*Free Radical Research*, February 2010; 44(2): 232–239

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healthcare

# Sildenafil protects epithelial cell through the inhibition of xanthine oxidase and the impairment of ROS production

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*(Received date: 10 July 2009 In revised form date: 1 October 2009)*



**Abstract**

Xanthine oxidase (XO) plays an important role in various forms of ischemic and vascular injuries, inflammatory diseases and chronic heart failure. The XO inhibitors allopurinol and oxypurinol held considerable promise in the treatment of these conditions both in experimental animals and in human clinical trials. More recently, an endothelium-based protective effect of sildenafil has been reported in preconditioning prior to ischemia/reperfusion in healthy human subjects. Based on the structural similarities between allopurinol and oxypurinol with sildenafil and with zaprinast the authors have investigated the potential effects of these latter compounds on the buttermilk XO and on non-tumourigenic (HMEC) and malignant (MCF7) human mammary epithelial cells. Both sildenafil and zaprinast induced a significant and consistent decrease of XO expression and activity in either cell line. In MCF7 cells only, this effect was associated with the abrogation of xanthine-induced cytotoxicity. Overall, the data suggest that the protective effect of sildenafil on epithelial cells is a consequence of the inhibition of the XO and of the resulting decrease of free oxygen radical production that may influence the expression of NADPH oxidase and PDE-5.

**Keywords:** *Xanthine oxidase, ROS production, oxidative stress inhibition, sildenafil, zaprinast, human mammary epithelial cells.*

## Introduction

Xanthine oxidoreductase (XOR) catalyses the hydroxylation of a wide variety of heterocyclic substrates, including purines, pyrimidines, pterins and, in addition, the oxidation of aldehydes to acids [1–5]. The active enzyme can exist in one of two interconvertible forms, the xanthine dehydrogenase (XDH; E.C. 1.17.1.4) and the xanthine oxidase (XO; E.C. 1.17.3.2) [6,7]. Both forms reduce $O_2$, although XO is more efficient in this respect, eventually leading to formation of reactive oxygen species (ROS) [8]. XO, detected on the outer surface of cultured endothelial and epithelial cells [9], has been implicated in the pathogenesis of ischemia/reperfusion injury [10,11], where XO-induced ROS generation may serve as the trigger for a microvascular inflammatory response [12]. Furthermore, under aerobic conditions, XO reduces oxygen to superoxide that can in turn react rapidly with NO to produce peroxynitrite, a potent oxidizing and hydroxylating agent [13]. The increased activity of XO and the increased production of ROS may be responsible for various pathological processes associated with oxidative stress, such as endothelial dysfunction in chronic heart failure, inflammatory diseases, LDL oxidation, atherosclerosis, hypertension, cancer, ageing, etc. [14–19]. Based on the above evidence, the inhibition of XO might be important to prevent or reduce the production of free radicals and/or urate.

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ISSN 1071-5762 print/ISSN 1029-2470 online 
DOI: 10.3109/10715760903431426

In this respect, a well-known XO inhibitor, allopurinol (and its bioactive derivative oxypurinol), is the most widely used drug to lower the blood concentrations of urate and, therefore, to decrease the number of repeated attacks of gout [20] and to protect endothelia and epithelia in pathological conditions such as chronic heart failure or inflammatory diseases [14–16,18,19].

Clinical trials [21–24] have shown the significant benefit of sildenafil, an inhibitor of the type 5 phosphodiesterase, in patients with pulmonary arterial hypertension. More recently, the enhancement of endothelium-dependent vasodilatation by sildenafil proved to be highly protective in preconditioning prior to ischemia/reperfusion in healthy human subjects [25]. In addition, sildenafil has a powerful effect in conduits that are useful in coronary artery bypass grafting [26–29].

Given the structural similarity of allopurinol and oxypurinol with sildenafil, which are characterized by pyrimidine and pyrazole rings (purine moiety), and with zaprinast featuring pyrimidine and triazolo rings (see Figure 1), which show protective and trophic effects on vascular endothelium and epithelia [15,16, 21–26], we conducted an initial study on the potential effects of these drugs on the buttermilk XO enzyme activity and subsequently on non-tumourigenic (HMEC) and malignant (MCF7) human mammary epithelial cells that express XDH and XO activities. The use of HMEC and MCF7 is due to the different expression of the two forms of the enzyme in these cell lines. In fact, while HMEC express the native dehydrogenase (XDH), the oxidase form (XO) is expressed in MCF7 cells [30]. In this paper we report that sildenafil is capable of inhibiting the xanthine oxidase bound to the external membrane of epithelial cells. We reckon that, as formerly reported in the literature for allopurinol and oxypurinol [15,16], the chronic administration of sildenafil might improve epithelial and endothelial cell function also through the inhibition of XO activity, decreasing its ability to produce superoxide and hydrogen peroxide.

## Materials and methods

### *Cell cultures*

MCF-7 (passage 150) human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD); HMEC normal human mammary epithelial cells (passage 11) were obtained from Clonetics (Lonza, Walkersville, MD). MCF-7 cell were grown in RPMI-1640 medium containing 5 μg/ml phenol red and supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin; both from Gibco-BRL, Uxbridge, UK). HMEC cell were grown in MEBM serum-free medium supplemented with EGF, hydrocortisone, insulin, gentamycin and BPE using Single quota reagent packs from Clonetics (Cambrex BioScience, Walkersville, MD). Cells were harvested using trypsin-EDTA (1:5, v/v; Gibco-BRL) and subcultured in 96-well microtiter plates at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$.

### *Chemicals*

All chemicals and zaprinast were from Sigma-Aldrich (Milan, Italy) except HPLC-grade acetonitrile, methanol and hexane that were from J.T. Baker. Centricon YM-50 membrane and Millex-$FG_{13}$ filter (0.22 μm pore size) were provided by Millipore.

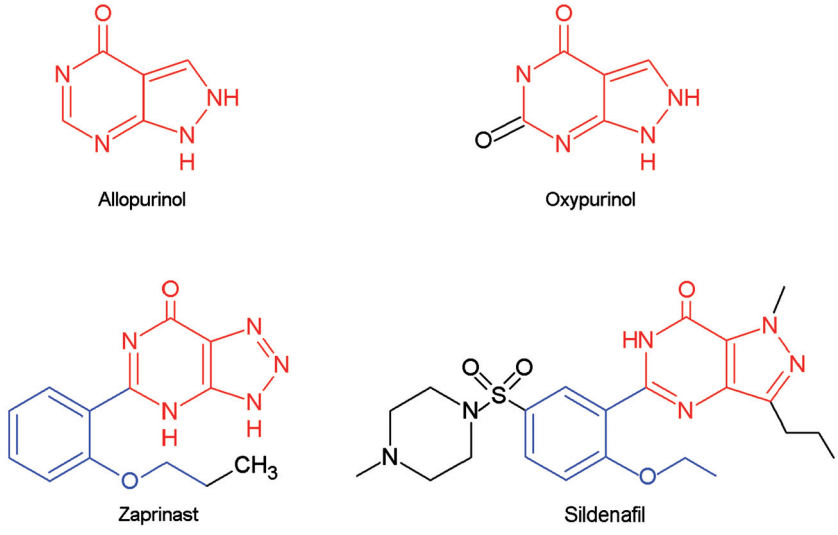


Figure 1. Structural similarities between allopurinol, oxypurinol, zaprinast and sildenafil. This figure provides structural formulas for the inhibitors of xanthine oxidase tested in this study. The aromatic ring analogy shared by the purinol derivatives and by zaprinast and sildenafil are shown in red.

Sildenafil citrate (Viagra tablet 100 mg) was a kind gift of Pfizer Italia.

*Instruments*

The Gilson analytical liquid chromatograph used throughout this work consisted of two Model 306 pumps, one 811B dynamic mixer, a Model 234 auto injector equipped with 20 µl injection loop, a Waters 2487 dual λ absorbance detector and Unipoint LC System Software (3.3) (Gilson Italia, Milan, Italy) for acquisition and elaboration of data. Separation and analysis of zaprinast and sildenafil were performed using a Varian RP-column (SP-C18-5, 4.5 µm; I.D. 4.0 mm × 15 cm). The Automated Micro Plate Reader Lambda E was furnished by Bio-Tek Instruments Coordination Center Europe (France).

*Sildenafil and zaprinast mixture solutions*

Considering a value for sildenafil citrate solubility of 3.5 mg/ml in water, at 20°C, one tablet of Viagra (100 mg) was homogenized in 30 ml of deionized water. The suspension, kept for a few minutes in warm water (30°C), was filtered through a vacuum pump using a 0.22 µm pore size membrane; after addition of 0.2 M Tris-HCl pH 7.8 (10 mL) it was kept at 4°C overnight to facilitate precipitation of the active ingredient. Then, after centrifugation at 5000 rpm for 10 min, the precipitate was washed twice with 10 mL of hexane. After hexane elimination, the white solid substance obtained was dried under nitrogen and subsequently saved at 4–8°C. Stock solution of sildenafil and zaprinast (1 µmol/mL) were prepared dissolving the compounds in 0.026 N NaOH. UV absorption spectra of the drugs were recorded using a Beckman DU8 spectrophotometer. It ought to be specified that the concentration reported for sildenafil might be over-estimated, because of the presence of an undefined number of crystallization water molecules in the preparation.

*Chromatographic analysis of sildenafil and zaprinast*

Prior to use, solvents were degassed by bubbling with helium. The solvent system used was a gradient of 50 mM phosphate buffer, pH 4.65 (solvent A) and methanol/50 mM phosphate buffer, pH 4.65; 80/20 v/v (solvent B). Compounds were eluted from 98% A (0–4 min) to 100% B (4–27 min) at room temperature. The gradient was then returned to 98% solvent A (28–30 min) and the initial conditions restored in 5 min. The flow-rate was 1 mL/min. Waters 2487 dual λ absorbance detector operating at 292 nm was used. Each compound was quantified using the calibration curves previously generated from standard solution. Each determination was performed in quadruplicate.

*Xanthine oxidase preparation*

Bovine buttermilk xanthine oxidase (2.5 µl; Sigma X-4500 grade III; 1–2 units/mg protein) were dissolved in 0.6 mL of 50 mM Tris-HCl pH 8.4, containing 1 mM EDTA (0.64 mg/mL). After stirring the sample was dialysed against the same buffer (5 L) for 2 h to remove ammonium sulphate and sodium salicylate. After filtration through a 0.22 µm pore size membrane the enzyme preparation was spectrophotometrically analysed and an $A_{280}/A_{450}$ ratio of 4.3 and an $A_{450}/A_{550}$ ratio of 3.9 were evaluated [31–33]. XO activity was determined by measuring the rate of oxidation of xanthine to uric acid spectrophotometrically at 295nm, using an absorption coefficient of 9.6 $mM^{-1}$ $cm^{-1}$ [34].

*Sildenafil and zaprinast effects on xanthine oxidase zymograms*

XO zymograms were prepared by loading the enzyme protein onto non-denaturing 7.5% polyacrylamide gels, following the indications reported in references [35,36]. A current of 20–25 mA was passed through the gels for 60 min. The gels were pre-incubated in the dark at 37°C for 10 min, with or without 50 µM zaprinast or 10 µM sildenafil, in 50 mL of a staining solution containing 50 mM Tris-HCl, pH 8.8, 17.5 mg of nitro blue tetrazolium (NBT) and 1 mg of phenazine methosulphate, as reported elsewhere [36]. Gels were finally incubated for 10 min in the presence of 50 µM and 10 µM hypoxanthine, respectively.

*XO enzyme assay with xanthine in the presence of zaprinast or sildenafil*

XO preparation (5 µL) was incubated with 20 µM xanthine in the presence of variable concentrations (2–20 µM) of zaprinast or sildenafil in 250 µL of 50 mM Tris-HCl, pH 8.4. After 10 min of incubation at 37°C the reaction mixture was stopped by dipping the sample into a cold bath. The uric acid formed was measured using the Roche kit UA plus. Uric acid calibration curve was in the range of 15–60 µmol/L. To calculate sildenafil inhibition constant (*Ki*), XO was assayed in a 250 µL medium containing 5 µL enzyme, 2.5–20 µM xanthine, at 2, 5, 20 or 20 µM sildenafil.

*MTT assay*

Cell viability was estimated by the quantification of the MTT reduction by cellular dehydrogenases [37]. In 90% confluent cultures of HMEC and MCF7 human mammary epithelial cells, routine medium was discarded and substituted with a new medium containing 10 µM xanthine, except controls. The cells were incubated for an additional 2 h at 37°C in a

humidified 5% $CO_2$ atmosphere. Some experimental series were simultaneously treated with 10 μM sildenafil, zaprinast or oxypurinol. At the end of treatments, the medium was discarded and a new medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) was added. The cells were incubated for additional 30 min at 37°C in a humidified 5% $CO_2$ atmosphere. After, medium was removed, cells were washed three times with PBS and DMSO was added for 10 min and the formazan salt formation was determined at 560 nm in a 96-well automated microplate reader. Data were expressed as a percentage of the formazan salt formation in the untreated cells.

### In vitro *assay for xanthine oxidase-mediated superoxide production*

Superoxide generation in xanthine oxidase-catalysed xanthine oxidation was determined spectrophotometrically by monitoring superoxide-dependent NBT reduction to the blue chromogen formazan at 560 nm with or without 10 μM sildenafil. In 90% confluent cultures of HMEC and MCF7 human mammary epithelial cells, routine medium was discarded and substituted with a new medium containing 0.5 mg/mL nitro blue tetrazolium (NBT) and 0.1 μl/ml phenazine methosulphate (PMS), with or without (controls) 10 μM xanthine. The cells were incubated for an additional 2 h at 37°C in a humidified 5% $CO_2$ atmosphere. Some experimental series were simultaneously treated with 10 μM sildenafil. After incubation the formazan crystals produced were solubilized in a 100 μL of DMSO and, after 10 min, the coloured solutions were quantified at 560 nm in a 96-well automated microplate reader. The rate of superoxide formation was calculated assuming an NBT absorption coefficient of 12.8 $mM^{-1}$ $cm^{-1}$ at 560 nm.

### *Kinetic data processing*

Leatherbarrow Eritacus Software made a provisional estimate of kinetic constants with the aid of the Grafit program. The appropriate velocity equations describing the kinetic behaviours [38] were verified. A non-linear iterative calculation using the Enzyfit program (Sigma-Aldrich) was also performed to calculate the kinetic parameters.

### *Protein quantification*

Protein content of each sample was measured by Sigma FluoroProfile Protein quantification Kit for data normalization.

### *Statistical analysis*

Results are presented as the mean ± SEM and were analysed using one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. Statistical significance was set at $p < 0.05$.

## Results

### *Chromatographic analysis of sildenafil preparation*

Based on UV absorption spectra of the extracted fractions, we established the maximum absorbance value for sildenafil and zaprinast at 292 nm. The degree of purification of the extract containing sildenafil was evaluated by a HPLC method developed by par-

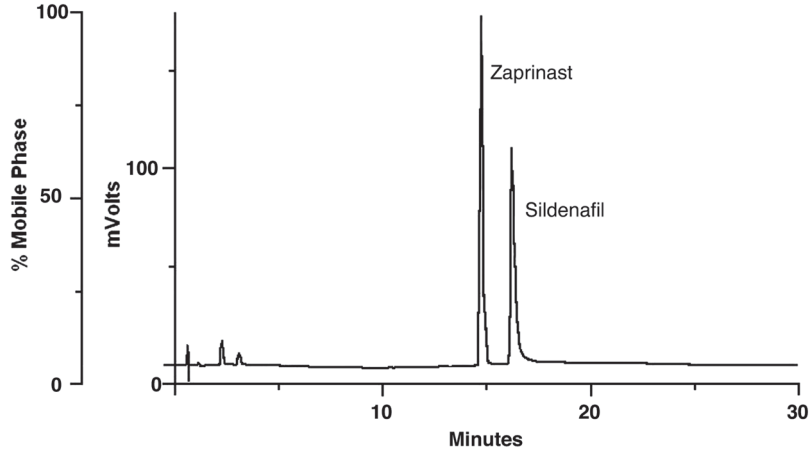


Figure 2. RP-HPLC analysis of zaprinast and sildenafil. The chromatographic pattern reported is related to 20 μl of a standard solution containing 1.28 nmoles of zaprinast and sildenafil. Chromatographic separation was performed with a gradient of solvent A (50 mM phosphate buffer, pH 4.65) and B (methanol/50 mM phosphate buffer, pH 4.65; 80/20 v/v) at a flow rate of 1 mL $min^{-1}$ and compounds were monitored at 292 nm.

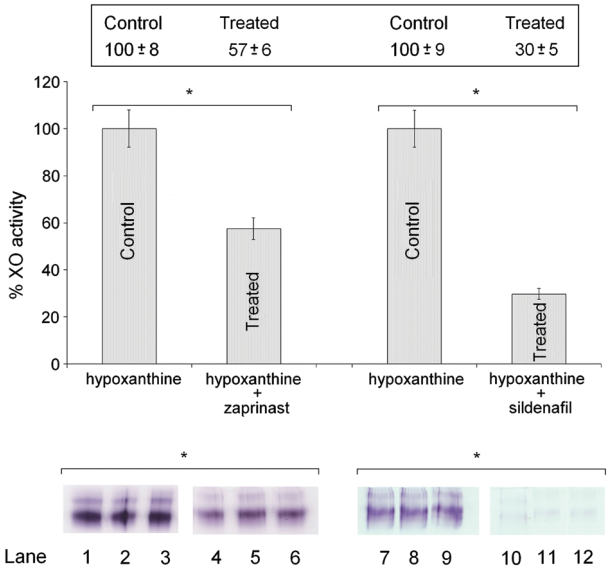


Figure 3. Inhibitory effects of Zaprinast and Sildenafil on xanthine oxidase activity assayed on electropherogram. The activity of xanthine oxidase (1.5 pmoles) assayed with 50 μM hypoxanthine (zymogram lanes 1-3) was significantly inhibited by 50 μM zaprinast (zymogram lanes 4-6). An inhibition value of about 40% respect to the control is reported in the upper panel where mean density values have been calculated. The same panel also reports an inhibition value of 70% relative to the marked effect of 10 μM sildenafil (zymogram lanes 10-12) compared to control assays, where XO were assayed with 10 μM hypoxanthine (zymogram lanes 7-9). Staining solution composition varied only for the kind of substrate used. Development times were 10 min. Data represent means ± SD from three independent experiments conducted in triplicate.

tial modification of that reported by Mei et al. [39]. Figure 2 shows the selective separation of a standard mixture of zaprinast and sildenafil.

### *Effects of zaprinast and sildenafil on XO zymograms*

Buttermilk xanthine oxidase, stained onto an electropherogram with 50 μM hypoxanthine, was able to produce superoxide anion, as measured by NBT reduction rates (Figure 3; Lanes 1–3). When zaprinast was added to the incubation mixture at the hypoxanthine equimolar concentration, a decrement of ~ 43% in hypoxanthine-induced superoxide production was observed (Figure 3; Lanes 4–6). More significant was the inhibitory effect exerted by sildenafil on xanthine oxidase activity, as shown in Figure 3 (Lanes 10–12), where an inhibition of 70% in hypoxanthine-induced superoxide production was observed.

Table I. Effect of zaprinast, sildenafil and oxypurinol on xanthine oxidase activity.

| Effector | Concentration | Xanthine oxidase % of activity |
|---|---|---|
| Zaprinast | 2 μM | 92 ± 6 |
| | 5 μM | 80 ± 7 |
| | 10 μM | 59 ± 6 |
| | 20 μM | 47 ± 4 |
| Sildenafil | 2 μM | 75 ± 4 |
| | 5 μM | 59 ± 7 |
| | 10 μM | 46 ± 4 |
| | 20 μM | 26 ± 6 |
| Oxypurinol | 2 μM | 70 ± 6 |
| | 5 μM | 50 ± 5 |
| | 10 μM | 36 ± 4 |
| | 20 μM | ND |

Percentage of activity is 4.56 nmol/min. 14.4 nM xanthine oxidase (29.16 μU) was assayed with 20 μM di xanthine, in presence of the inhibitors, at 37°C for 10 min. The results are the mean of at least four independent experiments.

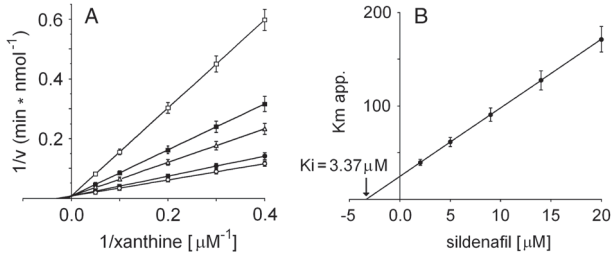


Figure 4. Sildenafil inhibition plot of xanthine oxidase. The basal activity was assayed with 14 nM enzyme in the range of xanthine concentration of 2.5-20 μM (○). The sildenafil concentrations were 2.5 μM (●), 5 μM (Δ), 10 μM (■) or 20 μM (□). The plot is representative of four separate experiments (Fig. 4A). The *Km* replot versus sildenafil concentration shows *Ki* the inhibition constant value (Fig. 4B).

### *Effects of zaprinast and sildenafil on XO enzyme activity*

In order to assess to what extent zaprinast or sildenafil can influence XO activity, dose–response experiments were carried out on XO enzyme with 20 μM xanthine in the presence of 5–20 μM zaprinast, sildenafil or oxypurinol (Table I). Uric acid formation was evaluated after 10 min of incubation, as reported in methods. Results showed that 5, 10 and 20 μM zaprinast significantly inhibited XO by 20 ± 6%, 41 ± 6% and 53% ± 5%, respectively. In the same assay conditions, the inhibitory effect of sildenafil was greater with a peak of 74% inhibition at 20 μM (Table I). As expected, oxypurinol exerted a significant inhibitory effect (30–64%) in the concentration range of 2–10 μM (Table I).

Competitive inhibition kinetics is shown in the plot of Figure 4A. Data were obtained assaying XO with 2, 5, 10 and 20 μM xanthine at fixed sildenafil concentrations (2, 5, 10 and 20 μM). The inhibition constant (*Ki*) determined for sildenafil was 3.37 ± 0.5 μM (Figure 4B).

### *Inhibition of XO reduces xanthine-induced ROS production and increases cell viability in normal and malignant human mammary epithelial cells*

Since our *in vitro* experiments indicate that ROS production by XO is inhibited by zaprinast and/or

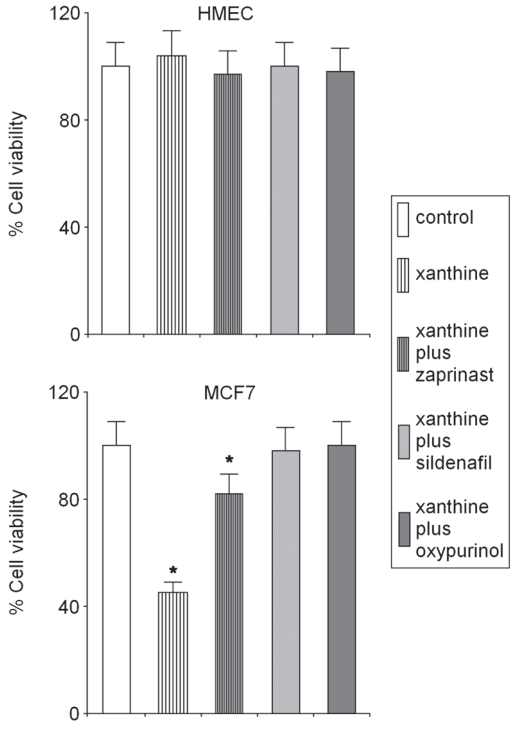


Figure 5. Effects of zaprinast, sildenafil and oxypurinol on cell viability in HMEC and MCF7 cells. Cells were incubated with or without (control) 10 μM xanthine for two hours with or without 10 μM zaprinast, sildenafil or oxypurinol. At the end of incubation cell viability was evaluated by MTT assay and data reported as percentage of basal values of untreated control cell cultures. Data represent means ± S.D. from two independent experiments conducted in triplicate.

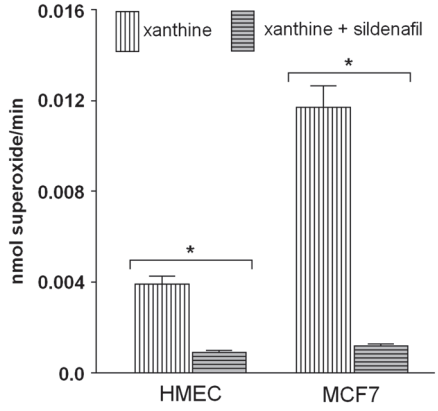


Figure 6. Inhibitory effect of sildenafil on xanthine oxidase-mediated ROS production in tumor and normal breast epithelial cells. HMEC and MCF7 cells were incubated in a medium containing 10 μM xanthine and NBT for two hours in presence or in absence of 10 μM sildenafil as reported in Materials and Methods (*see In vitro assay for xanthine oxidase-mediated superoxide production*). Reported values are net of ROS production by control cell samples, incubated without xanthine. Data represent means ± S.D. from two independent experiments conducted in triplicate.

sildenafil, we decided to investigate the potential effects of these drugs on the enzyme activity in cultured cells. HMEC and MCF7 cells (90% confluent) were treated with 10 μM xanthine for 2 h in the presence or absence of 10 μM zaprinast, sildenafil or oxypurinol. After incubation cell viability was evaluated using the MTT assay [37]. As shown in Figure 5, 10 μM xanthine did not cause any measurable decrease in cell viability of HMEC cells, while an inhibition of nearly 50% was observed in MCF7 cells. Treatment of MCF7 cells with zaprinast resulted in a recovery of 85% of the cell viability, while incubation with sildenafil or oxypurinol produced a 95–97% recovery. The evaluation of xanthine-induced ROS production in these cell lines was conducted using the NBT assay [36]. As expected, treatment with 10 μM sildenafil caused over 95% inhibition of the xanthine-induced ROS production in both cell lines (Figure 6).

## Discussion

Several reports support the involvement of XO in the oxidative stress that contributes to the endothelial dysfunction in chronic heart failure [14,15], chronic obstructive pulmonary disease, etc. In fact, in the vascular endothelium, XO is also present on the outer surface of epithelial and endothelial cell membranes [9] and is one of the main producers of superoxide anions. There is significant evidence for the pathogenetic role of XO in some murine experimental models of colitis and inflammatory bowel disease or duodenal ulceration [16,40,41]. There is also evidence for increased circulating levels of XO in plasma samples from patients with rheumatoid arthritis [17]. Some reports have proposed a potential role of XO in the pathogenesis of sickle cell disease, where a variety of components including inflammation, ischemia/reperfusion and also a high incidence of hyperuricemia and gout co-exist [40]. In all of these pathological conditions, a significant degree of conversion of XDH to XO and the release of XO into the systemic circulation [41,42] has been observed. Xanthine oxidoreductase (XOR) is increased in response to inflammatory cytokines, in human mammary epithelial cells, with a 2–2.5-fold increase being seen with TNF-$\alpha$ and IL-1$\beta$ and ~ 8-fold with INF-$\gamma$. These data strongly support a role for XOR in the inflammatory response of human mammary epithelial cells [18]. XO has also been implicated in a variety of toxic organ injury models, where allopurinol treatment has shown its efficacy. In many of these pathological conditions, allopurinol appears to reduce the symptoms and the effects of the disease [43–45]. Recently, effects akin to that of allopurinol have been ascribed to sildenafil, a drug that, like vardenafil and tadalafil, has been developed for its powerful effect on the corpus cavernosum for treatment of erectile dysfunction.

Sildenafil has also important vascular and myocardial protective effects, suggesting that this class of drugs may be useful in the treatment of patients with heart failure, pulmonary hypertension and coronary artery disease [20–29].

Considering the structural similarity between purine compounds and these drugs (Figure 1), in the present study we investigated whether the protective effect against the oxidative damage of these compounds may be mediated, at least in part, by their inhibitory effect on XO activity. Data reported herein demonstrate that zaprinast and sildenafil are both powerful inhibitors of XO, with sildenafil having a stronger inhibitory effect.

A kinetic study on the effects of sildenafil on XO activity, based on the Lineweaver-Burk plot of the velocity data, indicates a competitive inhibitory mechanism. Importantly, the *Ki* value of 3.37 μM, as obtained by replotting the apparent *Km* values vs sildenafil, is compatible with the plasma concentration of 1.5–3 μM after oral administration of sildenafil citrate 100 mg [46]. In fact, sildenafil which is extensively and rapidly absorbed, reaching maximum concentrations within 1 h, is distributed in humans in a volume of 1–2 L/kg [46].

Aiming to assess the effect of sildenafil and zaprinast on the oxidase activity of XOR *in vivo*, we selected non-tumourigenic (HMEC) and malignant (MCF7) human mammary epithelial cells, because we have previously observed a different expression of XDH and XO activities [30]. The higher XO activity determined in tumour epithelial cells with respect to normal cells led us to determine the xanthine oxidase-dependent ROS production and the associated cytotoxicity comparing MCF7 with the HMEC, where the native and $NAD^+$-dependent xanthine dehydrogenase is well expressed [30]. In particular, the evidence that incubation with xanthine produces a significant decrease of cell viability in MCF7 cells only and that the simultaneous addition of zaprinast, sildenafil or oxypurinol abrogates this effect and prevents ROS production in both cell lines clearly supports the concept that these drugs target the XO enzyme to protect either endothelial or epithelial cells from ROS-induced cell damage.

In conclusion, the present study provides clear-cut evidence that the protective effect exerted by sildenafil on epithelial cells is a consequence of the inhibition of the XO enzyme. This inhibition, resulting in the blockade of XO-driven superoxide production, also occurs with allopurinol and oxypurinol and may accomplish additive or synergic effects through the simultaneous inhibition of the phosphodiesterase type-5, with the resulting increment of bioavailable cGMP and nitric oxide (NO) and the potentiation of its physiological effects as well as the inhibited expression and activity of NADPH oxidase (NOX). This latter effect takes place in corpus cavernosal smooth muscle cells (CVSMC) where sildenafil inhibition of superoxide formation and $gp47^{phox}$ NOX expression, induced by the thromboxane $A_2$ mimetic, U46619, were observed [47]. Further studies should investigate whether the effect of sildenafil (and related drugs) on xanthine oxidase activity, as well as that ascertained on PDE5, may be correlated to the expression of NADPH oxidase in epithelial and in the endothelial cells [48]. Recently XO, as a ROS generating system in human vascular smooth muscle cell (hVSMCs), has been demonstrated to promote expression and activity of NOX-1 that up-regulates type 5 PDE expression [49].

***Declaration of interest:*** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 14 December 2009.