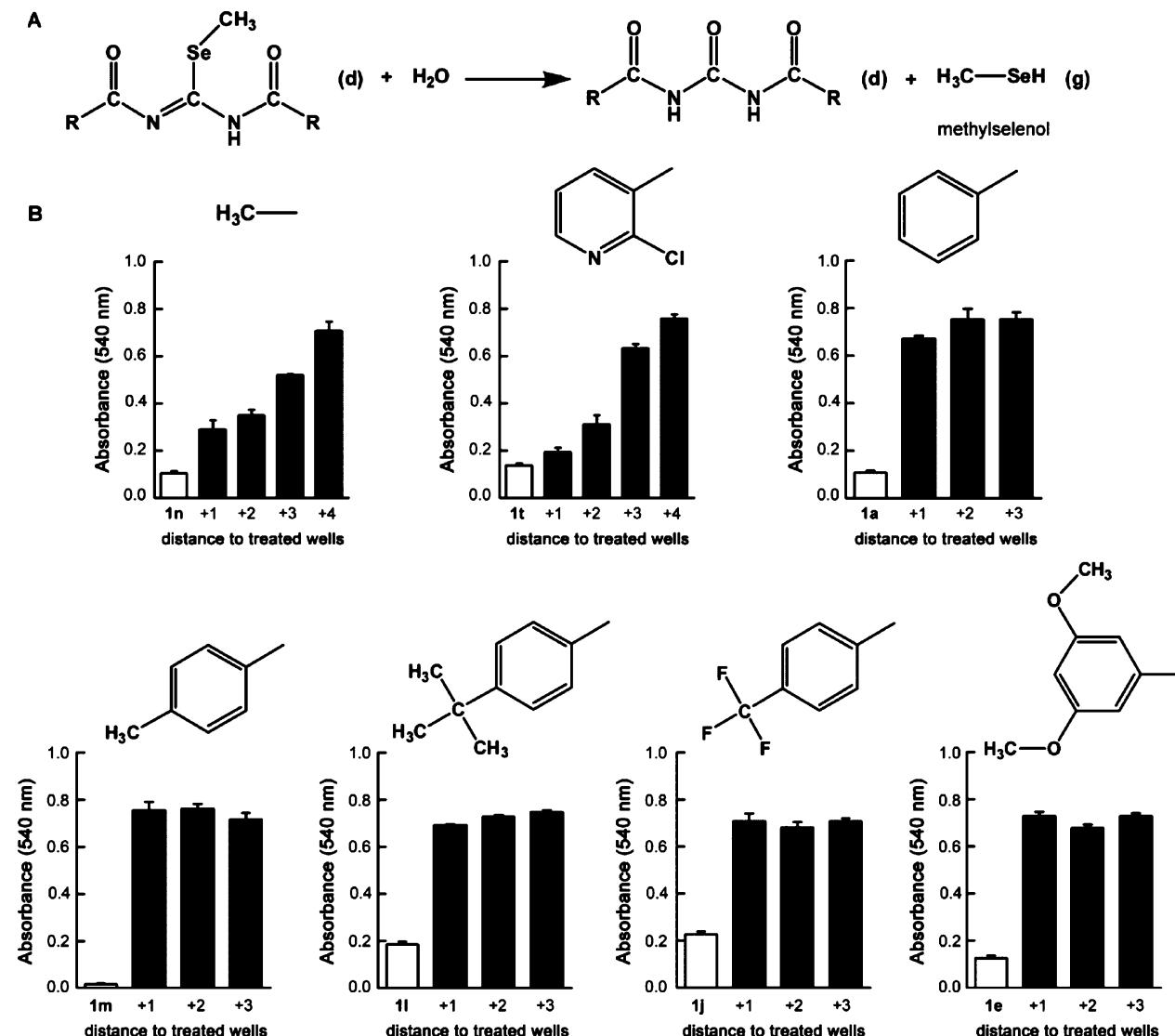
**Determination of Methylselenol by HS-GC-MS Analysis.** The release of methylselenol by methyl imidoselenocarbamate derivatives was analyzed by HS-GC-MS. First, we recorded mass spectra of standards of dimethyldiselenide (CH<sub>3</sub>—Se—Se—CH<sub>3</sub>) and methylselenol (CH<sub>3</sub>—SeH) by HS-GC-MS (Figure S1 in the Supporting Information). Commercially available dimethyldiselenide gives rise to a well-defined peak (retention time, 2.90 min) with a characteristic mass spectrum.<sup>18</sup> Methylselenol, produced in situ by reduction of dimethyldiselenide with sodium borohydride, gives two well-resolved peaks with retention times of 1.50 and 2.90 min, which correspond to methylselenol and dimethyldiselenide, respectively. The second peak gets more abundant over time, confirming that dimethyldiselenide is spontaneously formed by oxidation of methylselenol under aerobic conditions, as previously described.<sup>18</sup>

Release of methylselenol from imidoselenocarbamate derivatives was demonstrated by simple hydrolysis of compound **1n**. This molecule was dissolved in 5% DMSO/H<sub>2</sub>O, kept into a capped vial for 24 h, and analyzed by HS-GC-MS (Figure 6). Retention times and mass spectra obtained after this time confirmed the presence of methylselenol and dimethyldiselenide, which accumulates in the presence of oxygen.

**Kinetics of the Release of Methylselenol Correlated with Early Proapoptotic Activity.** We also tested whether the rate of release of methylselenol was related to the biological activity of these compounds. It has been previously shown that imidocarbamate derivatives induced apoptosis in tumor cells. Hence, we measured the induction of early apoptosis by several methylselenol precursors. Cells were treated for 5 h with methyl imidocarbamate derivatives, stained with annexin V and propidium iodide, and analyzed by flow cytometry. Figure 7 shows the percentage of damaged, necrotic, apoptotic, and live cells after treatment with a 10 μM concentration of each compound. Apart from **1l**, molecules that quickly release methylselenol (**1n**, **1r**, and **1t**) showed higher amounts of apoptotic cells than those that release it slowly (**1m** and **1a**) or release low levels that were undetected in the bioassay (**1e** and **1j**). The correlation between the rate of release of methylselenol and the induction of early apoptosis by imidoselenocarbamate derivatives suggests that this molecule was responsible for their proapoptotic activity.

## DISCUSSION

In this work, we show that certain imidoselenocarbamate derivatives inhibited VEGF expression induced by hypoxia in H157 cells. The most interesting compound was **1r**, because it inhibited VEGF expression ( $IC_{50} = 1.5 \mu\text{M}$ ) and cell proliferation ( $GI_{50} = 1.2 \mu\text{M}$ ) at low micromolar concentrations. Our results show that imidoselenocarbamate derivatives inhibited VEGF expression induced by hypoxia by a HIF-independent mechanism. Mechanistic studies performed with this molecule suggest, but do not demonstrate, that the inhibition of VEGF expression may be related with its ability to block STAT3 phosphorylation induced by hypoxia. This is a plausible hypothesis, and recently, several compounds have been shown to affect VEGF expression by a similar mechanism.<sup>14,23,24</sup> Hypoxia has been shown to induce STAT3 phosphorylation in many cell lines, and signaling pathways involving hypoxia-dependent activation of STAT3 have been



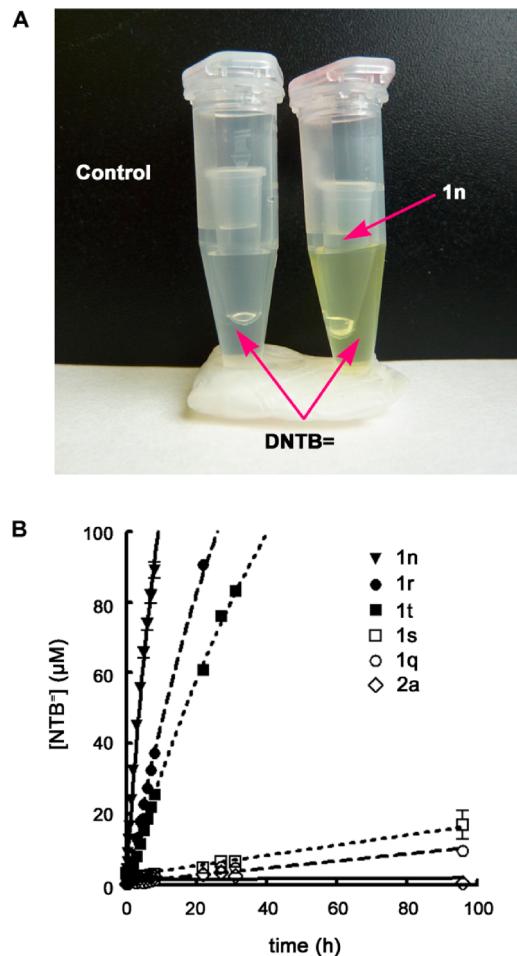
**Figure 4.** Release of methylselenol by methyl imidoselenocarbamates depends on R substituents. (A) Proposed reaction for the liberation of methylselenol by imidomethylselenocarbamates. (B) Effect of the indicated compounds on cell viability of adjacent wells measured under hypoxic conditions. Experiments were performed as shown in Figure 3 by adding a 40  $\mu\text{M}$  concentration of each compound to the treated wells.

well elucidated.<sup>25–27</sup> STAT3 is activated by dimerization upon tyrosine phosphorylation in response to various cytokines and growth factors, and it is also a target of the c-Src, a nonreceptor tyrosine kinase that is endogenously activated by hypoxia. Activation of STAT3 by tyrosine phosphorylation leads to its dimerization and translocation to the nucleus. HIF-1 $\alpha$  and STAT3 can bind simultaneously to the VEGF promoter, and both are required for maximum transcription of VEGF mRNA following hypoxia.<sup>25</sup>

While the anti-VEGF properties associated with these compounds may be relevant for their antitumoral properties, their associate toxicity limits their therapeutic use as pure antiangiogenic agents, and for this reason, anti-VEGF activity was not pursued further. Interestingly, we observed that under hypoxia, several methyl imidoselenocarbamate derivatives affected not only to the viability of the treated well but to the whole plate. Upon further analysis, it was found that several, but not all, monomethylated Se derivatives released a gas (methylselenol) that diffused across the plate, causing these effects. This discovery was accidental, since the diffusion of methylselenol between wells occurs only under hypoxic

conditions and is not detected in assays performed in normoxia. In the presence of oxygen, methylselenol readily oxidizes to the less volatile dimethylselenide ( $\text{CH}_3-\text{Se}-\text{Se}-\text{CH}_3$ ), which can be easily reduced to methylselenol within the cells.<sup>18,28</sup> The release of methylselenol did not depend on cellular activity, since it was also released when the product was added directly to the culture medium without cells or even to a neutral phosphate buffer. As far as we know, this is the first time that this behavior is described with any methylselenol precursor.

We performed a variety of experiments to verify that the diffusible gas was indeed methylselenol. First, we used a reaction that directly produces this substance. Esaki et al. demonstrated that bacterial L-selenomethionine  $\gamma$ -lyase (methioninase) generates methylselenol when selenomethionine was used as substrate.<sup>29</sup> Using this reaction, we found under hypoxic conditions that methylselenol was able to diffuse from one well to another, that the diffusion pattern observed in the plates was similar to those found with active methyl imidoselenocarbamates, and that methylselenol inhibited hypoxia-induced VEGF production, cell viability, and cell proliferation. What is more noteworthy, the concentrations of



**Figure 5.** Kinetics of the release of methylselenol and methylthiol from imidoselenocarbamates and imidothiocarbamates derivatives. Selenol and thiol groups were titrated by the method of Ellman. DTNB reacts with thiol or selenol groups to give TNB<sup>-</sup>, a bright yellow compound at neutral and alkaline pH. (A) This simple experiment demonstrates that **1n** releases a volatile selenol. Small PCR tubes (without caps) were inserted inside large Eppendorf tubes in a nitrogen atmosphere. Big tubes contained 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, and 100 μM DTNB at pH 8. Small tubes contained the same buffer plus 20 mM DTT, to avoid methylselenol oxidation. The small tube on the right also contained compound **1n**, a methylselenol precursor. The picture was taken after 14 h of **1n** addition. (B) Plots show time courses for the release of methylselenol and methylthiol as measured by the appearance of TNB<sup>-</sup>. Reactions were performed in normoxia at room temperature in 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, and 100 μM DTNB at pH 8. Measurements were done at 405 nm in the linear range of the Ellman reaction, using *N*-acetylcysteine (10–100 μM) as a standard. Experimental data were fitted using nonlinear regression to a first-order kinetic model. Results are means of triplicate values ± SDs. Compound **2a**, an imidoselenoacetic derivative unable to release selenols, was used as a negative control (see the Experimental Procedures).

methylselenol needed to produce these effects, measured as the concentration of its precursor selenomethionine, were similar to those found with the most potent imidoselenocarbamate derivatives. Moreover, Ellman's reaction confirmed the presence of volatile selenols upon addition of imidoselenocarbamates to an aqueous medium in the absence of O<sub>2</sub> (Figure 5A). Finally, HS-GC-MS directly confirmed that these compounds released methylselenol by hydrolysis and that this

molecule is easily oxidized to dimethylselenide under aerobic conditions.

Kinetic studies carried out indicate that not all methyl imidoselenocarbamate derivatives released methylselenol at the same speed. In fact, the rate of release of methylselenol can be controlled by modifying the chemical structure of the radical substituents. Kinetic analysis showed that sulfur analogues **1s** and **1q** also liberated free thiols (methanethiol), in a reaction similar to that shown in Figure 4A, but in that case, the rate of hydrolysis was very slow. Moreover, sulfur derivatives showed consistently far less biological activity than those of Se (Table 1). In the case of the pair **1s**–**1t**, this difference amounts to more than 100 times [GI<sub>50</sub> (**1t**) = 0.8 μM; GI<sub>50</sub> (**1s**) = 84 μM], reflecting the importance of Se in biological activity. Taking into account that Se and sulfur have similar covalent radii, it was concluded that the overall structure of the molecule plays a secondary role in the biological activity of these compounds with respect to the liberation of methylselenol. The remarkable increase in reactivity of the imidoselenocarbamate derivatives versus the corresponding imidothiocarbamate derivatives seems to be due to differences between the chemistry of sulfur and Se.<sup>10,30</sup> It is well-known that the C–Se bond is considerably weaker than the C–S bond<sup>30,31</sup> and that the –SeH group of selenocysteine (pK<sub>a</sub> = 5.2) is substantially more acidic than the –SH group of cysteine (pK<sub>a</sub> = 8.3).

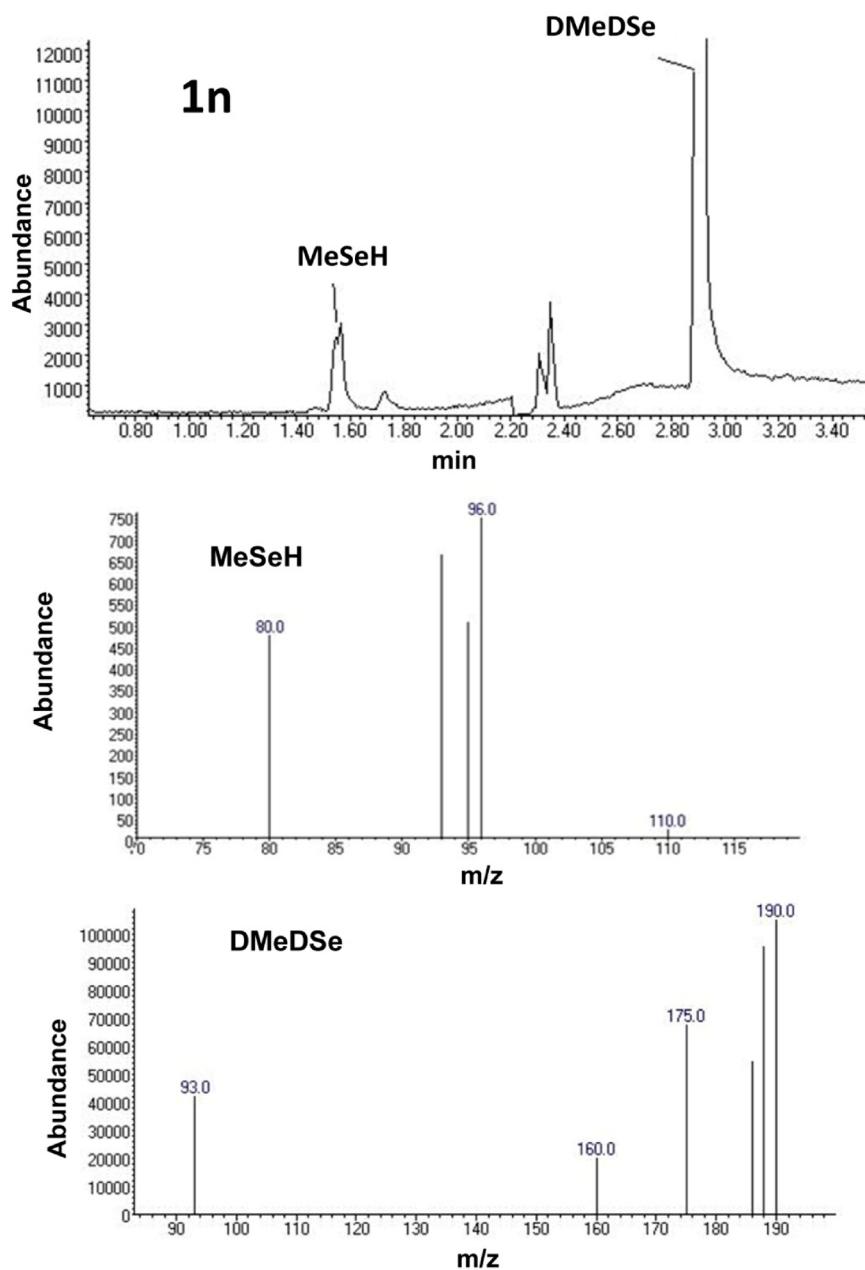
The results obtained in this work are consistent and shed light on those published previously with some of these derivatives in other pharmacological assays. Imidoselenocarbamates have been shown to possess a broad range of biological activities, including antiproliferative, proapoptotic, antitumoral,<sup>11,12</sup> and antileishmanial activities.<sup>13</sup> Accepting that methylselenol is the active metabolite of the molecule, it is easy to understand why molecules that release methylselenol, like **1t**, **1a**, or **1m**, are very cytotoxic in PC-3 prostate cancer cells.<sup>12</sup> Moreover, the effect of changing the alkyl chain length bound to the Se has also been tested in these cells, and the order of activity was found to be methyl > ethyl > isopropyl in all cases,<sup>12</sup> as one will expect from the activities of the corresponding alkylselenol derivatives (methyl-, ethyl- and propyl- selenol, respectively). Similar findings were found in antileishmanial assays, where compounds **1r** and **1t** were the most active among those tested.<sup>13</sup> The inhibition of VEGF expression induced by hypoxia by methyl imidoselenocarbamates also seems to be related to the release of methylselenol. In vivo studies have shown that methylselenol shows antiangiogenic properties, although the molecular mechanism implicated is not fully understood.<sup>9,32,33</sup> Moreover, it has also been described that methylselenol, but not selenol, inhibits VEGF expression in several cancer lines at lower concentrations than those needed for apoptosis induction.<sup>32</sup>

Organoselenium compounds have traditionally been regarded as chemopreventive substances, although given the interesting biological effects of methylselenol, it seems logical to regard this molecule as a promising antitumor agent. Methylselenol affects not just one key target but a variety of targets,<sup>34</sup> making it difficult for premalignant cells to escape the inhibitory effect of this compound. One of the main drawbacks for using methylselenol as a therapeutic agent is the method of administration, as methylselenol is an unstable and highly reactive gas. To date, the best way to analyze the biological effects of methylselenol is to generate it locally. Methylselenocysteine can be converted in vivo to methylselenol by β-lyases. However, mammary cell lines (rodent or human) have low β-

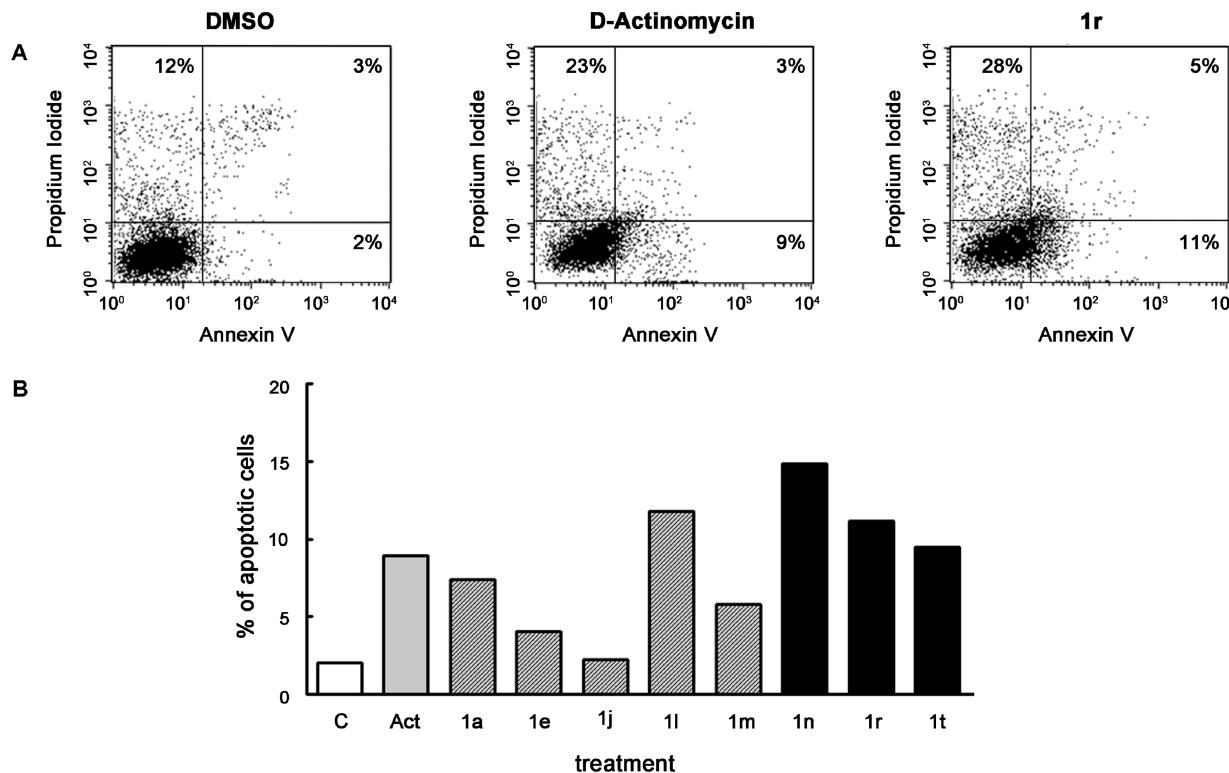
**Table 2.** Kinetics of Methylselenol Release from Imidoselenocarbamate Derivatives Measured in Phosphate Buffer and in RPMI Medium<sup>a</sup>

compd	phosphate buffer <sup>b</sup>			RPMI medium <sup>c</sup>		
	<i>k</i> (h <sup>-1</sup> )	R <sup>2</sup>	t <sub>1/2</sub> (h)	<i>k</i> (h <sup>-1</sup> )	R <sup>2</sup>	t <sub>1/2</sub> (h)
<b>1n</b>	0.075 ± 0.001	0.998	9.2	0.84 ± 0.07	0.973	0.82
<b>1r</b>	0.026 ± 0.001	0.996	26	0.24 ± 0.01	0.999	2.9
<b>1t</b>	0.017 ± 0.001	0.998	40	0.15 ± 0.01	0.992	4.7
<b>1s</b>	0.00075 ± 0.00004	0.962	917	0.023 ± 0.001	0.996	31
<b>1q</b>	0.00052 ± 0.00003	0.940	1333	0.033 ± 0.001	0.981	24

<sup>a</sup>The table shows the first-order rate constants for the release of methylselenol from imidoselenocarbamates measured with Ellman's reagent. Reactions were performed in normoxia at room temperature. *k* represents the first-order rate constant of the reaction; t<sub>1/2</sub>, the half-life of the reaction; and R<sup>2</sup>, the goodness of fit. <sup>b</sup>100 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, and 100 μM DTNB (pH 8). <sup>c</sup>Phenol red-free RPMI 1640 medium containing 100 μM DTNB.



**Figure 6.** HS-GC-MS of compound **1n** after 24 h in 5% DMSO. Compound **1n** was dissolved in 5% DMSO/H<sub>2</sub>O, and the vial was then capped using a rubber septum with a crimp cap and stored for 24 h at ambient temperature before the analysis. Head space GC-MS analysis showed the presence of methylselenol (MeSeH) at 1.56 min and dimethyldiselenide (DMeDSe) at 2.92 min (see Figure 1 in the Supporting Information). The ion monitoring (SIM) conditions were *m/z* 96, 95, 93, and 80 for methylselenol and *m/z* 190, 188, 186, 175, and 160 for dimethyldiselenide.<sup>18</sup>



**Figure 7.** Induction of apoptosis by methylselenol precursors in lung cancer cell line H157. Cells were treated with a 10  $\mu$ M concentration of the indicated compounds for 5 h in serum-free medium. (A) Representative flow cytogram of annexin V binding (abscissa) vs propidium iodide uptake (ordinate) for vehicle-treated cells, for D-actinomycin used as a positive control, and for 1r. The numbers in the upper left quadrant, upper right quadrant, lower left quadrant, and lower right quadrant represent the percentage of damaged cells (annexin V+/PI+), necrotic cells (annexin V+/PI+), live cells (annexin V-/PI-), and apoptotic cells (annexin V+/PI-), respectively. (B) Percentage of apoptotic cells after treatment with vehicle (0.5% DMSO), D-actinomycin (positive control), and several methylselenol precursors. Black bars show fast-releasing methylselenol compounds, while striped bars show those that either release methylselenol slowly or do not release it at all.

lyase activity.<sup>10</sup> Selenomethionine may also be transformed in methylselenol by the catalytic action of METase, but this activity is not present in mammalian cells.<sup>10</sup> It is possible to transform tumor cells in vitro to express this enzyme, although this approach is not applicable in vivo. In this work, we demonstrate that methyl imidoselenocarbamate derivatives released methylselenol by hydrolysis and that the kinetics of liberation depends on the radical substituents present in each molecule. From this point of view, imidoselenocarbamate derivatives provide an excellent opportunity for the rational design of new drugs that release methylselenol in a controlled way.

## ■ ASSOCIATED CONTENT

### S Supporting Information

Figure of analysis of standards of methylselenol and dimethylselenide by HSGC-MS. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid) or Ellman's reagent; EGCG, epigallocatechin-3-gallate; HIF-1, hypoxia-inducible factor 1; HREs, hypoxia-response elements; MSE, seleno-L-methionine; METase, L-methionine  $\gamma$ -lyase; Se, selenium; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor

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