Toxicity of pyrimidine derivatives under oxidative stress conditions: chemiluminescence-based assays in systems containing erythrocytes, mitochondria or blood plasma

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Abstract:
Participation of mono-, di-, and tricyclic pyrimidine derivatives in free radical processes was investigated with the use of luminol-enhanced chemiluminescence in measurements performed in vitro in systems containing erythrocytes, erythrocyte lysate, erythrocyte membranes, mitochondria, mitoplasts or blood plasma. The free radical processes were induced in the investigated systems by tert-butyl hydroperoxide (t-BuOOH) or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Amongst the examined compounds, only derivatives containing the thiol substituent were found to modify the course of chemiluminescence. Effects of both amplification and inhibition of light emission were observed to depend on the structure of a derivative and on the type of a biological (experimental) system. The light emission-amplifying activity was found to be characteristic of the system containing erythrocytes. The results of the investigations point out that the pyrimidine thio-derivatives prolonged the oxidative stress through some interactions mainly with (oxy)hemoglobin, which was related with specific toxicity against erythrocytes. Model investigations in the proposed experimental systems can serve as a useful tool at early stages of the drug discovery process when compounds involved in the interactions connected with the oxidative stress are being selected. On the other hand, this method allows to study mechanisms of the toxic action of xenobiotics on cells (erythrocytes) and organelles (mitochondria), strongly implicated in the free radical generation, and to examine the role of the extracellular liquid (plasma).

Key words: blood plasma, chemiluminescence, erythrocytes, mitochondria, oxidative stress, thiopyrimidine toxicity

Abbreviations: AAPH – 2,2’-azobis(2-amidinopropane) dihydrochloride, CL – (luminol-enhanced) chemiluminescence, CL% – relative chemiluminescence, CPS – counts per second, I0CL – AAPH induced chemiluminescence, I0CL% – relative chemiluminescence induced with AAPH, I1CL – t-BuOOH induced chemiluminescence, I1CL% – relative chemiluminescence induced with t-BuOOH, t-BuOOH – tert-butyl hydroperoxide, RBC(s) – red blood cell(s)

Introduction
At the beginning stage of drug discovery process, it is necessary to determine drug toxicity. A variety of assay methods and technologies for high-throughput screening in silico [5, 6, 35] and in vitro have been de-
Developed. Among others, cell cultures [15, 21, 32], ovary cells [11], erythrocytes [28], submitochondrial particles [12] and immunological molecules [30] have been applied in *in vitro* evaluation of chemicals’ interactions with biological systems.

Induction or expansion of oxidative stress connected mostly with lipid peroxidation in cytoplasmic membranes, cellular organelle membranes, and also lipoproteins in body fluids, resulting from free radical reactions, can be one of many forms of toxic activity of a potential drug. Various drugs, e.g. paracetamol, bleomycin, anthracyclines [38], and carazolol [22] cause tissue damage as a consequence of free radical and reactive oxygen species (ROS) generation. An imbalanced production of ROS and/or reactive nitrogen species (RNS) plays a role in the pathogenesis of a number of human diseases such as cardiac disease (ischemia/reperfusion injury, drug induced cardiotoxicity) [18], allergy, neurodegenerative diseases, atherosclerosis, cancer [26], and also in wound healing [34].

The aim of this work was to improve and demonstrate a practical usefulness of *in vitro* tests developed for selection of chemical compounds (potential drugs) revealing undesirable proprieties under oxidative stress condition.

During many years, several pyrimidine derivatives have been developed as drugs and have found wide clinical applications [14]. All the time, new pyrimidine-based compounds are in research as chemotherapeutic agents. In this work, oxidative interactions of the synthesized mono-, bi- and tricyclic pyrimidine derivatives were examined through luminol-enhanced chemiluminescence after induction of free radical processes by tert-butyl hydroperoxide (t-BuOOH) in the model systems containing isolated erythrocytes, erythrocyte membranes, mitochondria or mitoplasts, or in blood plasma in the presence of carbon-centred radicals generated during thermolysis of 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH).

### Chemical structure of the examined compounds

Chemical structures of the compounds used in this research are shown in Figure 1.

### Solutions of the investigated compounds

All derivatives were dissolved and diluted in dimethylsulfoxide (DMSO). Solutions of the tested derivatives (5 µl) were added to 3 ml of phosphate buffered saline (PBS, pH 7.4). Solubility (in the tested concentration range) in the buffer system was examined spectrophotometrically. An increase in absorbance within the characteristic UV bands was found to be proportional to the derivative concentration which was a proof of homogeneity of the solution (data are not presented).

### Red blood-cell preparations

Adult human blood was drawn into Na$_2$EDTA-containing tubes. A mean corpuscular hemoglobin concentration in RBC of individual adult donors was within the range of reference values (MCHC; 32–38 g/dl). After centrifugation (1000 × g at 4°C for 15 min) plasma and white cells (“buffy coat”) were removed and RBCs were washed three times with PBS. Hematocrit (Ht) were measured after a 5-minute spin in a Janetzki TH12 centrifuge. In the studies of interactions of pyrimidine derivatives, isolated erythrocytes with Ht = 2.4 × 10$^{-3}$% were used, which corresponded to the hypothetical number of 250 × 10$^3$/ml cells.

### Erythrocyte lysates

Erythrocyte suspension (Ht: 35–50%, 20 µl) was lysed in 2 ml of water.

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**Materials and Methods**

**Chemicals**

tert-Butyl hydroperoxide (t-BuOOH, 70% aqueous solution), bovine albumin (fraction V), mannitol, digitin (used to isolate mitochondria and mitoplasts) were obtained from Sigma, St. Louis, MO, USA. 2,2’-Azobis(2-methylpropionamide) dihydrochloride (AAPH) was from Sigma-Aldrich Chemie GmbH, Germany. Luminol was purchased from Koch Light Laboratories Ltd., UK. Pyrimidine derivatives (Fig. 1) were synthesized and characterized by Anna Długosz according to the methods described previously [8–10, 23]. All other chemicals were of analytical grade.
Erythrocyte membrane preparation

Erythrocyte membranes (ghosts) were prepared by a hypotonic lysis technique [20].

Isolation of mitochondria and mitoplasts

Mitochondria were isolated according to the method described by Radi et al. [31] from a fetal portion of normal human placenta obtained when normal labors had been completed.

Mitoplasts (mitochondria depleted of outer membrane) were prepared by removing the outer membrane by digitonin treatment [30].

Protein concentration

Protein concentrations in the erythrocyte membranes, mitochondria or mitoplasts were determined by the method of Lowry using bovine serum albumin as a standard.
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) [2].

Chemiluminescence measurement

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

Measurements of chemiluminescence induced by tert-butylhydroperoxide (t-BuOOH)

To sample whose total volume was 3 ml (PBS, pH = 7.4; 37°C) one of the following was added: erythrocytes whose count corresponded to the final hematocrit of 2.4 × 10–3%, lysate obtained from the equivalent number of erythrocytes, suspension of erythrocyte membranes, mitochondria or mitoplasts (0.5 mg/ml of protein). Subsequently, the examined derivative at the final concentration of up to 50/10^9 M was added. 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 has been recorded. The influence of a given derivative on the light emission was assessed by a comparison with the control sample containing DMSO (at the final concentration of 21 mM) and expressed as a relative chemiluminescence (CL%).

\[
CL\% = \frac{I_{\text{DERIV}}}{I_{\text{DMSO}}} \times 100
\]

where \( I_{\text{DERIV}} \) and \( I_{\text{DMSO}} \) are the integral intensities of chemiluminescence in the presence of an examined derivative or DMSO only, respectively,

\[
I_X = \sum_{t=1}^{T} n(t)
\]

where \( X = I_{\text{DERIV}}, I_{\text{DMSO}}, n(t) \) is the number of photon counts per second (CPS), \( t = 1,2,...,T (T = 10 \text{ min}) \).

Other control samples such as RBC, RBC membranes, mitochondria, mitoplasts treated with the derivatives in the presence of luminol, or the derivatives in the presence of t-BuOOH and luminol (but without RBC, RBCs membranes, mitochondria nor mitoplasts) did not produce any light emission.

Chemiluminescence induced by free radicals formed from 2,2’-azobis(amidinopropane) dihydrochloride (AAPH)

Investigations of the influence of pyrimidine derivatives on the system containing plasma were based on the total peroxyl radical-trapping potential (TRAP) measurement [1]. First, 250 μl of the basic AAPH solution (to the final concentration of 40 mM) and 100 μl of the basic luminol solution (to the final concentration of 1 mM) were added in sequence to 2.57 ml of PBS at 37°C. The sample was incubated at 37°C for 15 min. Next, 5 μl of the examined compound (diluted in DMSO) was introduced. Plasma (75 μl) was added in the next minute. Since that moment the chemiluminescence intensity has been recorded. The control contained DMSO at the final concentration of 21 mM, identically as in the samples. In addition chemiluminescence of the investigated compounds was measured in the PBS medium only (without plasma). The influence was estimated on the basis of two parameters: the relative chemiluminescence (CL%) according to the formula described above or a difference in the chemiluminescence time delay between the sample and the control as shown in Figure 4.

Results

Among the examined derivatives, the modifying effect on the AAPH-induced chemiluminescence (\( I_{\text{A CL}} \)) was found only for monocyclic pyrimidines that included the thiol group (\( I_a, I_b1, I_b2, I_b3 \)). The remaining derivatives \( I_c, I_d, I_e, I_I, I_{II} \) did not change significantly the chemiluminescence time course.

In Figure 2, the \( I_{\text{A CL}} \) kinetics, representing the influence of \( I_a \) in the PBS medium, is compared with that for glutathione (GSH), a well-known antioxidant. Inhibition of the light intensity was nearly stable throughout the whole test period and was found to be proportional to \( I_a \) concentration. The influence of thiol derivatives from \( I_b \) class was analogous (data are not shown). \( I_{\text{A CL}} \) kinetics in the presence of GSH
of IACL% inhibitory effect (Pinh = 1/IC50) ranged in the following order according to the power stimulatory effect (Pstim) (Fig. 4). The test compounds can be arranged in the following class: Ib1, Ib2, Ib3, class Ib was very weak.

Concentrations corresponding to IC50 values were used in further investigations of interactions of thioderivatives in the system containing blood plasma. IACL kinetics is shown in Figure 4. At 1 µM concentration the Ia compound inhibited the photon emission approximately 3 times stronger than Ib class derivatives, whereas the differentiation within the compared class was very weak.

The test compounds can be arranged in the following order according to the power stimulatory effect (Pstim) = 1/IC50):

\[ I_a > (I_b2 > I_b3 > I_b1) \]

\[ I_b1 > I_b2 > I_b3 > I_b1 \]

Thiopyrimidinecarboxylic acid derivative (Ia) intensified chemiluminescence about 8 times more strongly than thiopyrimidine derivatives with the phenyl group (Ib). Among Ib group derivatives, Ib1 stimu-
lated the light emission of cells by about 45% more strongly than the remaining substances of this group (statistically significant difference at p < 0.05). The activity of Ib3 was slightly greater (8%) than that of Ib2.

The concentrations corresponding to SC50 values of pyrimidine thiol derivatives were used in further experiments with RBC membranes, RBC lysates, isolated mitochondria and mitoplasts. The results of the investigations were assembled in Table 1. Ia and Ib1 stimulated the lysate chemiluminescence almost by about 40% more strongly than in the system with RBCs, whereas no significant differences in lgCL% value were observed under the influence of Ib2 and Ib3. Light emission from the erythrocyte membranes under the influence of Ia did not differ significantly from the values measured for RBCs. IgCL% of the membranes, treated with Ib1 or Ib2, oscillated around 100% and did not differ significantly from those for the control tests, while Ib3 inhibited photon emission in the RBC membrane suspensions by 38%. Ia compound did not change significantly mitochondrial chemiluminescence, however, Ib group compounds lowered IgCL% slightly (15–22%). Ia and Ib2 did not modify significantly the mitoplast chemiluminescence. The influence of Ib1 and Ib3 produced 30–34% inhibition of photon emission.

The other compounds Ic, Id, Ie, II, III did not generate significant changes in the chemiluminescence intensity in the same experimental systems.

Discussion

The inhibition of AAPH-induced luminol chemiluminescence caused by the monocyclic thioderivatives of pyrimidine (Ia, Ib1, Ib2, Ib3) in PBS medium seems to indicate their capability to neutralize free radicals (Fig. 3). Also effects of the dependence of inhibition on the compound structure are visible. On one hand, different IACL inhibition is exerted by different derivatives (Fig. 3), and on the other, one can observe different kinetics of the photon emission inhibition by different compounds in comparison with that for glutathione (Fig. 2).

Potential antioxidant properties of thiol pyrimidine derivatives did not find a faithful analogy in the plasma system. For compounds from Ib group that enhance the IACL delay time (with an average of 1–3 min), an antioxidant action can be suggested, whereas Ia influence was found to be ambivalent: on one hand, one could conclude a prolongation of Δτ, and on the other one could infer an increase in the light intensity pointing to an increase in free radical reactions (Fig. 4).

t-BuOOH-induced stimulation of the chemiluminescence intensity in the presence of all thiol derivatives was proven in the system containing erythro-
cytes (Fig. 5). Exposure of erythrocytes to organic hydroperoxides (ROOH) causes irreversible lipid and protein damage. Scission of the ROOH by hemoproteins generates alkoxy and peroxy radicals which initiate membrane unsaturated fatty acids peroxidation [36]. The influence of thiol derivatives on lysates and erythrocyte membranes was estimated in order to describe and partly explain their mechanism of action.

In hemolysate, there is a free access to both sides of cellular membranes as well as to the examined compounds as t-BuOOH and hemoglobin which initiate free radical processes. Simultaneously, compartmentalization of different elements of antioxidant systems (peroxide dismutase, catalase, glutathione peroxidase, glutathione) occurs. Greater stimulation of chemiluminescence by Ia and Ib1 in comparison to RBCs (Tab. 1) can be the product of complex activities indicating the above-mentioned factors. A greater ability of these derivatives to generate free radical processes in comparison to Ib2 and Ib3, determined by their structure, has also been confirmed. The influence of thiol compounds on I BCL of the isolated erythrocyte membranes was heterogeneous: Ia increased CL, Ib1 and Ib2 lacked any essential action, and Ib3 had the inhibitory effect. Comparing these facts with different features of interference in the systems containing erythrocytes or lysate, one can infer that hemoglobin mediates mainly the reactions of free radical transformations generated through pyrimidine thiols. According to Smith et al. [33] isolated erythrocyte membranes (ghost) can contain catalytically-accessible heme iron, which can explain the CL increasing effect of (the most active) compound Ia in the absence of cytosolic antioxidants. Next, about 38% decrease in light emission from membrane preparation elicited by Ib3 points to the ability of scavenging of certain forms of free radicals, visible only in the absence of dominating reaction being catalyzed by hemoglobin.

Investigations in the system containing mitochondria confirmed indirectly reactions of thiol derivatives with hemoglobin. Unlike the systems with erythrocytes or lysates, the level of mitochondrial I BCL was not changed by Ia, whereas in the presence of the Ib group derivatives that level decreased by about 20% (Tab. 1). Cytochromes or iron-sulfur proteins act as catalysts in the mitochondrial t-BuOOH-dependent lipid peroxidation [31]. Human placental mitochondria contain cytochrome P-450SSC (side-chain cleavage cytochrome P-450) in the inner mitochondrial membrane which may also participate in the free radical generation from synthetic and natural hydroperoxides [17]. A little higher I BCL of mitoplasts (127 ± 7%) than that of mitochondria (109 ± 6%) under Ia action suggests possibility of the reaction, to a weak degree, through cytochromes, resulting especially from better penetration after the removal of external mitochondrial membrane. However, analogous effect

<table>
<thead>
<tr>
<th>System</th>
<th>Thiol derivative</th>
<th>Relative chemiluminescence (CL%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Ia</td>
<td>Ib1</td>
</tr>
<tr>
<td>RBC</td>
<td>196 ± 4</td>
<td>149 ± 11</td>
</tr>
<tr>
<td>RBC lysate</td>
<td>139 ± 5</td>
<td>94 ± 9</td>
</tr>
<tr>
<td>RBC membrane</td>
<td>109 ± 6</td>
<td>85 ± 3</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>127 ± 7</td>
<td>66 ± 4</td>
</tr>
<tr>
<td>Mitoplast</td>
<td></td>
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</tbody>
</table>

Experimental conditions and the CL% calculation are described in Materials and Methods. Data represent the mean ± SD calculated for five different RBC preparations and their lysate, or three preparations of mitochondria and their mitoplasts isolated from different placentas. SC50 is the coefficient representing the concentration of each derivative causing the 50% increase in the light emission intensity in the system with the RBCs (see legend to Fig. 5). SC50 was calculated on the basis of the regression functions. * Indicates statistically significant differences (p < 0.05, Student’s t-test) in comparison with RBC. ** Statistically significant differences (p < 0.05, Student’s t-test) in comparison with the control sample without the thiol derivative (CL% = 100%)
does not occur in the presence of \( \text{Ib} \) group derivatives, what indicates that this process is very complex. The induction of free radical transformations would interlock with scavenging of some free radical forms, similarly as it was shown for the system containing erythrocyte membranes.

Thiols (biothiols as well as pharmaceuticals) are generally known as protecting agents against the damaging effects of xenobiotics, reactive oxygen species and free radicals in biological systems. This protection is based on the ability of thiols (RSH) to repair free radical sites. Since S-H bond strength is lower than that of many C-H bonds, numerous carbon-centred radicals are repaired by donation of a hydrogen atom or an electron from sulfur followed by a proton. This process is called thiol pumping what is defined as the oxidation of a thiol compound by another free radical metabolite that results in formation of a parent molecule and a thyl free radical, or a radical derived therefrom [3, 25]. Of the biothiols, by far the most important are the aminothiols, particularly cysteine and glutathione [37]. Drugs containing thiol e.g. N-acetylcysteine [7], mesna [24], captopril, tiopronin [16], methimazole [4] are used in the treatment of a variety of diseases sharing alternations of the redox status. In contrast to the beneficial actions associated with the aliphatic aminothiols, simple aliphatic (methanethiol), aromatic (benzenethiol) and heterocyclic (furan-3-thiol) thiols are, to a greater or lesser degree, poisonous to animals, selectively toxic to erythrocytes, and cause hemolytic anemia [27].

It has been proposed that hemoglobin reacts with \( t\text{-BuOOH} \) to produce tert-butylperoxyl radical which, through secondary reactions, produces tert-butoxy and methyl radicals that are able to oxidize thiols to their corresponding thyl radicals [13]. The results of investigations on pyrimidine thiol derivatives indicated that increased chemiluminescence intensity in the systems containing (oxy)hemoglobin were perhaps the effect of thyl radicals formation. However, it is difficult to explain the mechanism of the observed increase in the chemiluminescence intensity under the influence of \( \text{Ia} \) in the presence of plasma occurring after the delay phase \( I_{\text{A CL}} \) (Fig. 4) using only the performed experiment.

A large array of pyrimidine drugs possess a variety of medicinal properties. These properties include anti-cancer, antibacterial, antiprotozoal, antimicrobial, antiviral, antihypertensive, anti-inflammatory, diuretic, uricosuric action [14].

In silico investigations (SPECS and BioSPECS software programs) showed that \( \text{Ia} \) compound was characterized by a moderate-low probability (0.5 < \( p < 0.7 \)) as a radioprotector, MAO inhibitor, antiviral and an uricosuric compound with a moderate probability (0.7 < \( p < 0.9 \)). The in vitro investigations presented in this work, however, allowed us to eliminate the pyrimidine thiol derivatives from further pre-clinical trials because of some possible interactions in free radical processes and, particularly, strong toxicity to erythrocytes.

The remaining monocyclic derivatives (\( \text{Ic, Id, Ie} \)), dicyclic (\( \text{II} \)) and tricyclic (\( \text{III} \)) ones, in the light of the performed investigations, did not reveal any toxic activity under conditions of oxidative stress. If necessary, these compounds can be investigated in respect to their individual effects e.g. \( \text{Id, Ie} \) as uricosuric, and \( \text{III} \) – topoisomerase II inhibitor whose probability was calculated as moderate.

The results of the present investigations show that the proposed model with human-derived in vitro systems can be used for preliminary selection of compounds participating in interactions connected with oxidative stress, suggesting simultaneously some mechanisms of their action on cells (erythrocytes), organelles (mitochondria) most implicated in the generation of free radicals and also on extracellular liquid (plasma). Data derived from this trial can validate computational methods to predict interactions. The easily accessible material for investigation and the possibility of automatization of chemiluminescence measurements create conditions for including it into the High Throughput Screening in Toxicology Programme.

Acknowledgements:
The work was supported by grant GU 987 from Wroclaw University of Medicine. The author wishes to thank Professor Anna Długosz from the Department of Toxicology, Wroclaw University of Medicine, for providing the pyrimidine derivatives, and Mrs Mia Keizer from the Compound Acquisition Department at SPECS, Rijswijk, The Netherlands, for the in silico evaluation of possible biological activities of the pyrimidine derivatives.

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Received: July 29, 2005 in revised form: November 14, 2006.