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ORIGINAL ARTICLE

Ebselen is a potent non-competitive inhibitor of extracellular nucleoside diphosphokinase

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Abstract Nucleoside di- and triphosphates and adenosine regulate several components of the mucocilairy clearance process (MCC) that protects the lung against infections, via activation of epithelial purinergic receptors. However, assessing the contribution of individual nucleotides to MCC functions remains difficult due to the complexity of the mechanisms of nucleotide release and metabolism. Enzymatic activities involved in the metabolism of extracellular nucleotides include ecto-ATPases and secreted nucleoside diphosphokinase (NDPK) and adenyl kinase, but potent and selective inhibitors of these activities are sparse. In the present study, we discovered that ebselen markedly reduced NDPK activity while having negligible effect on ecto-ATPase and adenyl kinase activities. Addition of radiotracer [γ^{32}P]ATP to human bronchial epithelial (HBE) cells resulted in rapid and robust accumulation of [^{32}P]-inorganic phosphate (^{32}Pi). Inclusion of UDP in the incubation medium resulted in conversion of [γ^{32}P]ATP to [^{32}P]UTP, while inclusion of AMP resulted in conversion of [γ^{32}P]ATP to [^{32}P]ADP. Ebselen markedly reduced [^{32}P]UTP formation but displayed negligible effect on ^{32}Pi or [^{32}P]ADP accumulations. Incubation of HBE cells with unlabeled UTP and ADP resulted in robust ebselen-sensitive formation of ATP (IC_{50}=6.9±2 μM). This NDPK activity was largely recovered in HBE cell secretions and supernatants from lung epithelial A549 cells. Kinetic analysis of NDPK activity indicated that ebselen reduced the V_{max} of the reaction (K_i=7.6±3 μM), having negligible effect on K_M values. Our study demonstrates that ebselen is a potent non-competitive inhibitor of extracellular NDPK.

Keywords Nucleoside diphosphokinase · Ebselen · Extracellular nucleotides · Nucleotide release · Lung epithelial cells

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Introduction

Nucleotides and nucleosides within the airway surface liquid (ASL) regulate key components of the mucociliary clearance (MCC) process that removes foreign particles and pathogens from the lung [1–3]. Adenosine-5′-triphosphate (ATP) and uridine-5′-triphosphate (UTP) activate the G_q-coupled $P2Y_2$ receptor ($P2Y_2$-R) that promotes mucin secretion from goblet cells [2]. The $P2Y_2$-R expressed on ciliated cells, promotes ciliary beat frequency, protein kinase C-enhanced cystic fibrosis transmembrane conductance regulator (CFTR)-mediated Cl^- secretion, and Ca^{2+}-activated Cl^- channel (CaCC) activity [3]. In addition, $P2Y_2$-R activation results in inhibition of the epithelial sodium channel ENaC [3]. UDP promotes CaCC activity via activation of the $P2Y_6$-R expressed on ciliated cells [3]. Adenosine generated from the hydrolysis of ATP in ASL activates the G_s-coupled A_{2B} receptor (A_{2B}-R) that promotes cyclic AMP-regulated CFTR activity and increases cilia beat frequency [3–5]. In the distal lung, ATP/UTP and/or adenosine (mainly via $P2Y_2$-R and A_{2B}-R, respectively) stimulate type II cell surfactant secretion [6], regulate alveolar ion transport and fluid clearance [7, 8], and contribute to alveolar remodeling and inflammation [9, 10].

Nucleotide/nucleoside levels in ASL and lung secretions reflect a balance between cellular nucleotide release and extracellular metabolism [11]. Early studies suggested that ATP, and to a lesser extent UTP and other nucleoside

triphosphates (NTPs), are released fromairway epithelial cells [11–13] and subsequently hydrolyzed to nucleoside di- and monophosphate (NDP and NMP, respectively) and nucleosides (e.g., adenosine) by action of cell surface NTPDases, NTP pyrophosphatase, alkaline phosphatase, and 5′-nucleotidase [11, 14, 15]. In addition, NDP kinase (NDPK) and adenyl kinase provide nucleotide interconversion activities in ASL [11, 16, 17].

Recently, a mathematical model based on the rates of ATP release and metabolism and adenosine accumulation rates in ASL predicted that adenosine-5′-diphosphate (ADP) and/or monophosphate (AMP) are released from airway epithelial cells, in addition to ATP [18]. Consistent with this prediction, we have recently demonstrated the presence of a nucleotide pool within secretory granules isolated from goblet cells, which was represented (in abundance order) by ADP>AMP >>ATP [19]. Direct release of ADP/AMP to ASL would selectively promote liquid secretion from ciliated cells, via adenosine formation and A_{2B}-R activation. In addition, our recent demonstration that airway epithelial and other cells release UDP-sugars from Golgi-derived vesicles [20] implies that UDP, which accumulates in the Golgi as product of UDP-sugar-based glycosylation reactions, is released from cells via the secretory pathway [21, 22]. Since the $P2Y_6$-R is expressed on ciliated cells (but not on goblet cells) [2], UDP release would selectively promote CaCC-mediated electrolyte transport and ciliary beat frequency without enhancing mucin secretion.

While ATP, UTP, ADP, UDP, AMP, and adenosine are naturally occurring molecules in ASL [13, 23–26], assessing the exact contribution of individual nucleotides to MCC functions remains difficult, due in part to the complexity of the mechanisms that control nucleotide release and concentrations in ASL. In particular, strong NDPK activity has been reported in conditioned medium from airway epithelial cells [11, 27]. The activity of extracellular NDPK, which catalyzes transphosphorylation reactions such as the reversible phosphorylation of UDP by ATP (ATP+UDP= ADP+UTP), surmounts that of nucleotidases under a number of conditions. NDPK-catalyzed transphosphorylating reactions provide a confusing factor in the assessment of the primary source of ASL nucleotides. Measuring the rates of release of NTPs, NDPs, and NMPs has been dampened by the lack of non-nucleotide molecules to inhibit nucleotide interconversion.

The organoselenium compound ebselen [1, 2-phenyl-1, 2-benzisoselenazol-3(2H)-1] has been reported to inhibit, albeit partially (~60% inhibition, <100 μM ebselen), the hydrolysis of extracellular ATP in rat platelets [28]. More recently, we have illustrated that ebselen slightly delayed ATP metabolism on primary cultures of human airway epithelial cells [25], but the identity of the ATP metabolizing ecto-activity present in airway epithelia targeted by ebselen is not known. In the present study, by examining the effect of ebselen on the metabolism of extracellular ATP on airway epithelial cells, we discovered that ebselen is a potent, non-competitive full inhibitor of NDPK.

Materials and methods

Reagents ADP, UDP, 2-phenyl-1, 2-benzisoselenazol-3(2H)-1 (ebselen), and luciferase from *Photinus pyralis* were obtained from Sigma (St. Louis, MO). For experiments using ebselen derivatives, ebselen and its analogues 2, 2′-diseleno benzanilide ($EbSe_2$), 2, 2′-ditelluro benzanilide ($EbTe_2$), 2-*methyl* seleno benzanilide (EbMe), and 2-benzyl seleno benzanilide (EbBz) were obtained as previously described [29]. Luciferin was obtained from BD PharMingen (Franklin Lakes, NJ). ATP and UTP were purchased from GE Healthcare (Hillsborough, NC). [γ^{32}P]ATP was obtained from Amersham Biosciences (Piscataway, NJ). All other reagents were from sources previously reported [11, 13, 20].

Cell culture and incubations Polarized cultures of well-differentiated primary HBE cells (provided by the UNC Cystic Fibrosis Center Center Tissue Culture Core Lab) and A549 lung epithelial cells were grown on 12-mm Transwell supports (Costar) and on 24-well plastic plates, respectively, as previously described [13, 25, 30]. The cells were rinsed and 300 μl serum-free Dulbecco's modified Eagle's medium were added to the mucosal compartment of HBE cells or to A549 cell culture wells. After a 1 h pre-incubation period, cell cultures where incubated with drugs, as indicated below. Alternatively, pre-incubation medium was collected, centrifuged, and the supernatant used within 2 h to assess nucleotide metabolism activities in conditioned medium. Incubations were initiated by the addition of the indicated reagent to cultures or to 100-μl conditioned medium. After the desired incubation time at 37°C, samples from cultures and conditioned medium were heated for 2 min at 95°C to inactivate enzyme activities.

NDPK and adenyl kinase activities NDPK catalyzes the reversible phosphorylation of NDPs by NTPs, while adenyl kinase catalyzes the reversible phosphorylation of AMP by ATP. NDPK activity was assessed via two alternative protocols, (reaction 1) as a function of UDP-dependent conversion of [γ^{32}P]ATP to [^{32}P]UTP, or (reaction 2) as a function of UTP- and ADP-dependent formation of ATP:

$$\text{Reaction 1}: \left[\gamma^{32}\text{P}\right]\text{ATP} + \text{UDP} \rightleftharpoons \text{ADP} + \left[^{32}\text{P}\right]\text{UTP}$$

$$\text{Reaction 2}: \text{UTP} + \text{ADP} \rightleftharpoons \text{UDP} + \text{ATP}$$

Adenyl kinase activity was assessed either as a function of AMP-dependent conversion of [γ^{32}P]ATP to [^{32}P]ADP (reaction 3) or as a function of ADP conversion to ATP (reaction 4):

$$\text{Reaction 3}: \left[\gamma^{32}\text{P}\right]\text{ATP} + \text{AMP} \rightleftharpoons 2\text{ADP} + \left[^{32}\text{P}\right]\text{ADP}$$

$$\text{Reaction 4}: 2\text{ADP} \rightleftharpoons \text{AMP} + \text{ATP}$$

The resulting ^{32}P-labeled species were quantified by high-performance liquid chromatography (HPLC). ATP mass formation was assessed by the luciferin/luciferase assay. In experiments where NDPK activity was assessed as a function of UTP-dependent phosphorylation of ADP (reaction 2), ATP formation values obtained in the absence of UTP (as in reaction 4) were subtracted from those obtained in the presence of UTP.

HPLC analysis [^{32}P]ATP, [^{32}P]UTP, [^{32}P]ADP, and ^{32}P-inorganic phosphate (^{32}Pi) were separated by HPLC (Shimatzu) using 10-μm Hamilton PRP-X100 anion exchange column. The mobile phase (1 ml/min; 30% methanol, solvent A; 0.5 M NH_4HCO_3 (pH 8.5) in 30% methanol, solvent B) developed as follows: 75% A and 25% B from 0 to 5 min, 33% A and 67% B from 5 to 15 min, and the column was re-equilibrated to the initial conditions for additional 10 min. ^{32}P-species were quantified on-line with a FLO-ONE 500TR Radiomatic analyzer (Packard), as described previously [11].

Measurement of ATP mass ATP measurements were performed via a LB953 AutoLumat luminometer (Berthold), as previously described [11]. Calibration curves were generated at the end of each experiment, using known concentrations of ATP. None of the reagents used during incubations interfered with the luciferase reaction.

Data analysis Kinetic parameters from substrate concentration–response relationships and inhibition constants were calculated using Sigma Plot v.10 data fitting analysis.

Results

Ebselen inhibits the UDP-dependent conversion of [γ^{32}P]ATP to [^{32}P]UTP on WD-HBE cells The fate of trace amounts of [γ^{32}P]ATP added to the mucosal medium bathing WD-HBE cell cultures was assessed by HPLC. Addition of [γ^{32}P]ATP to cells resulted in rapid and robust release (~50% after 3 min) of the radiolabel as ^{32}Pi (Fig. 1a (i and ii)). ATP hydrolysis was not affected by the inclusion of 30 μM ebselen in the incubation mix (Fig. 1a (iii)). Previously, we reported the presence of ecto-NDPK activity on various cell types, including airway epithelial cells [11, 27, 31]. Consistent with these reports, addition of radiotracer [γ^{32}P]ATP and excess mass of UDP (10 μM UDP) to WD-HBE cells resulted in the formation of [^{32}P]UTP (Fig. 1a (iv)). The conversion of [γ^{32}P]ATP to [^{32}P]UTP was markedly reduced by the presence of 30 μM ebselen (Fig. 1a (v)).

Ebselen-sensitive NDPK activity is recovered in the conditioned medium ATP hydrolysis on airway epithelial cells reflected the action of cell surface ecto-ATPases [11]. Accordingly, release of ^{32}Pi from [γ^{32}P]ATP was rapid and robust when the radiotracer was added to cells, but was negligible in the WD-HBE cell-conditioned medium (Fig. 1b (i)). Consistent with the data in Fig. 1a, ebselen had no effect on [γ^{32}P]ATP hydrolysis (Fig. 1b (i)). In contrast to ATPase activity, NDPK activity could be substantially recovered as a soluble enzyme, i.e., in the cell conditioned medium (Fig. 1b (ii), and [11]). Ebselen (30 μM) markedly reduced the conversion of [γ^{32}P]ATP to [^{32}P]UTP (Fig. 1b (ii)).

In addition to NTPDase and NDPK activities, airway epithelia express ecto-adenyl kinase activity [16, 17], which reversibly phosphorylates AMP (as described above in reaction 3). Addition of [γ^{32}P]ATP together with 10 μM AMP resulted in rapid formation of [^{32}P]ADP on both WD-HBE cell cultures and cell-conditioned medium (Fig. 1b (iii)). The adenyl kinase activity was markedly less robust than the NDPK activity and was not affected by ebselen (Fig. 1b (iii)).

Altogether, the data indicate that NDPK activity (1) is markedly inhibited by ebselen and (2) can be assessed in cell-free-conditioned medium.

Ebselen inhibits the UTP-dependent conversion of ADP to ATP To further investigate the action of ebselen on NDPK activity, we adopted a non-radioactive protocol that quantifies the formation of ATP as a function of UTP and ADP in conditioned medium (see reaction 2 in "Materials and methods"). Conditioned medium from WD-HBE cell cultures contains low nanomolar concentrations of ATP (~6 nM, Fig. 2a), which reflects the presence of endogenous ATP released from cells [13]. Addition of 10 μM ADP in the absence of UTP resulted in formation of ATP (~50 nM ATP, Fig. 2a), consistent with an adenyl kinase-catalyzed reaction (see reaction 4 in "Materials and methods"). The ADP-dependent ATP formation (observed in the absence of exogenous UTP) was not affected by ebselen (Fig. 2a). Addition of 10 μM UTP to the medium in the absence of exogenous ADP resulted in minor ebselen-sensitive formation of ATP (~20 nM ATP, Fig. 2a), likely

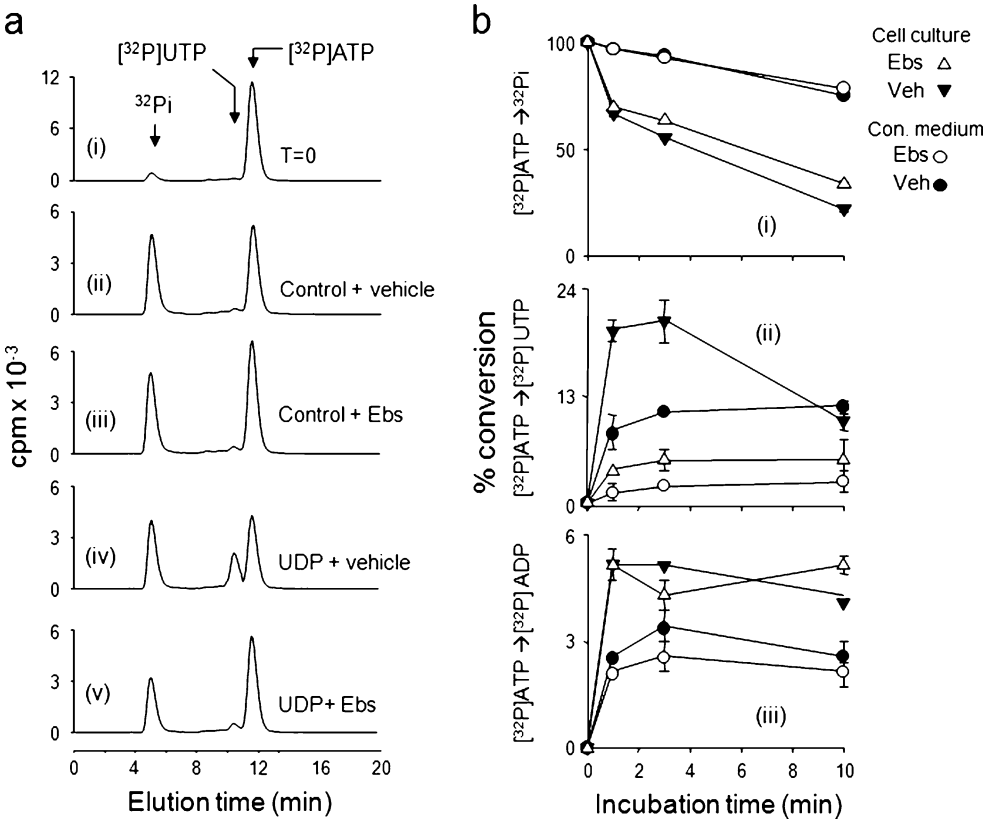

Fig. 1 Ebselen inhibits the UDP-dependent conversion of [γ^{32}P]ATP to [^{32}P]UTP on WD-HBE cells. HPLC analysis of [γ^{32}P]ATP metabolism on WD-HBE cells. Radiotracer [γ^{32}P]ATP (~0.1 μCi) was added to cultures (mucosal addition) and the resulting [^{32}P]-labeled species separated and analyzed by HPLC, as indicated in "Materials and methods". **a** Representative HPLC tracings (n=4) corresponding to samples incubated for T=0 min (*i*), or 3 min in the presence of vehicle (*ii*), 30 μM ebselen (*Ebs*) (*iii*), 10 μM UDP (*iv*), or 30 μM ebselen and 10 μM UDP (*v*). The tracings represent the radioactivity (*cpm*) eluting from the HPLC column, recorded at 10-s intervals. **b** [γ^{32}P]ATP alone (*i*), [γ^{32}P]ATP and 10 μM UDP (*ii*), or [γ^{32}P]ATP and 10 μM AMP (*iii*) was added to cell cultures or to conditioned medium (Con. medium) for the indicated times, and the formation of ^{32}Pi (i), [^{32}P]UTP (*ii*), and [^{32}P]ADP (*iii*) were quantified by HPLC (mean ± SD from two experiments performed in triplicates). The results are expressed as percent conversion of [^{32}P]ATP to [^{32}P]-product, relative to T=0". The area of each radioactive peak was calculated using the HPLC analysis software provided by the manufacturer

reflecting phosphorylation of endogenous ADP by NDPK. Furthermore, a marked formation of ATP (~200 nM) was observed when UTP and ADP were added together (Fig. 2a). Formation of ATP in the presence of both UTP and ADP was nearly abolished by ebselen (Fig. 2a). Figure 2b indicates that ebselen dose-dependently inhibited NDPK activity in WD-HBE cell secretions, displaying an IC_{50} value of 6.9±2 μM.

Ebselen is a potent, non-competitive inhibitor of NDPK To characterize in greater detail the nature of ebselen inhibition on NDPK, conditioned medium from lung epithelial A549 cells was obtained. We chose using this fast-growing cell line as a source of secreted NDPK to bypass the limitations inherent to the low availability of primary cultures of HBE cells. Thus, protocols described above were adapted to non-polarized cultures of lung epithelial A549 cells. An initial assessment indicated that, like primary HBE cells, the conditioned medium of A549 cells displays robust NDPK activity that catalyzes the phosphorylation of UDP (10 μM) by [γ^{32}P]ATP in an ebselen-sensitive manner (Fig. 3). Also like in HBE cells, [γ^{32}P]ATP hydrolysis by A549 cell-conditioned medium was minor (<20% ^{32}Pi accumulation) and only slightly affected by ebselen (Fig. 3).

Next, the effect of ebselen on the substrate concentration dependence for UTP- and ADP-promoted formation of ATP was assessed. Figure 4a illustrates the effect of UTP concentration on ATP formation, using 20 μM ADP as acceptor substrate for the NDPK activity present in the A549 cell-conditioned medium. In the absence of ebselen, the reaction displayed an apparent K_M value of 18 μM UTP, and maximal ATP formation (6.8 μM/10 min) occurred with an apparent V_{max}=68±6 pmoles/min. Ebselen dose-dependently decreased the V_{max} of the reaction (Fig. 4a) while displaying a minor effect on the

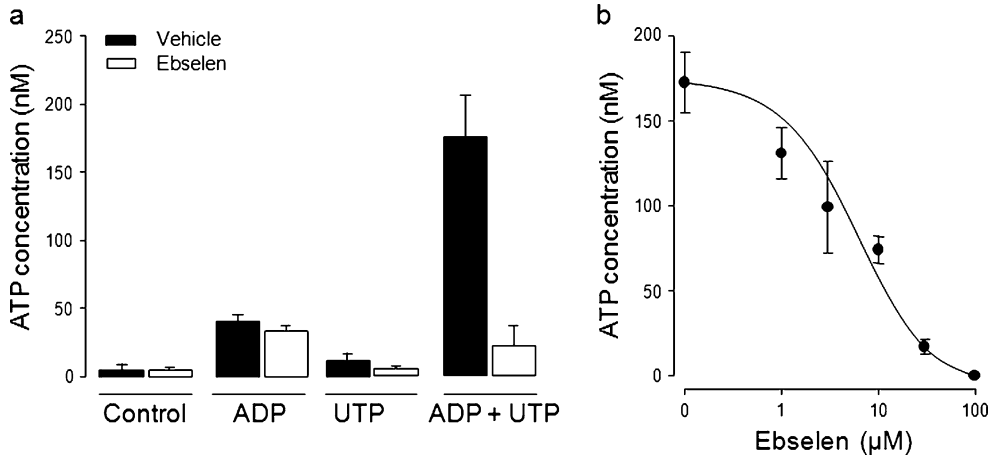

Fig. 2 Ebselen inhibits the UTP-dependent phosphorylation of ADP in conditioned medium from WD-HBE cells. **a** Conditioned medium from WD-HBE cells was incubated for 5 min with either vehicle or 30 μM ebselen in the absence (*control*) or presence of 10 μM ADP and/or 10 μM UTP. The formation of ATP was assessed by the luciferin–luciferase assay, as described in Methods. **b** Concentration–effect relationship for ebselen-inhibited UTP-dependent ATP formation (mean ± SD, n=4); ATP values obtained in the absence of UTP were subtracted from the corresponding ADP/UTP data point

UTP K_M value (Table 1). ADP concentration–effect relationships generated in the presence of 100 μM UTP indicated K_M and V_{max} values of 1.3 μM and 47+ 4 pmoles/min, respectively (Fig. 4b and Table 1). Ebselen reduced the V_{max} (Fig. 4b) but had no effect on the ADP K_M value (Table 1). The data suggest that ebselen behaves as a non-competitive inhibitor. Dixon Plot analysis [32] of the data indicated a K_i value for ebselen-inhibited NDPK activity of 7.6±3 μM. In the absence of exogenous UTP, some ATP formation was observed, which was likely mediated by adenyl kinase and represented approximately 5% of that attained in the presence of 100 μM UTP (compare Figs. 4c vs. 4b). In contrast to the robust inhibition of NDPK activity, ebselen (30 μM) had negligible effect on the adenyl kinase-mediated formation of ATP (Fig. 4c).

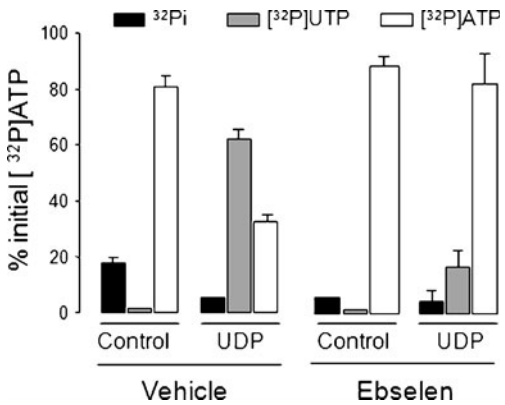

Fig. 3 Ebselen inhibits the phosphorylation of UDP in conditioned medium from A549 cells. A549 cell-conditioned medium was incubated with [γ^{32}P]ATP in the absence (*control*) or presence of 10 μM UDP. The percent conversion of [γ^{32}P]ATP to ^{32}Pi or [^{32}P] UTP was assessed as in Fig. 1. Ebselen (30 μM) or vehicle was added to samples 1 min prior incubations, as indicated. The data represent the mean (±SD) from two experiments performed in triplicate

Several ebselen derivatives have been recently used to assess the redox properties of selenium compounds [29]. Concentration–effect curves generated for a series of ebselen analogues indicated that the organotellurium $EbTe_2$ reduced UTP-dependent formation of ATP in conditioned medium, but the inhibitory effect of $EbTe_2$ on ATP formation was markedly weaker than that of ebselen (Fig. 5). Se-methylated (EbMe) and Se-benzylated (EbBz) derivatives of ebselen and the diselenide $EbSe_2$ had negligible effect on ATP formation (Fig. 5). These results were consistent with the notion that structural modification in organoselenium compounds alter their redox activities [29].

Discussion

Our present study demonstrates that ebselen is a potent non-competitive inhibitor of NDPK. NDPK (also known as Nm23) is a housekeeping enzyme catalyzing the phosphorylation of NDPs, utilizing NTPs as terminal phosphate donor. Intracellular NDPK fulfills a crucial role in maintaining the high energy phosphate bond in ATP as part of the citric acid chain. NDPK also has been proposed to play a major role in maintaining a relative balance in the concentrations of cellular NTPs. In addition to its central role in cellular metabolism, NDPK is released from cells to catalyzed transphosphorylation reactions between extracellular nucleotides. Robust NDPK activity has been reported in conditioned medium from astrocytes, glioma, and airway

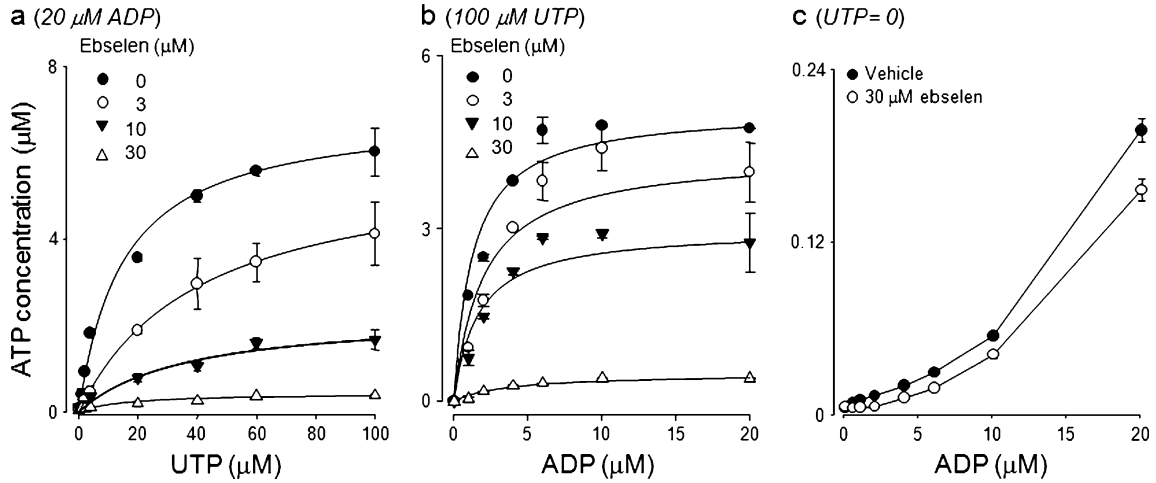

Fig. 4 ATP formation as a function of substrate concentration. Effect of ebselen. Concentration–effect relationships for UTP- and ADP-dependent formation of ATP were established in A549 cell-conditioned medium. **a** UTP concentration vs. ATP formation was assessed in the presence of 20 μM ADP and the indicated concentration of ebselen. **b** ADP concentration vs. ATP formation in the presence of 100 μM UTP and the indicated concentration of ebselen. **c** Eebselen (30 μM) exhibited negligible effect on the ADP-dependent ATP formation in the absence of exogenous UTP. The data represent the mean (±SD) from one experiment performed in triplicate; similar results were obtained with three independent preparations of A549 cells. ATP values observed in the absence of UTP [as shown in (**c**)] were subtracted from the corresponding ADP/UTP data point in (**a**) and (**b**). All incubations were for 10 min at 37°C

epithelial cells, endothelial cells, osteoblasts, and keratinocytes [11, 31, 33–35].

Local transphosporylation complicates the task of accurately quantifying nucleotide concentrations at the cell surface upon release. For example, Buxton et al. [33] reported the involvement of bradykinin receptors in the release of ATP in coronary endothelium and also illustrated that although application of ADP resulted in an increase in ATP levels, this likely occurred as a consequence of phosphorylation of ADP by NDPK rather than stimulation of $P2Y_1$ receptors. Bradykinin and phenylephrine promoted ATP release in Madin–Darby canine kidney, simian COS-7, and human embryonic kidney (HEK)-293 cells [36]. UTP also enhanced extracellular ATP accumulation in COS-7 and HEK-293 cells, but the effect of UTP probably reflected competitive inhibition of ATP hydrolysis and phosphorylation of endogenous ADP by NDPK rather than $P2Y_2$ receptor-stimulated ATP release [36]. In a study designed to investigate the potential role of connexin hemichannels in ATP release, Cotrina et al. reported that stimulation of purinergic receptors with 100 μM UTP in HeLa cells, C6 glioma cells, and U373 glioblastoma cells

Table 1 Effect of ebselen on KM values for the UTP-dependent phosphorylation of ADP in A549 cell secretions

Ebselen (μM)	K_M (μM)	
	UTP	ADP
0	18	1.3
1	21	1.7
3	40	1.9
10	35	1.6
30	14	3.6

Concentration–effect relationships for UTP- and ADP-promoted ATP formation were established in A549 cell-conditioned medium incubated for 5 min in the presence of the indicated concentration of ebselen, as described in Fig. 4

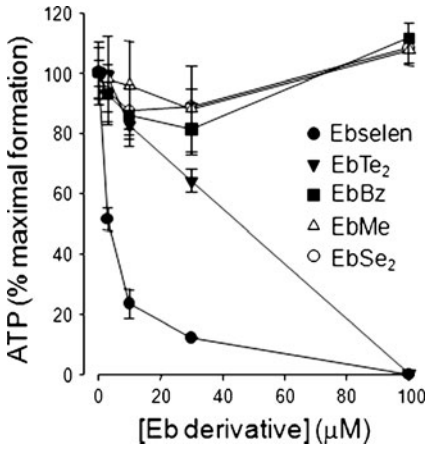

Fig. 5 Effect of ebselen derivatives on the UTP- and ADP-dependent formation of ATP. A549 cell-conditioned medium was incubated for 5 min at 37°C in the presence of 60 μM UTP, 20 μM ADP, and the indicated concentration of ebselen or ebselen derivative. The data represent the percent value (mean ± SD, n=4) relative to the ATP formation observed in the obscene of inhibitor

resulted in a Ca^{2+}-dependent increase of extracellular ATP concentrations. However, other Ca^{2+}-mobilizing agents such as bradykinin, endothelin, and the calcium ionophore A2317 had little or no effect on extracellular ATP levels [37]. Because the likely contribution of NDPK in the phosphorylation of endogenous ADP was not examined, the significance of the ATP measurements in response to exogenous UTP remains unclear. Our present finding that ebselen inhibits NDPK activity provides a tool to assess the potential contribution of NDPK to nucleotide-promoted ATP release.

It has been previously reported that ebselen exhibits both glutathione peroxidase activity and antioxidant activity and that ebselen inhibits several redox-sensitive enzymes such as constitutive endothelial nitric oxide synthase, lipoxygenases, nicotinamide adenine dinucleotide phosphate oxidase, and other activities (Table 2 and [29, 38–43]). Furthermore, organoselenium compounds in the form of seleninic acids can couple with thiols on catalytically important cysteine residues (e.g., of phosphotyrosine phosphatase), resulting in enzyme inhibition [44]. It has been suggested that cysteine residues sensitive to redox conditions are involved in the functional regulation of NDPK [45]. Our data indicating that structural modifications affecting the redox properties of the organoselenium compound resulted in reduced NDPK inhibition (Fig. 5) are consistent with the notion that oxidative modifications regulate NDPK functions.

Two functionally active human NDPK isoenzymes have been characterized: NDPK-A (Nm23-H1) and NDPK-B (Nm23-H2) [46, 47]. In addition, four putative additional NDPK genes have been identified: DR-nm23, nm23-H4, nm23-H5, and nm23-H6, however, the presence of NDP kinase activity in these gene products has not been unambiguously demonstrated [48–51].

Recent studies with guinea pig endothelial cells and human breast carcinoma MDA-MB-435 cells have identified NDPK-A and NDPK-B as the major NDPK enzymes secreted from these cells, respectively [33, 52]. Whether NDPK-A, NDPK-B, or both are released from lung epithelial cells and what is the physiological role of such an activity remains to be elucidated. However, an important role of secreted NDPK may reside in regulating purinergic signaling, by affecting the extracellular concentrations of ATP, ADP, UTP, and UDP [31, 33, 53]. By regulating ATP levels locally, secreted NDPK-B supports angiogenesis, via endothelial cell P2Y receptor activation [52]. The mechanism of NDPK secretion is not understood. Based on the lack of secretion signal sequence of NDPK-A and NDPK-B, it has been proposed that NDPK may be secreted via non-classical export mechanisms similar to those involved in the release of fibroblast growth factors 1 and 2 [52].

Irrespective of the NDPK release mechanism, identification of potent and selective NDPK inhibitors would be relevant to studies of P2Y and P2X receptors and, potentially, may provide useful in elucidating the role of secreted NDPKs in angiogenesis and tumor development. Previously, cyclic AMP analogues were shown to inhibit NDPK activity, with IC_{50} values in the 100–500 μM range [54]. More recently, angiostatin (a proteolytic fragment of plasminogen), polyphenols, and adenosine 3′-phosphate 5′-phosphosulfate were shown to inhibit secreted NDPK-B, but only one of these compounds, ellagic acid, displayed inhibitory effect in the low micromolar concentration range (IC_{50} ~ 10 μM [55]).

By illustrating that ebselen non-competitively inhibits extracellular lung epithelial cell NDPK activity with a <10 μM K_i value, our present study suggest a valuable tool to assess the contribution of nucleotide release to lung epithelial cell functions. In addition, while ebselen has broad substrate selectivity, it may be a candidate structure for the development of highly potent and selective NDPK inhibitors useful to studies of the physiological consequences of extracellular NDPK activity.

Table 2 Ebselen inhibits various biological activities

Activity	IC_{50} (μM)	Reference
NOS	8.5	38
15-Lipoxygenase	0.17	39
Prostaglandin endoperoxide synthase-1	37.7	39
NADPH oxidase	0.5–1.0	40
PKC (partially purified)	1.0	40
Phorbol ester-stimulated PKC in intact cells	50	40
Horseradish peroxidase	16.9	29
Lipid peroxidation	2.5	29
Yeast plasma membrane H^+-ATPase	4.5	42
Yeast growth	2–5	43
DMT1-mediated iron uptake	0.2	41

NOS nitric oxide synthase, *PKC* protein kinase C, *DMT1* divalent metal transporter-1

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