Toxicology in Vitro 29 (2015) 148–154

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Comparative study on thiol drugs' effect on *tert*-butyl hydroperoxide induced luminol chemiluminescence in human erythrocyte lysate and hemoglobin oxidation

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ARTICLE INFO

Article history:
Received 3 September 2013
Accepted 5 September 2014
Available online 13 October 2014

Keywords:
Chemiluminescence
Erythrocyte lysate
Hemichromes
Methemoglobin
Principal Component Analysis
Thiol drugs

ABSTRACT

The current studies have investigated the effect of heterocyclic drugs with the single thiol group (thiamazole, mercaptopurine) and dithiol aliphatic drugs (dimercaptosuccinic acid, dithiothreitol) under oxidative stress conditions, using *tert*-butyl hydroperoxide (*t*-BuOOH), in human erythrocyte lysate with the luminol-enhanced chemiluminescence technique. Knowing that oxidative processes induced by *t*-BuOOH are triggered by (oxy)hemoglobin (Hb), the effect of different thiol drugs (RSH) on isolated human Hb oxidation to methemoglobin (MHb) and hemichromes (HChr) was further considered. Three types of chemiluminescence curves, fitting to logistic-exponential model, have been revealed under influence of RSH. Structure of the data (MHb and HChr production, and free radical activity of RSH) in Principal Component Analysis visualization and kinetic profiles of chemiluminescence integrate information in terms of the diversity of RSH reaction mechanisms depending on the specific molecular context of the given thiol: aliphatic or aromatic nature as well as the number and position of the –SH groups in the molecule. The study conducted in presented *in vitro* systems indicates the potential role of thiol drugs mediated toxicity in an oxidative stress dependent mechanism.

1. Introduction

The ability of thiol compounds (thiol drugs – RSH) to function as either anti- or prooxidants is determined by the type of a stress oxidant and the physiological/experimental circumstances (structural and conformational features of RSH, RSH concentration, p*K* value of the sulfhydryl group, polarity of the environment, presence of transitory metal ions) (Asmus, 1990). The prooxidant effects induced by RSH have been associated with the formation of reactive oxygen species (ROS) i.e.: superoxide, hydrogen peroxide, hydroxyl radicals and the sulfur-centered free radicals in chain reactions (Netto and Stadtman, 1996).

In previous studies (Sajewicz, 2010) bimodal luminol chemiluminescence curve was detected in oxidative stress conditions under the influence of aliphatic drugs with the single –SH group in the system containing red blood cells (RBC) or RBC lysate (LYS). The kinetic solution of these bimodal chemiluminescence demonstrated that changes appeared under the RSH influence may have resulted from associated processes – scavenging by RSH of the *t*-BuOOH-induced free radicals and simultaneous generation of thiol-derived secondary free radicals. Chemiluminescence (CL) modifications in the systems with RBC and LYS were significantly different. In interactions of RSH in RBC system the essential role could be attributed to crossing the cellular membrane and distribution in each cellular compartment. In the system with LYS there was a free access to the cellular components.

The cascade of oxidative processes induced by *t*-BuOOH in RBC/LYS was triggered by (oxy)hemoglobin (Hb) (Smith et al., 1992). The radical effects involve both heme and globin moieties and lead to the formation of the ferric and ferryl forms of hemoglobin derivatives (Van der Zee, 1996; Domanski et al., 2004; Svistunenko, 2005). As has been shown, hydrogen peroxide triggers a redox cycle between ferric and ferryl Hb leading to the formation of transient protein radicals, covalent hemoprotein cross-links and induces structural changes (Jia et al., 2007).

Abbreviations: CL, (luminol-enhanced) chemiluminescence; CTR, control probe; DIM 1 or 2, Dimension 1 or 2; DMS, dimercaptosuccinic acid; DTT, dithiothreitol; Hb, (oxy)hemoglobin; HChr, hemichromes; LYS, erythrocyte (RBC) lysate; MHb, methemoglobin; MPU, mercaptopurine; MES, mesna; NAC, N-acetylcysteine; NAP, N-acetylpenicillamine; PCA, Principal Component Analysis; PEN, penicillamine; RBC(s), red blood cell(s); ROS, reactive oxygen species; RSH, thiol (drug(s)); *t*-BuOOH, *tert*-butyl hydroperoxide; TZL, thiamazole; TPR, tiopronin.

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http://dx.doi.org/10.1016/j.tiv.2014.09.001
0887-2333/

The current studies have shown the action of heterocyclic drugs with the single –SH group (thiamazole, mercaptopurine) and dithiol aliphatic drugs (dithiothreitol, dimercaptosuccinic acid) in RBC lysate determined by the chemiluminescence technique used previously (Sajewicz, 2010). The studies have investigated the RSH effect on Hb ($HbFe^{II}$) oxidation to methemoglobin (MHb, $HbFe^{III}$) and hemichromes (HChr) in comparison to the free radical activity of RSH in system with LYS, as well. MHb formation has been employed as an index of RSH-derived interaction to heme moiety in redox processes $HbFe^{II} \leftrightarrow HbFe^{III}$ whereas HChr as an index of the globin moiety structure changes (destabilization) in transformation MHb ↔ HChr with the protein as a target for the generated free radicals. The data analyzed with Principal Component Analysis (PCA) in comparison with the CL kinetic profiles has demonstrated distinguished/similar patterns of each RSH derivative action. The aim of this study was to approach some mechanisms and pathways of RSH potentially toxic reactions passing through the Hb and developing oxidative stress reflected in bimodal chemiluminescence.

2. Materials and methods

2.1. Chemicals

All used thiol compounds (RSH), dimethylsulfoxide (DMSO), and *tert*-butyl hydroperoxide (*t*-BuOOH, 70% aqueous solution) were obtained from Sigma–Aldrich Co. Ltd. Luminol was purchased from Koch Light Laboratories Ltd., UK. DEAE Sephadex A-50 – Pharmacia Fine Chemicals Inc., Uppsala, Sweden.

2.2. Effects of heterocyclic drugs with the single –SH group and dithiol aliphatic drugs on t-BuOOH-induced oxidative stress in human erythrocyte lysate

2.2.1. Red-cell and erythrocyte lysate preparations

Human blood from three healthy donors was drawn separately into Na_2EDTA-containing tubes. The mean corpuscular hemoglobin concentration in RBC from individual adult donors was within the range of reference values (MCHC; 32–38 g/dl). Plasma and white cells ("buffy coat") were removed after centrifugation (1000×*g* at 4 °C for 15 min) and RBCs were washed three times with PBS. Hematocrits (Ht) were measured after a 5-min spin in a Janetzki TH12 centrifuge. Erythrocyte suspension (Ht: 35–50%) 20 μl was lysed in 2 ml of water (Sajewicz, 2010).

2.2.2. Solutions of the investigated thiol compounds

TZL and DTT were dissolved in phosphate buffered saline (PBS, pH 7.4) whereas MPU and DMS in dimethylsulfoxide DMSO. Solutions of the tested thiol derivatives (5 μl) were added to 3 ml of phosphate buffered saline (PBS, pH 7.4). In order to maintain standard conditions in chemiluminescence measurements, 5 μl of DMSO was added to samples containing thiols dissolved in PBS and to the control (final concentration 21 mM). Equivalent concentrations of thiol derivatives were used in the investigations, which caused 10-min delay of chemiluminescence induced by 1 mM AAPH – 2,2′-azobis (2-amidino -propane) dihydrochloride as a water-soluble free radical source (Sajewicz, 2010). The final concentrations for the measurement of chemiluminescence in erythrocyte lysate (LYS) were as follows: mercaptopurine (MPU) – 2.9 μM, thiamazole (TZL) – 1.3 μM, dimercaptosuccinic acid (DMS) – 21.0 μM, dithiothreitol (DTT) – 10.5 μM.

2.2.3. Luminol

Luminol was dissolved in 0.1 M NaOH to obtain the concentration of 75 mM and then diluted in PBS to 30 mM. The concentration was controlled spectrophotometrically (λ = 347 nm) (Sajewicz, 2010).

2.2.4. Luminol-enhanced chemiluminescence measurements

Chemiluminescence (CL) was determined with a photometric unit (Photoamplifier M12 FQC 51, Germany). Chemiluminescence intensity (CL(t)) was measured by counting single photons in 1 s time intervals (counts per second – CPS) separated by 1 min intervals.

To the sample with a total volume of 3 ml (PBS, pH = 7.4; 37 °C) LYS was added which obtained from the equivalent number of erythrocytes (RBC) corresponding to the final hematocrit of 2.4×10^{-3}% (hypothetical number of 250×10^3/ml cells). Subsequently, the investigated thiol derivative was added at the final concentration above-mentioned. The mixture was incubated for 5 min at 37 °C and luminol was introduced (at the final concentration of 0.2 mM). After next 5 min of incubation at 37 °C *t*-BuOOH was added (at the final concentration of 1.0 mM). Since that moment luminescence intensity was recorded. Control sample did not contain thiol derivative (Sajewicz, 2010).

2.2.4.1. Estimation of chemiluminescence parameters. Estimation of some parameters such as kinetic curves of chemiluminescence intensity and free radical activity coefficient (A_{FR}) for thiol drugs was performed as previously described (Sajewicz, 2010).

The bimodal chemiluminescence kinetics ($CL_\sigma(t)$) was considered as the linear combination (sum) of two parallel processes $CL_\alpha(t)$ and $CL_\beta(t)$:

$$CL_\sigma(t) = CL_\alpha(t) + CL_\beta(t) = \frac{D_\alpha \cdot \exp(-k_\alpha t)}{1+\exp(a_\alpha + b_\alpha t)} + \frac{D_\beta \cdot \exp(-k_\beta t)}{1+\exp(a_\beta + b_\beta t)} \quad (1)$$

The kinetic curves of components $CL_\alpha(t)$ and $CL_\beta(t)$ have been fitted to experimental data points ($CL_\sigma(t)$).

Moreover the influence of a given thiol derivative on the light emission was assessed by comparing with the control sample without RSH and expressed as a free radical activity coefficient,

$$A_{FR}(\text{cps} \cdot \text{l}/\mu\text{mol}) = (ICL_{RSH} - ICL_{CTR})/c_{RSH} \quad (2)$$

where ICL_{RSH} and ICL_{CTR} are the integral intensities of chemiluminescence in the presence of the investigated derivative in concentration c_{RSH} (μmol/l) or in absence of one, respectively,

$$I_X = \sum_{t=2}^{25} n(t) \quad (2a)$$

where $X = CL_{RSH}$ or CL_{CTR}, $n(t)$ is the number of photon counts per second (cps), t = 2, 3…25 min.

Values of A_{FR} for N-acetylcysteine (NAC), mesna (MES), penicillamine (PEN), N-acetylpenicillamine (NAP) and tiopronin (TPR) in a system with LYS have been taken from studies published in (Sajewicz, 2010) and compared with results obtained in current studies.

2.3. Effects of thiol derivatives on t-BuOOH-induced oxyhemoglobin (OxyHb) oxidation

2.3.1. Preparation of human OxyHb

Human blood from healthy donors was drawn into Na_2EDTA-containing tubes. After centrifugation (1000×*g* at 4 °C for 15 min), plasma, platelets and white cells ("buffy coat") were removed and RBCs were washed three times with PBS. The erythrocyte suspension was lysed in 3 volumes of distilled water for 15 min. The hemolysate was centrifuged (20,000×*g* at 4 °C for 30 min) to remove cellular debris. OxyHb was purified by one-step procedure on a DEAE-Sephadex column chromatography of