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Original research paper

α_1-Adrenoceptor agonist methoxamine inhibits base excision repair *via* inhibition of apurinic/apyrimidinic endonuclease 1 (APE1)

ANETA KOHUTOVA[1]
DITA MÜNZOVA[1]
MARTIN PEŠL[1,2]
VLADIMIR ROTREKL[1,2,*]

[1] *Masaryk University, Faculty of Medicine, Department of Biology 625 00, Brno, Czech Republic*

[2] *International Clinical Research Center (ICRC), St.Anne's University hospital in Brno, 625 00, Brno, Czech Republic*

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ABSTRACT

Methoxamine (Mox) is a well-known α1-adrenoceptor agonist, clinically used as a longer-acting analogue of epinephrine. 1*R*,2*S*-Mox (NRL001) has been also undergoing clinical testing to increase the canal resting pressure in patients with bowel incontinence. Here we show, that Mox hydrochloride acts as an inhibitor of base excision repair (BER). The effect is mediated by the inhibition of apurinic/apyrimidinic endonuclease APE1. We link this observation to our previous report showing the biologically relevant effect of Mox on BER – prevention of converting oxidative DNA base damage to double-stranded breaks. We demonstrate that its effect is weaker, but still significant when compared to a known BER inhibitor methoxyamine (MX). We further determined Mox's relative IC_{50} at 19 mmol L^{-1}, demonstrating a significant effect of Mox on APE1 activity in clinically relevant concentrations.

Keywords: methoxamine, base excision repair, apurinic/apyrimidinic endonuclease APE1, α1-adrenoceptor agonist

Methoxamine (Mox) became known originally in the 1960s for its inotropic effect mediated by α-adrenergic receptors in the ventricular myocardium (1). As a less potent but longer-acting analogue of phenylephrine (generic name Vasoxyl), it has been used as a peripheral vasoconstrictor (blood-pressure-increasing drug) during various surgeries, for example, to improve cognitive dysfunction and blood TNF α-levels during hip replacement surgery in elderly patients (2, 3) or as nasal decongestant (4). Importantly, Mox was used in combination with atropine to maintain blood pressure in pregnant women undergoing cesarean delivery (5) in addition to ephedrine (6). Apart from these short-term treatments, Mox has recently been tested in a clinical trial for its capability to increase the anal canal resting pressure in patients with bowel incontinence (7). Patients suffering from faecal incontinence are predetermined to long-term treatment and hence theoretical continuous Mox exposition. Therefore, the importance to investigate Mox's off-target effects caused by involvement in other biological processes arises, stressing the consequences of

* Correspondence; e-mail: vrotrekl@med.muni.cz

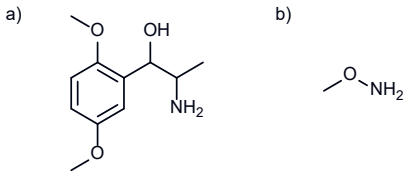

Fig. 1. Chemical structure of: a) α1-adrenoreceptor agonist methoxamine (Mox), and b) known BER inhibitor methoxyamine (MX), which blocks apurinic/apyrimidinic sites against APE1 cleavage.

chronic application, but also due to unknown placental crossing and unclear lactation transfer the fetal influence of Mox shall be studied. We have previously reported that somewhat surprisingly Mox can prevent the formation of DNA double-stranded breaks (8) and increase mutant frequency (9) upon treatment of human embryonic stem cells with ionizing radiation. We attributed it to the ability of Mox to inhibit base excision repair (BER) similarly to methoxyamine (MX; here we ask the reader not to confuse methoxamine and methoxyamine, see Fig. 1). BER is an essential DNA repair mechanism found ubiquitously in all cell types in the mammalian body and it is essential for correct early embryonic development (10). It is responsible for the repair of base lesions, one of the most common endogenous DNA damages occurring in the cells. While most of the enzymes and proteins participating in BER (*e.g.*, specific glycosylases, polymerases and ligases) are redundant to a certain degree the apurinic/apyrimidinic endonuclease APE1 is unique. Some of the somatic cell types present very low BER activity usually due to low expression of one or more of the BER enzymes, *e.g.* low activity of BER in mouse liver due to low expression of polymerase β (11) and mouse brain limited by low expression of XRCC1 (12). Yet all these tissues present relatively high expression of APE1 (12).

BER has been shown to be highly active in germ cells of young mice (13) and also in human pluripotent stem cells (8). APE1 expression has been shown to play a central role in limiting the BER efficacy in old murine germ cells (13) and adapted human pluripotent stem cells (8). Increased APE1 expression was shown to confer resistance against mutagenesis in murine germ cells and its decrease seems to contribute to the parental age effect (14). We have also previously shown that a decrease in BER activity upon prolonged cultivation of human pluripotent stem cells is mediated by decreased expression of APE1 (8), which results in less DNA double-strand break release and detection (15) resulting in elevated mutant frequency (9).

In our previous report, we showed that Mox can prevent the conversion of the clustered oxidative damage induced by ionizing radiation to double-stranded breaks by BER in human embryonic stem cells (8). Considering the wide range of biological processes regulated by APE1 and potentially long-term treatment of patients using Mox we considered it of utmost importance to decipher the effect of methoxamine in BER activity, namely inhibition of APE1 activity by Mox.

EXPERIMENTAL

APE1 inhibition assay

The *in vitro* APE1 activity assay was reconstituted as previously described (16–18) with modifications as follows. 1 unit of recombinant human APE1 (New England Biolabs,

Table I. Sequence of THF-containing substrate

Sense DNA sequence	5′ GCTTGCATGCCTGCAGGTCTGA THF TCTAGAGGATCCCCGGG-TACCGAGCTCGA 3′
Antisense DNA sequence	5′ TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCAGA CCTGCAGGCATGCAAGC 3′

M0282S) was used for each reaction in the reaction buffer. Fluorescently labelled double-stranded oligomer (25 nmol L^{-1}, Sigma Aldrich, labelled by fluorescein isothiocyanate (FITC) on 5′ end of sense strand) containing tetrahydrofuran (THF) was used as a substrate for the reaction (Table I).

Increasing concentrations of Mox (methoxamine hydrochloride; Sigma Aldrich, M6524) were used to test the relative IC_{50}. Concentration ranged from 5 to 225 mmol L^{-1} of Mox. Methoxyamine (MX; methoxyamine hydrochloride; Sigma Aldrich, 226904), a previously described inhibitor of BER (19, 20) was used at a concentration of 8 mmol L^{-1} as a positive control for inhibition of APE1 activity. Reaction in a total volume of 10 μL containing APE buffer (New England Biolabs, supplied with APE1) was run at 37 °C for 1 h. The reaction was stopped by the addition of an equal volume of 1 % SDS and placed on ice. Samples were then placed at 95 °C for 20 min prior to loading onto 20 % polyacrylamide (40 % Acrylamide/Bis Solution, 19:1, Bio-Rad, 1610144) gel with 7 mol L^{-1} urea (Pentachemicals, 21420-31000). The gel was run at 100 V, 14 mA for 90 min in 0.5xTBE (0.05 mol L^{-1} Tris-HCl, 0.05 mol L^{-1} boric acid, 1 mmol L^{-1} EDTA, pH 7.4) heated to 55 °C. Gels were visualized on FLA-9000 Station (FujiFilm, Life Science) using the LPB filter module and LD473 laser. The bands' intensity was analyzed by ImageJ (Fiji) (21). Intensities of uncut *versus* cut oligomers were evaluated as % of substrate digestion. % of APE1 activity for all concentrations of inhibitors were calculated as ratios of substrate digestion in the presence or absence of inhibitor at a particular concentration. Inhibition constant relative IC_{50} (concentration of inhibitor corresponding to the oligomer cleavage reaching 50 % between lower and upper plateau on the graph – maximum dose of inhibitor and no inhibitor, respectively) was calculated using AAT Bioquest *Quest Graph*™ IC_{50} *Calculator (*Retrieved from https://www.aatbio.com/tools/ic50-calculatoronline software, access date September 23, 2020).

RESULTS AND DISCUSSION

In order to decipher the effect of Mox we reconstituted the APE1 catalyzed endonuclease reaction *in vitro* using double-stranded oligonucleotide carrying THF as apurinic/apyrimidinic site (AP) site analogue. The THF-containing oligonucleotide was labelled with FITC allowing for the detection of uncut oligonucleotide as well as the product of APE1-mediated endonuclease reaction (Fig. 2). MX was used as a known inhibitor for comparison with Mox in a concentration in which it completely abolishes APE1 mediated digestion (Fig. 3 and Supplementary Fig. 1).

We successfully reconstituted APE1 activity *in vitro*. THF-containing 52-mer was quantitively digested by APE1 as 97.21 ± 3.5 % of the oligonucleotide was digested (Fig. 3).

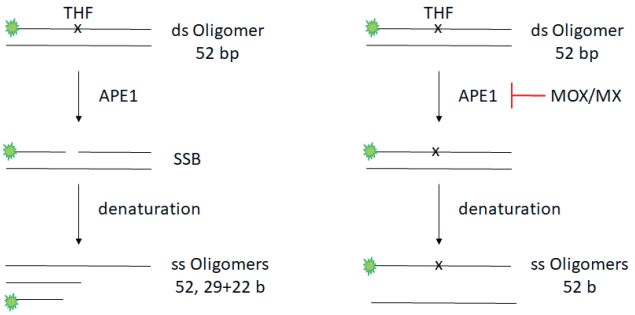

Fig. 2. Graphical representation of the experimental design. The double-stranded 52-mer oligonucleotide was used as the reaction substrate. One strand of the oligonucleotide contained THF on position 23 to mimic the AP site. The THF-containing single-stranded oligonucleotide was labelled with FITC on 5′-end to allow for fluorescent detection of both, the undigested 52-mer as well as the digested 23-mer. Methoxamine (Mox) or methoxyamine (MX) were used to inhibit the APE1-catalyzed DNA digestion.

Spontaneous degradation of THF-containing 52-mer oligonucleotide was excluded by control reaction without APE1 protein. In order to demonstrate the endonuclease digestion inhibition specificity, we used MX, a known inhibitor of APE1. MX at a concentration of 8 mmol L^{-1} completely inhibited endonuclease digestion of the THF-containing 52-mer, as no digestion product could be detected after the reaction. In comparison, 5 mmol L^{-1} Mox weakly attenuated the digestion allowing an average of 94.37 ± 3.35 % digestion. Increasing the Mox's concentration, inhibition of APE1 activity was observed, reaching a plateau at 42.54 % digestion.

Mean values from 4 repetitions were used to create a dose-response curve in the graph (Fig. 4).

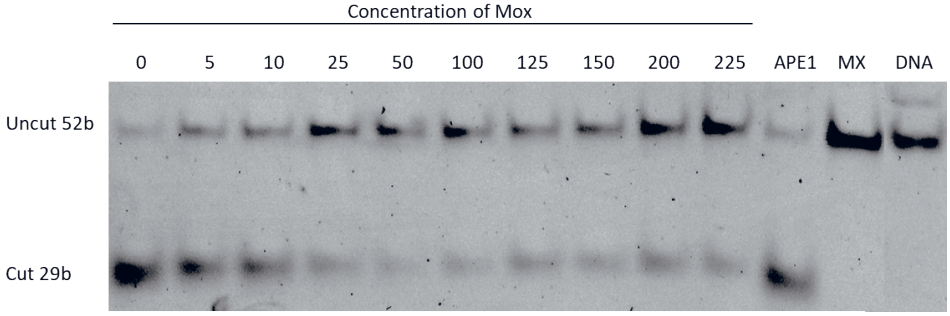

Fig. 3. Representative photo of polyacrylamide gel detection of APE1 activity. The APE1 endonuclease reaction was reconstituted resulting in cleavage of uncut 52 bp FITC-labelled oligonucleotide to FITC-labelled 23 bp and non-labelled 29 bp long fragments (lane labelled as APE1). The inhibitory effect of Mox at increasing concentrations (0 to 225 mmol L^{-1}) was compared to the known inhibitor MX and control reaction without APE1 (DNA). The products were separated using PAGE under denaturing conditions. The bands were visualized and quantified using FITC fluorescence.

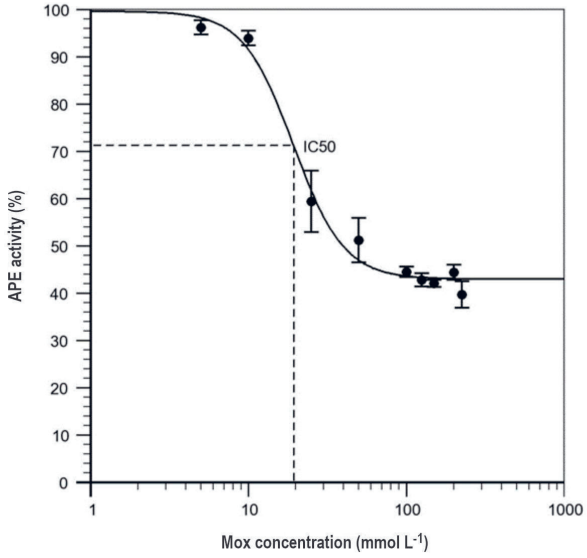

Fig. 4. Mox inhibition curve in the *in vitro* APE1 assay. Mean values of the digestion percentage were obtained by dividing cut oligonucleotide density by the sum of cut and uncut oligonucleotide, relativized to uninhibited reaction (APE alone, 100 %). The mean values of digestion percentage were plotted against the concentration of Mox. The experimental data were fitted with an exponential curve using the online tool IC_{50} calculator. Dots represent means with a standard deviation of means.

Based on the experimental data the inhibition constant for Mox was calculated, resulting in a relative IC_{50} value of 19.34 ± 1.94 mmol L^{-1}.

Mox is a clinically relevant α1-adrenoreceptor agonist considered to be a long-term pharmaceutical approach for various medical conditions (7). We have previously shown that Mox presence prior to ionizing radiation treatment of human embryonic stem cells results in elevated mutant frequency similar to APE1 downregulation (8, 9). We attributed this phenomenon to Mox's ability to inhibit BER and thus inhibit the conversion of ionizing radiation-induced clustered base damage to double-stranded breaks (8), which are essential to trigger proper checkpoint response and maintain low mutant frequency (15). Here we show, that Mox possesses the ability to inhibit APE1, a key non-redundant BER enzyme. Considering the inhibition of APE1 in patients, it is important to note that APE1 knockout is embryonically lethal in mice (22, 23), conditional knockout induced at embryonic day 7 resulted in 84 % prenatal mortality (24) and in remaining mice, similar to mice with APE1 knocked out postnatally suffered large amounts of senescence in various tissues leading ultimately to the premature ageing and cell death associated with elevated DNA damage and excessive telomere erosion (24). These data suggest that sufficient level of APE1 activity is essential in order to maintain proper development and possibly might have adverse effect on developing embryo as well as on tissue homeostasis and ageing.

BER protein expression was shown to be modulated both ways in numerous types of cancers. For example, while elevated expression of thymine glycol DNA glycosylase (NTHL1) induces hallmarks of cancer (25) and a subset of human lung cancer tumours

display elevated expression of 8-oxoguanine DNA glycosylase (OGG1) (26), some gastric cancers were shown to have lost NTHL1 expression at all (27). Importantly polymorphisms in the APE1 region are associated with an increased risk of cancer (*e.g.* 28). Thus, chronic APE1 activity downregulation caused by prolonged exposure to Mox might render the surrounding tissue more prone to carcinogenesis and/or senescence. APE1 endonuclease becomes a strategic therapeutic target as the BER is responsible for the amelioration of major damage caused by anticancer therapy, becoming a chemotherapeutic prognostic marker (*e.g.* 29) and target during radiotherapy (*e.g.* 30). As such it interferes with the efficacy of these therapeutic approaches by inducing survival effects *via* activation of transcriptional activities of NF-kappaB and HIF-1α (31) and it is also considered an anticancer target *per se* (*e.g.* 32). Yet no data on the effect of Mox on these APE1 protein-protein interactions are available. We have tested the effect of Mox only on the DNA repair function (endonuclease) of APE1 so we cannot comment on the APE1 role in multiprotein complex binding minimal hypoxic response element (33) which seems to be important for angiogenesis as APE1 downregulation led to inhibited angiogenesis in lung cancer (34). Yet to our knowledge no reports are available describing problems with angiogenesis, such as for example in patients with lower limb ischemia or angina pectoris, which might be again potentially contraindicative to Mox treatment and reflects in caution warning for concomitant use of Mox with the myocardial disease, arteriosclerosis (6).

Even therapeutically in cancer clinical trials, inhibition of APE1 (*e.g.* by MX) is usually considered for either short-term or one-time administration due to its pharmacokinetic profile with a half-life of around 43 hours (35). In this light, attention should be paid to the long-term downregulation of APE caused by chronic Mox treatment.

APE-mediated oxidative DNA damage repair seems to be essential for telomere length maintenance (36) as an important mechanism of genome stability maintenance. BER has been also shown to be highly active in germ cells of young mice (13) and also in human pluripotent stem cells (8) where it safeguards genomic integrity. Thus, possible contraindications for pregnant women on Mox treatment should be reassessed.

APE1 expression has been shown to play a central role in limiting the BER efficacy in old murine germ cells (13) and adapted human pluripotent stem cells (8) while loss of APE1 leads to the parental age effect in mice (14). These data suggest that sufficient APE1 activity is indispensable for stem cell homeostasis maintenance. Moreover, APE1 deficiency induced by the deletion in both alleles in experimental mice led to the promotion of cellular senescence resulting in cell death in differentiated cells and premature ageing phenotype (24). Higher APE1 expression levels and its interactions with multiple proteins seems to play also a role in protection of neurons against hypoxia, oxidative stress and neuronal loss (37) in Alzheimer patients (38–40) and amyotrophic lateral sclerosis (41) gain rising possible contraindications for patients considered for long term Mox treatment.

MOX was used in clinical trials as an intravenous infusion during surgery to increase blood pressure for maintaining intraoperative hemodynamics (2, 3), where a dose of 5 to 15 mg was administered to the patients intravenously. Such doses present roughly 14 nmol L^{-1} concentration in the blood (providing a pool of 5 L of blood and limited lipid binding). In a clinical trial phase-II as a potential drug for faecal incontinence (NCT00857467, NCT01656720) (42–45), patients were treated with a dose reaching 15 mg of Mox administered to rectal suppositories, likely to result in a high local concentration of Mox in the rectum. Considering that intravascular administration was so far limited to a maximum

of 14 nmol L^{-1} of Mox and the IC_{50} is three orders of magnitude higher (19 mmol L^{-1}) it is not very likely that APE1 will be significantly inhibited in a systemic manner, but local concentrations in used in trials for long-term administration might lead to local inhibition of APE1 in surrounding tissue. It must be mentioned that 1S,2R-methoxamine was used in the clinical study while we demonstrate the APE1 inhibitory effect using Mox hydrochloride, which is an undefined mixture of Mox optical isomers.

As for the molecular mechanism of Mox inhibition, we can only speculate based on both the inhibition curve and structural similarity to other compounds. Mox is an analogue of catecholamine/norepinephrine and similar compounds which also induce oxidative DNA damage (substrate for BER and APE1) as was shown previously both *in vitro* on ovarian cancer cells (46) as well as *in vivo* for example in blood cells (47). These compounds were recently shown to act *via* beta-2-adrenergic receptors/adenylate cyclase/cAMP/PKA leading to upregulation of ROS by downregulating Hif-1α (*e.g.* 48) and to P53 downregulation *via* AKT-mediated Mdm-2 activation (*e.g.* 49). On the other side, we are dealing with reconstituted APE1 reaction missing cellular components such as adrenergic receptors. Mox inhibitory effect seems to plateau around 40 % activity which might suggest either competitive inhibition with relatively high Ki, possibly *via* non-covalent modification of the AP site or some sort of allosteric inhibition targeting APE1, on the other side presence of ionized amine group similar to MX might suggest AP site covalent binding similar to MX (50, 51). Further, it might involve the interaction of Mox, AP site and APE1 together as an analogy to topoisomerase II nicking the DNA in the vicinity of AP sites covalently modified by MX (20, 52).

When considering inhibition of APE1 in animal models or tissue cultures main advantages of MOX compared to MX are its lower cytotoxicity even in comparably higher concentrations and easier and safer manipulation. Even when considering the animal model exposure the Mox toxicity in mice (LD_{50} mouse *i.p.* 92 mg kg^{-1}) is considerably lower compared to MX (LD_{50} mouse *i.v.* 5030 μg kg^{-1}) (53).

CONCLUSIONS

In summary, we show that α1-adrenoreceptor agonist Mox inhibits key DNA repair enzyme APE1. The clinically relevant concentrations of Mox have a significant effect on APE1 enzymatic properties *in vitro*. Thus, we suggest caution when administering the Mox to patients, especially those who might be at risk due to a family history of cancer and pregnant women. More research in terms of cancer development, Mox placental permeability, mammary gland secretion, and embryonic and fetal development should be conducted.

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Conflicts of interest. – The authors declare no conflict of interest.

Authors contributions. – Conceptualization, V.R. and M.P.; methodology, A.K. and D.M.; analysis A.K.; writing, original draft preparation, A.K.; writing, review and editing, V.R and A.K. All authors have read and agreed to the published version of the manuscript.

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