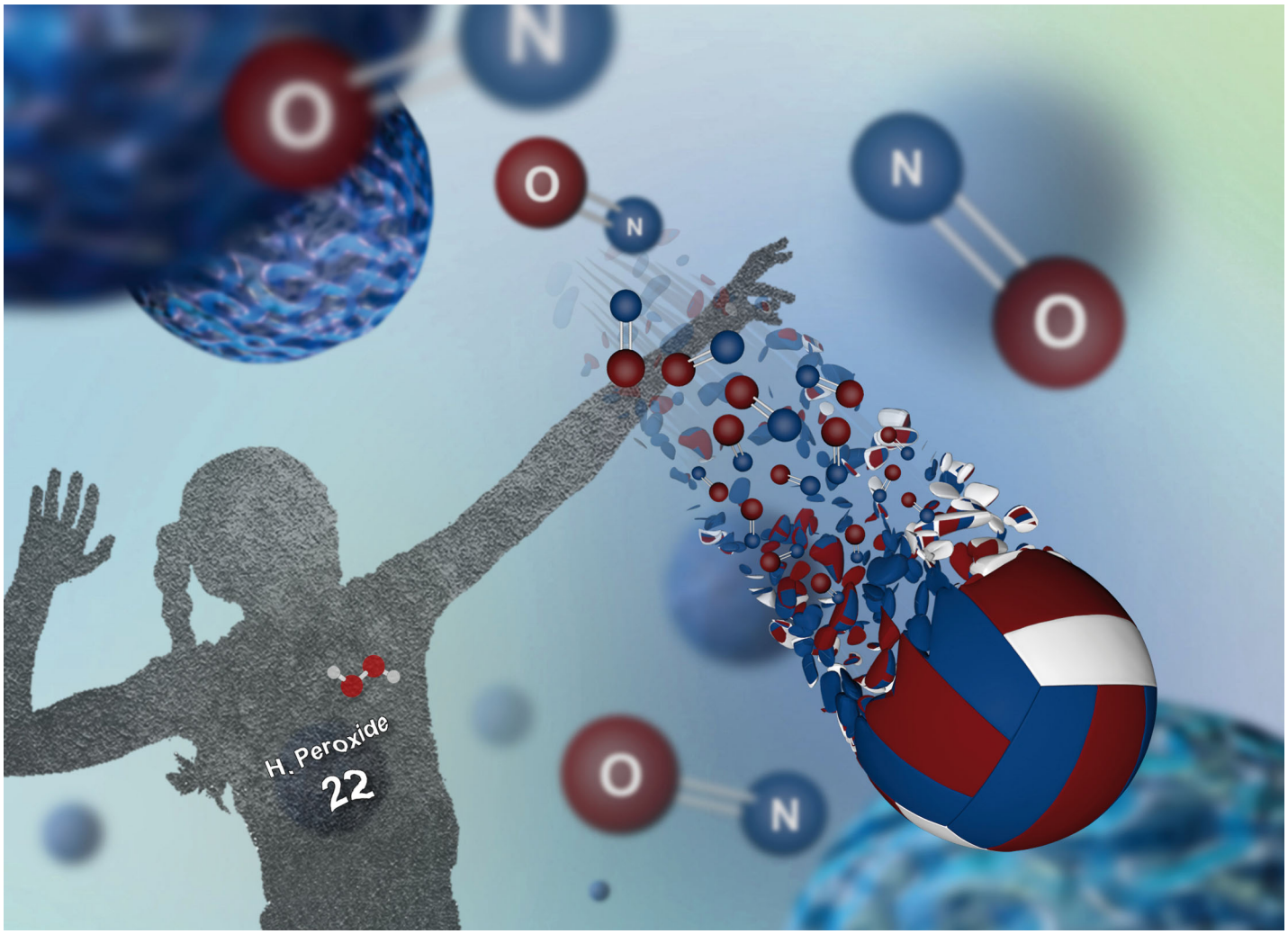

Showcasing research from Professor Jian Yin's laboratory, School of Biotechnology, Jiangnan University, Wuxi, China.

Novel α-ketoamide based diazeniumdiolates as hydrogen peroxide responsive nitric oxide donors with anti-lung cancer activity

KA-NO, a novel type of hydrogen peroxide (H_2O_2)-activated diazeniumdiolate based on an α-ketoamide moiety was developed as nitric oxide (NO) donors. KA-NO-4 inhibited lung cancer cells with submicromolar activity.

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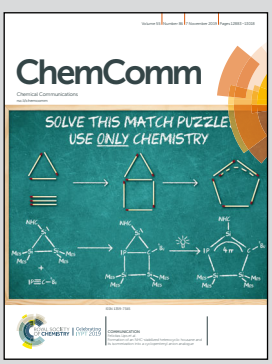

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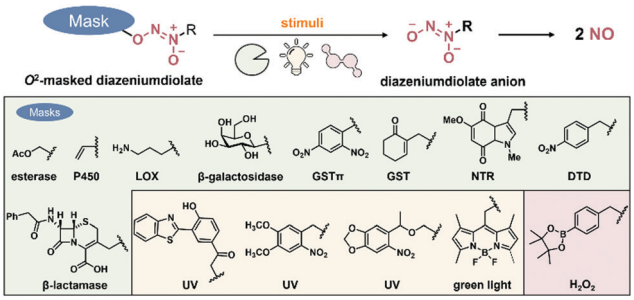
Novel α-ketoamide based diazeniumdiolates as hydrogen peroxide responsive nitric oxide donors with anti-lung cancer activity†

Junjie Fu, ‡[a] Jing Han, ‡[b] Tingting Meng,[a] Jing Hu[ac] and Jian Yin*[a]

A novel type of hydrogen peroxide (H_2O_2)-activated diazeniumdiolate based on an α-ketoamide moietey was developed as a nitric oxide (NO) donor. KA-NO-4 inhibited lung cancer cells with submicromolar activity. The H_2O_2-responsive behaviour of KA-NO-4 was thoroughly investigated. The NO-centered mechanism of action of KA-NO-4 was intracellularly studied.

Nitric oxide (NO), once treated simply as an atmospheric pollutant, was revolutionarily recognized as the endothelium-derived relaxing factor (EDRF) in 1986 and is now at the forefront of medicinal chemistry research. The important and multifunctional roles of NO in biological systems have provoked the rethinking of many old compounds, such as glyceryl trinitrate and isosorbide dinitrate, as useful medications due to their ability to release NO.[1] Diazeniumdiolates (NONOates), first reported nearly 60 years ago, have been developed into promising NO-releasing molecules pioneered by Keefer *et al.*[2] The most intriguing property of diazeniumdiolates is the synthetic accessibility to modify them as O^2-masked prodrugs, which are stable but can be triggered by different stimuli to decompose into diazeniumdiolate anions and liberate NO (Scheme 1). Numerous masking moieties responsive to different stimuli can be employed to construct O^2-masked diazeniumdiolates, the number of which is increasing robustly. The majority of reported diazeniumdiolate prodrugs are activated by endogenous enzymes such as esterase, cytochrome P450 (CYP),[3] lysyloxidase (LOX),[4] β-galactosidase,[5] glutathione transferase (GST),[6–8] DT-diaphorase (DTD),[9] nitroreductase (NTR),[10] β-lactamase,[11] (Scheme 1) *etc.* Of particular importance is that many of these enzymes are known to be specifically or highly expressed in cancer cells. As a result, the liberation of highly diffusible NO can be spatially controlled. Moreover, several photocaged diazeniumdiolates have been documented (Scheme 1).[12–15] Light-triggered activation of these prodrugs makes the spatiotemporal control of NO release possible.



Scheme 1 Reported O^2-masked diazeniumdiolates activated by different stimuli (labelled under each structure) and the general mechanism to release NO.

Hydrogen peroxide (H_2O_2)-activated promoieties are hotly pursued in the fields of medicinal chemistry, analytical chemistry and nanomaterials.[16] Compared with the above-mentioned stimuli such as enzymes and light, there are several unique advantages of utilizing H_2O_2 as a trigger. Firstly, the levels of H_2O_2 are significantly higher in cancer cells (5–1000 μM)[17] than in normal cells (0.001–0.7 μM).[18] Secondly, H_2O_2 is a hallmark not only in cancer, but also in inflammatory diseases and cardiovascular disorders. Therefore, H_2O_2-activated prodrugs can be potentially applicable in multiple pathological conditions.

The Chakrapani group initiated the synthesis of arylboronate ester based diazeniumdiolates as H_2O_2-inducible NO donors in bacteria and in cancer cells.[19,20] Until now, this remains the only type of H_2O_2-responsive diazeniumdiolate, although other O^2-masked diazeniumdiolates keep emerging. This is because arylboronate ester is almost the sole structure, which can be used to construct a H_2O_2-responsive prodrug. Encouragingly, we recently demonstrated that in addition to arylboronate ester, α-ketoamide can be another H_2O_2-specific switch for prodrug design.[21] Herein,

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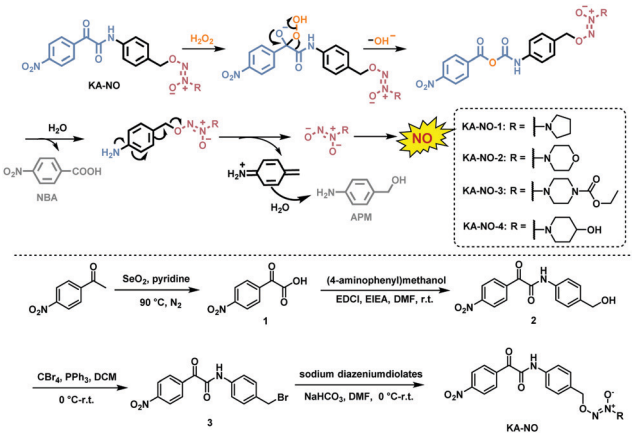
‡ These authors contributed equally.



Scheme 2 The mechanism of H_2O_2-responsive activation of KA-NO to release NO (top) and the synthetic route of KA-NO (bottom).

we are highly motivated to see if the α-ketoamide structure can be employed to develop novel H_2O_2-inducible NO donors. This will not only expand the family of diazeniumdiolates, but also further testify the universality of α-ketoamide as a prodrug handle.

The general structure of α-ketoamide based NO donors (KA-NO) is shown in Scheme 2. The α-ketoamide moiety is liable to the attack of H_2O_2 and the intermediate resulting from nucleophilic addition quickly rearranges to an anhydride *via* the Baeyer–Villiger reaction.[22] Further hydrolysis generates an aniline, which undergoes a 1,6-elimination[23] to liberate the diazeniumdiolate anion, accompanied by 4-nitrobenzoic acid (NBA) and 4-aminophenyl methanol (APM). Finally, diazeniumdiolate anions decompose quickly, although with various half-lives (Table 1), to yield NO.

The synthetic route to KA-NO is quite straightforward (Scheme 2). 4-Nitroacetophenone was oxidized with SeO_2 in pyridine to give 4-nitrophenylglyoxalic acid **1**. (4-Aminophenyl)methanol was coupled with acid **1** in EDCI and DIEA to afford α-ketoamide intermediates **2**. After bromination of the hydroxyl group using CBr_4 and PPh_3, the active benzyl bromide derivative **3** underwent a SN2 nucleophilic attack by sodium diazeniumdiolates at low temperature to give the final products. Detailed synthetic procedures are shown in the ESI.†

Considering the high amount of H_2O_2 specifically present in cancer cells, the cytotoxicity of KA-NO prodrugs was evaluated by measuring their IC_{50} values in four H_2O_2-overproducing cancer cells lines (A549, HL-60, HCT-116, and B16)[17,18,24] plus one normal cell line (CCD 841 CoN). JS-K, a well-known O^2-2,4-dinitrophenyl diazeniumdiolate with a broad-spectrum antitumor activity was selected as the positive control.[25] The four KA-NO prodrugs exhibited potent cytotoxicity comparable or superior to that of JS-K against cancer cell lines (Table 1). Meanwhile, compromised inhibitory effects were observed for all the tested compounds in the normal cell line. What particularly impressed us is the submicromolar IC_{50} value (0.88 μM) of KA-NO-4 in lung cancer A549 cells, representing a 40-fold selectivity when compared with its IC_{50} in normal cells (35.31 μM). Three additional lung cancer cell lines were chosen to further test the anticancer activity of KA-NO-4. Micromolar IC_{50} values were observed in NCI-H446 and NCI-H209 cells (5.76 and 8.95 μM, respectively). A decent activity (14.47 μM) was measured in cisplatin-resistant lung cancer cell line A549/DDP. It is noteworthy that compound **3**, NBA and APM showed negligible cytotoxicity in A549 cells at a concentration up to 50 μM, strongly indicating that NO dominantly contributed to the anticancer activity of KA-NO-4. This was further supported by the fact that the NO scavenger, C-PTIO diminished the cytotoxicity of KA-NO-4 in A549 cells (Fig. S1, ESI†).

To confirm the H_2O_2-induced activation behaviour, KA-NO-4 was treated with excess amount of H_2O_2 and the reaction mixture was monitored by NMR and HPLC. KA-NO-4 decomposed quickly upon H_2O_2 addition in an equivalent dependent manner (Fig. S2, ESI†). In accordance with the proposed mechanism in Scheme 2, the by-products NBA and APM appeared on NMR (Fig. 1A), HPLC (Fig. S3, ESI†) and mass spectra (Fig. S4, ESI†) after H_2O_2 treatment. In sharp contrast, KA-NO-4 was very stable in PBS buffer (pH = 7.4) and bovine plasma in the absence of H_2O_2. More than 90% KA-NO-4 survived even after 48 h (Fig. S5, ESI†). The nitro group within the α-ketoamide fragment is requisite for H_2O_2-initiated nucleophilic attack.[22] Therefore, KA-NO-5 was synthesized (ESI†) as a non-nitro counterpart of KA-NO-4 for the following studies. MTT assays revealed that KA-NO-5 inhibited A549 cells to a much less extent than KA-NO-4 at the same concentration (Fig. 1C). Importantly, pre-incubation of A549 cells with the H_2O_2 scavenger, *N*-acetyl-cysteine (NAC) remarkably diminished the cytotoxicity of KA-NO-4, but not KA-NO-5. As expected, KA-NO-5 was totally resistant to 10 eq. of H_2O_2 (Fig. 1D). Taken together, these results strongly supported that H_2O_2-triggered activation was vital for the anticancer activity of KA-NO. Moreover, a group of reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulfur species (RSS) were employed to examine the H_2O_2-specific response of KA-NO-4 (Fig. 1E). It was found that only $HO^•$, $ONOO^-$ and GSH slightly induced the

Table 1 IC_{50} values of KA-NO prodrugs on cancer and normal cells[a]

Compounds	$t_{1/2}$[b] (s)	IC_{50} (μM) A549	HL-60	HCT-116	B16	CCD 841 CoN
KA-NO-1	<10	6.24 ± 0.33	4.87 ± 0.23	16.19 ± 1.06	7.52 ± 0.41	63.99 ± 4.19
KA-NO-2	132 ± 7	19.60 ± 1.57	9.88 ± 0.68	7.58 ± 0.67	4.27 ± 0.23	238.10 ± 16.41
KA-NO-3	304 ± 9	3.21 ± 0.18	6.50 ± 0.32	11.55 ± 0.73	10.23 ± 0.67	56.94 ± 3.97
KA-NO-4	169 ± 7	0.88 ± 0.05	23.08 ± 1.57	8.34 ± 0.44	17.89 ± 1.27	35.31 ± 1.69
JS-K	304 ± 9	5.45 ± 0.32	17.66 ± 1.09	25.26 ± 1.67	12.58 ± 0.96	101.4 ± 8.32

[a] Cells were incubated with the indicated compounds at different concentrations for 72 h, and IC_{50} values were measured by MTT and CCK-8 assays. Mean ± SD, n = 3. [b] The half-lives of the corresponding sodium diazeniumdiolates to decompose at pH 7.4 and 25 °C to release NO were assessed by UV spectra. Mean ± SD, n = 3.

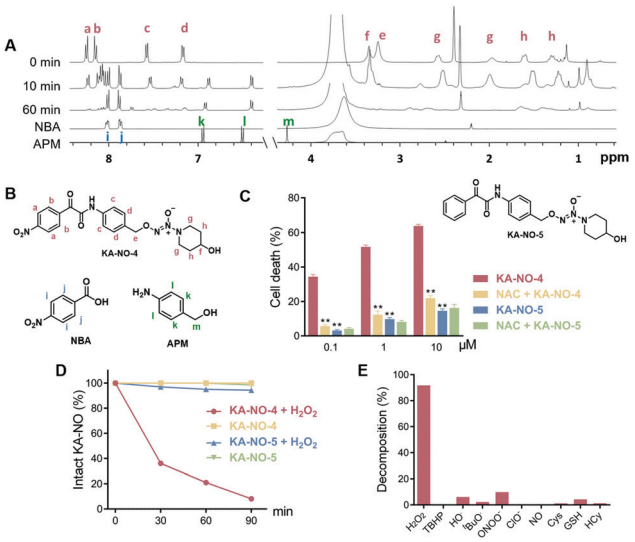


Fig. 1 H_2O_2-induced activation of KA-NO-4. (A) ^{1}H NMR analysis of KA-NO-4 (40 mM) degradation in a mixture of d_6-DMSO (550 μL) and D_2O (50 μL) in the presence of H_2O_2 (10 eq.) at 37 °C. (B) Proton assignments of KA-NO-4, NBA and APM. (C) The effects of NAC on the cytotoxicity of KA-NO-4 and KA-NO-5 in A549 cells. Mean ± SD, n = 3, $^{**}p < 0.01$ *vs.* KA-NO-4. (D) Decomposition of KA-NO-4 and KA-NO-5 with or without H_2O_2 (10 eq.) as measured by HPLC (254 nm). (E) Selective response of KA-NO-4 toward H_2O_2.

decomposition of KA-NO-4. All the other species had no effects on the activation of KA-NO-4.

An extracellular Griess assay was performed to evaluate the amount of NO generation. Comparable nitrite levels were detected from the four H_2O_2-responsive prodrugs, but not KA-NO-5, after treatment with H_2O_2 for 8 h (Fig. 2A). Interestingly, the intracellular Griess assay indicated that the highest nitrite level in A549 cells was generated by KA-NO-4 treatment, followed by KA-NO-1 and KA-NO-3, and KA-NO-2 yielded the least nitrite amount (Fig. 2B). This correlates well to the IC_{50} values in Table 1. NAC pretreatment diminished the intracellular NO production of KA-NO-4. Collectively, we assume that the four prodrugs own similar NO release behaviour extracellularly. However, their intracellular NO donating ability might vary possibly due to different cell membrane permeability, leading to unequal cytotoxicity. The ability of KA-NO prodrugs to release NO in A549 cells was also investigated using a fluorophore probe DAF-FM DA.[26] KA-NO prodrugs generated modest to high fluorescence in A549 cells compared with the blank control. Moreover, KA-NO-4 induced fluorescence in a dose-dependent manner. Either NAC or C-PTIO pretreatment attenuated the fluorescence from KA-NO-4 (Fig. S6, ESI†).

Once released, NO is known to react with the superoxide anion ($O_2^{\cdot-}$) to afford peroxynitrite ($ONOO^-$), which is capable of nitrating many proteins including cytochrome *C* (Cyt-*c*). The nitration occurs at tyrosine residues to form 3-nitrotyrosine (3-NT). Therefore, the induced expression of 3-NT protein can be regarded as a unique indicator of NO generation and function.[27] The ability of KA-NO-4 to nitrate Cyt-*c* in A549 cells was investigated by Western blot (Fig. 2C). Clearly, KA-NO-4 increased the level of 3-NT Cyt-*c* in a dose- and time-dependent

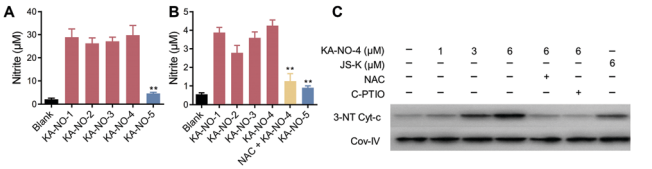


Fig. 2 NO release and function from KA-NO prodrugs. (A) KA-NO prodrugs (50 μM) in PBS (pH 7.4, 10 mM)/MeCN (v/v, 80/20) solution were treated with 10 eq. of H_2O_2 at 37 °C for 8 h. The nitrite level in each group was detected by Griess assay. Mean ± SD, n = 3, $^{**}p < 0.01$ *vs.* KA-NO-4. (B) A549 cells were treated with 6 μM of the indicated compounds for 8 h, or pretreated with NAC (20 mM) for 1 h, followed by treatment with KA-NO-4 for 8 h. Intracellular nitrite levels were detected by Griess assay. Mean ± SD, n = 3, $^{**}p < 0.01$ *vs.* KA-NO-4. (C) A549 cells were treated with the indicated concentration of KA-NO-4 for 24 h, or pretreated with NAC (20 mM) or C-PTIO (50 μM) for 1 h, followed by treatment with KA-NO-4 for 24 h. The levels of 3-NT Cyt-*c* were detected by Western blot.

way. NAC or C-PTIO preincubation hampered intracellular NO generation indirectly or directly, and therefore reversed the effect of KA-NO-4 on 3-NT Cyt-*c* formation.

The nitration of mitochondrial proteins is supposed to lead to alteration of protein function and cell apoptosis. The apoptotic effects of KA-NO-4 were thus studied in A549 cells. KA-NO-4 dose-dependently induced apoptosis of A549 cells (Fig. 3A and Fig. S7, ESI†). Pretreatment of NAC or C-PTIO led to resistance of cancer cells to KA-NO-4 induced apoptosis. Compound **3**, NBA and APM had no apoptosis-inducing activity (Fig. S8, ESI†). Moreover, KA-NO-4 arrested the cell cycle of A549 cells at G2/M phase (Fig. 3B and Fig. S9, ESI†), decreased the membrane potential of mitochondria ($\Delta\Psi_m$) (Fig. 3C and Fig. S10, ESI†), induced the cleavage of Caspase 3 and Caspase 9, increased the expression of Bax, and lowered the level of Bcl-2 (Fig. 3D and Fig. S11, ESI†). All these results indicated that KA-NO-4 inhibited the growth of A549 cells *via* a mitochondrial-dependent apoptosis-inducing pathway, which was blocked by NAC or C-PTIO due to H_2O_2 or NO scavenge.

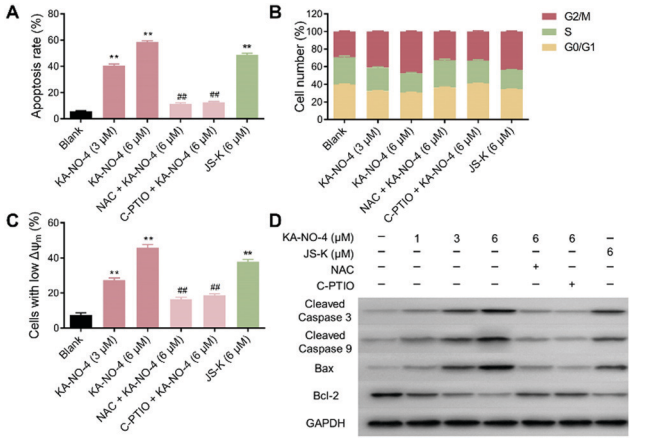


Fig. 3 Apoptotic effects of KA-NO-4. A549 cells were treated with compounds at indicated concentrations for 24 h, or pre-incubated with NAC (20 mM) or C-PTIO (50 μM) for 1 h, followed by treatment with 6 μM KA-NO-4 for 24 h. Apoptosis rates (A), cell cycle phase distributions (B) and $\Delta\Psi_m$ (C) were measured by flow cytometry. Mean ± SD, n = 3, $^{**}p < 0.01$ *vs.* blank, $^{\#\#}p < 0.01$ *vs.* KA-NO-4 (6 μM). (D) The expressions of apoptosis-related proteins were visualized using Western blot.

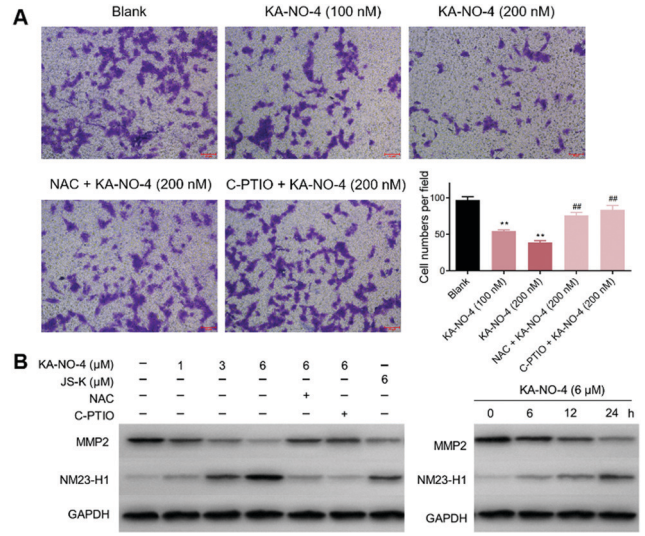


Fig. 4 Effects of KA-NO-4 on A549 cell invasion. (A) Transwell assay. A549 cells seeded on Matrigel-coated chambers were treated with 100 or 200 nM KA-NO-4 for 48 h, or pretreated with NAC (20 mM) or C-PTIO (50 μM) for 1 h, followed by treatment with KA-NO-4 for 48 h. Cells that migrated through the Matrigel-coated chambers were stained with crystal violet. Mean ± SD, $n = 3$, $^{**}p < 0.01$ *vs.* blank, $^{\#\#}p < 0.01$ *vs.* KA-NO-4 (200 nM). (B) A549 cells were treated with the indicated concentration of KA-NO-4 for 24 h, or pretreated with NAC (20 mM) or C-PTIO (50 μM) for 1 h, followed by treatment with KA-NO-4 for 24 h. The levels of MMP-2 and NM23-H1 were detected by Western blot.

Metastasis is the main cause of death for lung cancer patients. NO has been reported to be able to prevent cancer metastasis by its direct vasodilation and inhibition of cell adhesion molecules (CAMs).[28] Transwell assays found that KA-NO-4 dose-dependently decreased the cell invasiveness (Fig. 4A) as reflected from the number of A549 cells that migrated through the Matrigel-coated chamber. H_2O_2 and NO are requisite for the invasion inhibition as demonstrated from the NAC and C-PTIO pretreatment groups. Matrix metalloproteinase (MMP)-2 is a notable factor in the development of tumor metastasis, and NME/NM23 nucleoside diphosphate kinase 1 (NM23-H1) is a tumor metastasis suppressor, which reduces MMP-2 expression.[29] Western blot assays disclosed that KA-NO-4 decreased the level of MMP-2 while it increased the level of NM23-H1 in A549 cells (Fig. 4B).

In summary, a series of O^2-masked diazeniumdiolates based on a H_2O_2-responsive α-ketoamide structure (KA-NO) was unprecedentedly reported. KA-NO-4, which decomposed quickly and selectively in the presence of H_2O_2 to release NO, showed submicromolar inhibitory activity in A549 cells. The intracellularly released NO from KA-NO-4 nitrated tyrosine residues of Cyt-*c*, led to mitochondrial-dependent cell apoptosis, and inhibited the invasiveness of lung cancer cells. Previously, the overwhelming majority of H_2O_2-acitivated prodrugs were based on the arylboronic acid/ester moiety. As an extension of our recent discovery,[21] this work developed a novel type of H_2O_2-inducible NO donor with demonstrated anticancer activity. Considering the multiple roles of NO and H_2O_2 in pathological conditions, other bioactivities of KA-NO are also expected and are currently under investigation.

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Conflicts of interest

There are no conflicts to declare.

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