DNA Cleavage and Trypanosomes Death by a Combination of Alamar Blue and Au(III)

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Summary: Alamar Blue test indicates toxicity of Au(III), but Au(III) ion itself is not toxic *in vitro* and does not cleave DNA. However, combination of Alamar Blue and Au(III) cleaved DNA through formation of $O_2^{-\bullet}$ and H_2O_2 and showed high toxicity on *Trypanosoma brucei brucei* that causes African Sleeping Sickness (Trypanosomiasis) *in vitro* and *in vivo* tests. The DNA cleavage study was performed by gel electrophoresis and circular dichroism (CD) techniques. *In vitro* study was carried out with tissue cultured adapted *T. b. brucei* 427 strain blood-stream form parasites which were grown in HMI-9 medium with and without Au(III) ion and incubated at 37 °C in 5% CO_2. Toxicity of the mixture of Alamar Blue and Au(III) ion was also tested *in vivo* with mice which were infected intraperitoneally with 4.0×10^4 trypanosomes. Mechanism of DNA cleavage and of toxicity *in vitro* test is discussed for the mixture of Alamar Blue and Au(III) ion.

Alamar Blue has long been used for cell proliferation. Recently it has been found that Alamar Blue induces cleavage of DNA in the presence of Au(III) through the formation of $O_2^{-\bullet}$ and H_2O_2. On the other hand, Alamar Blue alone or Au(III) ion is incapable of DNA cleavage. It has also been observed that in the presence of certain metal ions such as Pd(II), Zn(II), Cd(II) and Pb(II), Alamar Blue is incapable of DNA cleavage. The cleavage study was carried by gel electrophoresis and circular dichroism (CD) techniques. It was observed that Form I and Form II of the pBluescript plasmid DNA were converted into Form III and DNA fragment by gel electrophoresis. The intensity of Form II was gradually decreased with increasing concentration of Au(III) with the completed disappearance at its higher concentration. The inhibitory effect by the superoxide dismutase (SOD) and catalase confirmed the formation of $O_2^{-\bullet}$ and H_2O_2 respectively in the presence of Alamar Blue and Au(III) ion. The drastic change in CD spectra from positive ellipticity to negative ellipticity at 270 nm is also suggested the cleavage of DNA with increasing concentration of Au(III) in the presence of Alamar Blue. The toxic effect of the mixture of Alamar Blue and Au(III) on *Trypanosoma brucei brucei* [that causes African Sleeping Sickness (Trypanosomiasis)] was investigated and found the IC_{50} value for aqueous Au(III) was 3.8×10^{-7} M while that for Pd(II) was 1.3×10^{-5} M. However, Alamar Blue alone or Au(III) ion did not show any significant toxic effect on the parasite. The toxic effect of the mixture of Alamar Blue and Au(III) ion was also tested *in vivo* with mice which were infected intraperitoneally with 4.0×10^4 trypanosomes. Mechanism of DNA cleavage and of toxicity *in vitro* test is discussed for the mixture of Alamar Blue and Au(III) ion.

Key Words: Alamar Blue/Au(III); Reactive Oxygen Species; DNA cleavage; Toxicity; *Trypanosoma brucei brucei.*

Introduction

It has long been a concern to find out commonly used chemicals (both inorganic, mainly metal ion and organic compounds) in different laboratories such as chemistry, biochemistry, biology etc and also in many industries which show carcinogenic or mutagenic effects to the living body [1-7]. The occurrence of carcinogenicity or mutagenicity induced by the chemicals or metal ions is mainly due to formation of free radicals *in situ* resulting in cleaving the biomolecules e.g., nucleic acids, proteins, peptides etc [6, 8, 9]. A particular compound/metal ion, however, has no or little toxic effect but the combination with non-toxic compound/metal ion shows toxicity to the living body. Scientist Norman Good and his colleagues synthesized twenty buffering agents for biochemical and biological research which is known as Good's buffers [10]. It was assumed believed that all these buffering agents are quite stable in biochemical or biological system with a tendency to generate free radicals from the piperazine ring-based Good's buffers such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EPPS (4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid) and PIPES (1,4-piperazinediethanesulfonic acid), but not from MES (4-morpholineethanesulfonic acid) which contains a morpholine ring in the presence of ferritin, an iron containing blood cell protein [11]. However, Tabata and his co-workers found that both the piperazine and

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morpholine ring-based Good's buffers in combination with Au(III) ion generate nitrogen-centered cationic free radicals which cause DNA cleavage [12, 13]. Tabata et al. also reported that no free radicals were formed from both the piperazine and morpholine ring-based Good's buffers in the presence of even iron along with other metal ions, for example, Co(II), Ni(II), Zn(II), Pd(II), Cd(II), Hg(II), and Pb(II).

Tabata and his co-workers reported that metalloderivatives of free base porphyrins, H_2TMPyP^{4+} (tetrakis(1-methylpyridinium-4-yl)porphyrin) with Cd(II)-, Pb(II)-, Hg(II)-, Au(III)- and Pd(II) ions showed toxic effects on *Trypanosoma brucei brucei* which causes African trypanosomiasis [14, 15]. The toxic effects by the metalloporphyrins on the parasite were higher than their counterpart free metal ions. Parasites intake metalloporphyrins more efficiently for growth than their counterpart metal ions because of their structural similarity with heme which is one of the essential biomolecules of living organism. Toxicities arise due to interruption of biochemical pathways by the metalloporphyrins for the formation and utilization of heme [16]. However, Au(III) ion showed higher toxicity on the parasite than its metalloporphyrin ($Au(III)TMPyP^{5+}$) , in *in vitro* test by using HMI-9 medium. This is due to generation of free radicals from HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) which is used in HMI-9 medium to maintain physiological pH, in combination with the Au(III) ion [10]. No such radical was formed from the medium HMI-9 in the presence of other metal ions such as Pb(II), Cd(II) or Pd(II) and/or their metalloporphyrins [15].

The main component of Alamar Blue is resazurin, a fluorescent dye which is mainly responsible for showing metabolic activities of microorganisms through changing its color [17]. The Alamar Blue mixture is non-toxic, soluble and stable in tissue culture medium, so it has been used with tritiatedthymidine for quantitative measurement of proliferation of various types of cells, for example, human and animal cells, bacteria, mycobacteria, fungi etc [18-21]. Alamar Blue has also been used to develop viability assay for cell screening of *Trypanosoma brucei brucei* bloodstream form strain 427 [22]. Moreover, the Alamar Blue mixture is used as an alternative to the MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [23, 24]. The Alamar Blue assay can also be used in many other cases, for example, for cell suspensions/attachment [25-27], monitoring of various growth factors [28], cytokines [29] etc.

We present here toxicity of a combination of Alamar Blue and Au(III). Alamar Blue test suggested toxicity of Au(III) but Au(III) itself did not cleave DNA and affect anything in *in vitro* study using *Trypanosoma brucei brucei* which causes African trypanosomiasis. However, the combination of Alamar Blue and Au(III) cleaved DNA as well as killed the parasites of *Trypanosoma brucei brucei*.

Experimental

Materials

Alamar Blue was purchased from Bioscience, Camarillo, CA (a fluorometric/colorimetric growth indicator). H_2TMPyP^{4+} and $Na_2H_2edta{\cdot}2H_2O$ (ethylenediamine-*N*,*N*,N',*N*'-tetraacetic acid, disodium salt, dihydrate) were purchased from Dojindo laboratories Ltd., Japan; phosphate buffered saline was purchased from Sigma-Aldrich; sodium tetrachloroaurate(III) dihydrate; sodium chloride, sodium hydroxide, ethanol, dimethyl sulfoxide (DMSO), superoxide dismutase (SOD), catalase, tris(hydroxymethyl)aminomethane, mercury(II) nitrate, iron(III) chloride, cobalt(II) chloride, nickel(II) chloride, zinc(II) chloride and palladium(II) chloride were purchased from Wako Chemicals Co. (Osaka, Japan); manganese(II) chloride, cadmium(II) nitrate and lead(II) nitrate were purchased from Katayama Chemical Co. (Japan). A standard procedure was used to extract pBluescript II plasmid DNA from plasmid bearing *Escherichia coli* strain form bacteria [30]. After that the plasmid DNA was dissolved in sterilized doubly deionized water and used it as a stock solution. Molar extinction coefficient value ($\varepsilon = 1.32 \times 10^4$ mol^{-1} dm^3 cm^{-1} at 260 nm) was used to calculate the concentration of DNA in base pairs [31]. Sterilized doubly deionized water was used throughout the experiments.

Methods and Apparatus

Preparation of porphyrin complexes

A standard Cu(II) solution was used to standardize the free base porphyrin (H_2TMPyP^{4+}) solution [32]. For preparation of the metalloporphyrin complexes, a requisite volume of the porphyrin solution was mixed with the target metal salt and then refluxed [33]. The metalloporphyrins were removed from the system through precipitation upon addition of $NaClO_4$ and purified via recrystallization. Perchlorate (ClO_4^-) form-metalloporphyrins were passed through chloride form-ion-exchange column for solubilization. A UV-visible spectrophotometer (Shimadzu UV 2100) was used to confirm the purity of the synthesized porphyrins through observing their Soret maxima. The absorption maxima of the synthesized metalloporphyrins were found to be in excellent agreement with the literature

values (Au(III)porphyrin [34] and Pd(II)porphyrin [35]. Stability of the synthesized metalloporphyrins was confirmed by dissolving them in 1 M HCl separately and found to be no dissociation at all. A flame atomic absorption spectrophotometer (AAS, PERKIN ELMER Analyst 100 AA) was used to determine the concentrations of Au(III) and Pd(II) ions.

DNA cleavage by the mixture of Alamar Blue and Au(III) ion

Gel electrophoresis experiments were done to investigate the DNA cleavage induced by the mixtures of Alamar Blue and metal ions. Before run the gel for electrophoresis, the plasmid DNA (2.00×10^{-4} M in base pairs) was mixed with Alamar Blue (4%), NaCl (0.10 M), phosphate buffered saline (pH 7.2) and target metal ion (2.00×10^{-4} M) in an Eppendorf tube (1.5 cm^3). The DNA mixture was then incubated at 37 °C for 60 min in a constant temperature bath (Yamato, Japan). The incubated DNA samples were then stained with 1 μdm^3 of a loading buffer (containing 30 % glycerol, 0.1 M EDTA, 0.25% xylene cyanol and 0.25% bromophenol blue) and then run in 1% neutral agarose slab horizontal gel containing a TAE buffer solution of pH 8.30 [tris(hydroxymethyl)aminomethane), 2.40 g; $Na_2H_2EDTA \cdot 2H_2O$, 0.37 g; glacial acetic acid, 0.57 cm^3 (99.7%) in 500 cm^3 doubly deionized water] for 32 min using a Mupid-2 Cosmo Bio Company apparatus (Japan). Since DNA is non-fluorescent so the gel was stained with a fluorescent dye solution (ethidium bromide, 0.50μg/mL) for 35 min. A Polaroid MP-4 land camera with a Polapan black and white coatless film was used to take photograph of the gel under trans-illuminating condition. Similar gel electrophoresis experiments were conducted using 4% Alamar Blue to investigate as follows: (i) the effects of the concentration of Au(III) ion and (ii) the inhibitory effects by the scavengers of the reactive oxygen species (ROS) (e.g., ethanol, DMSO, superoxide dismutase and catalase) on DNA cleavage. Sodium chloride (0.10 M) was used to keep constant ionic strength for all the metal ions while 0.10 M $NaNO_3$ was for Hg(II). All the experiments were carried out conducted under room light.

DNA conformational change by the mixture of Alamar Blue and Au(III) ion

DNA conformational changes were investigated by using circular dichroism (CD) technique (Jasco J-720 spectropolarimeter, Japan) upon addition of standard Au(III) solution in the presence of Alamar Blue (4%) at physiological pH (phosphate buffered saline, pH = 7.2) and 37 °C where NaCl concentration was 0.10 M. For each addition of Au(III) solution into the DNA solution (1.50×10^{-4} M in base pairs), a CD spectrum was recorded with five times scanned and then averaged.

In vitro cultivation and toxicity tests of Trypanosomes.

A detail *in vitro* and toxicity tests of *Trypanosomes* was reported previously described in the past [12, 13, 36]. Briefly, optimum concentrations of Alamar Blue, metal ions and metalloporphyrins were introduced into HMI-9 medium which contained tissue cultured trypanosomes (4.0×10^5 mL^{-1}). The mixture was then incubated at 37 °C with 5% CO_2 for 24 h. Alamar Blue indicator was used to monitor the growth of the trypanosomes by changing its color [14, 37]. The minimum effective concentration to inhibit the growth of the parasites was determined through keeping the parasities for a period of time in the HMI-9 medium containing Alamar Blue, metal ions/metalloporphyrins. IC_{50} values were calculated by linear interpolation selecting values above and below the 50% mark [38]. Minor deviations of the IC_{50} calculated by linear interpolation from the IC_{50} value calculated by logit analysis were negligible. Confirmation of the IC_{50} concentrations determined by the dye-method was done by light microscopy by direct counting of parasite number using a hemocytometer. Measurements of absorbance/optical densities were determined within the incubation time (0-24 h) using a microplate reader (Benchmark, BIO-RAD, Japan) with a computer attachment. The observed absorbance was the difference between the absorbances of Alamar Blue at the two wavelengths of 570 nm (reduced form) and 630 nm (oxidized form).

In vivo test of the mixture of Alamar Blue and Au(III).

The mixture of Alamar Blue and Au(III) was used to investigate the *in vivo* test by this mixture as follows: (i) infect the mice with 4.0×10^4 trypanosomes intraperitoneally and (ii) various doses of the mixture of Alamar Blue and Au(III) were administered into the infected and non-infected mice through injections.

Results and Discussion

DNA cleavage by Alamar Blue-Au(III) system

Fig. 1 shows the gel electrophoresis of the plasmid DNA incubated with Alamar Blue and metal ions where lane 1 stands for control (only Alamar Blue) and lanes 2-6 stand for metal ions and Alamar Blue. As seen from the Fig. 1, the intensities of Form-1 (supercoiled) and Form-II (circular) are decreased and a fragment is appeared in lane 2 (Alamar Blue-Au(III) system). But gels of lanes 3-6 did not change and were comparable to that of lane 1. The results indicates the mixture of Alamar Blue and Au(III) ion cleave DNA

while mixture of Alamar Blue and either Pd(II) or Zn(II) or Cd(II) or Pb(II) did not cleave DNA.

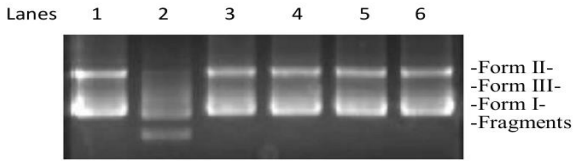


Fig. 1: Gel electrophoresis of plasmid DNA (2.00×10^{-4} M base pairs) in the presence of Alamar Blue and metal ions. Lane 1, DNA alone; Lane 2, Au(III); Lane 3, Pd(II); Lane 4, Zn(II); Lane 5, Cd(II); Lane 6, Pb(II). Alamar Blue was maintained at 4% of pH 7.20 (phosphate buffered saline). Concentrations of metal ions = 2.00×10^{-4} M; [Metalloporphyrins] = 2.00×10^{-4} M. Incubation temperature: 37 °C; Incubation time : 60 min

Fig. 2 shows the gel electrophoresis of plasmid DNA with different concentrations of Au(III) ion in the presence of 4% Alamar Blue. Here, lane 1 stands for DNA alone and lanes 2-6 indicate the effects of increasing concentrations of Au(III) from 2.00×10^{-6} M (lane 2), 2.00×10^{-5} M (lane 3), 2.00×10^{-4} M (lane 4), 6.00×10^{-4} M (lane 5) and 1.00×10^{-3} M (lane 6) in the presence of Alamar Blue (4%). The results show that concentration of the Au(III) ion is enhancing the degree of DNA cleavage. DNA cleavage was not observed clearly at the concentration of 10^{-6} M of Au(III), but for lanes 3 the intensity of Form II is decreased (2.00×10^{-5} M) and completely disappeared for lane 4 (2.00×10^{-4} M). A fragment peak appeared for lanes 4-6 and the intensities of Forms I and II gradually weakened from lanes 4 to 6. These results indicate the DNA cleavage occurs at specific site which leads to forming a given fragment. In our previous work, a half molecular weight of DNA fragment was confirmed for DNA cleavage by the combination of Au(III) and Good's Buffer [12, 13]. DNA was not completely cleaved to till small molecules.

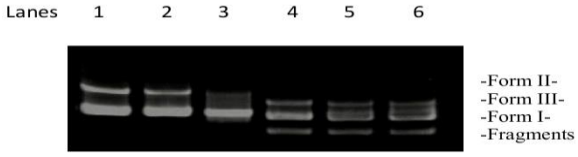


Fig. 2: Gel electrophoresis of plasmid DNA (2.00×10^{-4} M base pairs) with different concentrations of Au(III) ion in the presence of 4% Alamar Blue (pH 7.20). Lane 1, DNA alone; Lane 2, Alamar Blue + 2.00×10^{-6} M; Lane 3, Alamar Blue + 2.00×10^{-5} M; Lane 4, Alamar Blue + 2.00×10^{-4} M; Lane 5, Alamar Blue + 6.00×10^{-4} M; Lane 6, Alamar Blue + 1.00×10^{-3} M Au(III) ion. Incubation temperature and time were maintained at 37 °C and 60 min., respectively.

Fig. 3 shows DNA cleavage in the presence of scavengers of the reactive oxygen species (ROS), for example, $O_2^{-\bullet}$ and H_2O_2, for the mixture of Alamar Blue (4%) and Au(III) (10^{-4} M) at pH 7.20. Comparing of the gel of lane 2 with those of lanes 3 and 4, superoxide dismutase (SOD of 100 unit, lane 3) and catalase (100 unit, lane 4), suppressed the formation of the DNA fragment and the intensity of Form I increased by a little. The results show that SOD and catalase inhibited the DNA cleavage. In these experiments phosphate buffer was used to adjust solution pH, this is because mixture of Good's buffer and Au(III) generates free radical which cleave DNA while phosphate buffer-Au(III) system does not generate any free radical resulting in no DNA cleavage [12, 13].

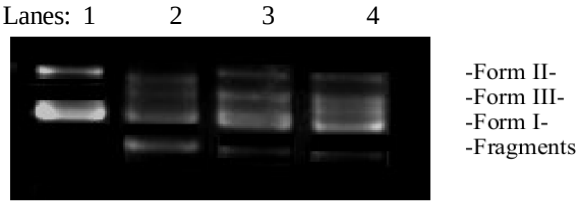


Fig. 3: Effects of scavengers of ROS e.g., $O_2^{-\bullet}$ (SOD) and H_2O_2 (catalase), on DNA damage induced by Alamar Blue (4%, pH 7.20) in the presence of Au(III). Lane 1, DNA alone; Lane 2, Alamar Blue + Au(III); Lane 3, Alamar Blue + Au(III) + 100 unit superoxide dismutase (SOD); Lane 4, Alamar Blue + Au(III) + 100 unit catalase. Incubation temperature and time were maintained at 37 °C and 60 min., respectively. [Au(III)] = 2.00×10^{-4} M; [DNA] = 2.00×10^{-4} M base pairs

DNA conformation change in the presence of Alamar Blue with Au(III)

Circular dichroism (CD) technique was used to elucidate the structural changes of the plasmid DNA with increasing concentrations of Au(III) ion. As seen from the Fig. 4 (a), drastic spectral changes were observed with titration of Au(III) of $(1.50\text{-}7.00) \times 10^{-4}$ M and reached to constant spectra at more than 1.00×10^{-5} M. On the other hand, only a small hypochromicity in CD spectra was observed for Pd(II), Zn(II), Cd(II) and Pb(II) ions and metalloporphyrins of Au(III) and Pd(II) (Fig. 4 b). The drastic spectral change in CD spectra from positive to negative ellipticity at 270 nm upon addition of Au(III) is also confirmed the cleavage of DNA [12, 13]. The CD spectra exhibited an irregular spectral pattern which suggesting the formation of unwound DNA fragments [12, 13]. These alternations in the CD spectra is due to the change in the conformation of DNA from B-form to Z-form and through the cleavage of DNA by the free radicals generated from the Alamar Blue-gold(III) system.

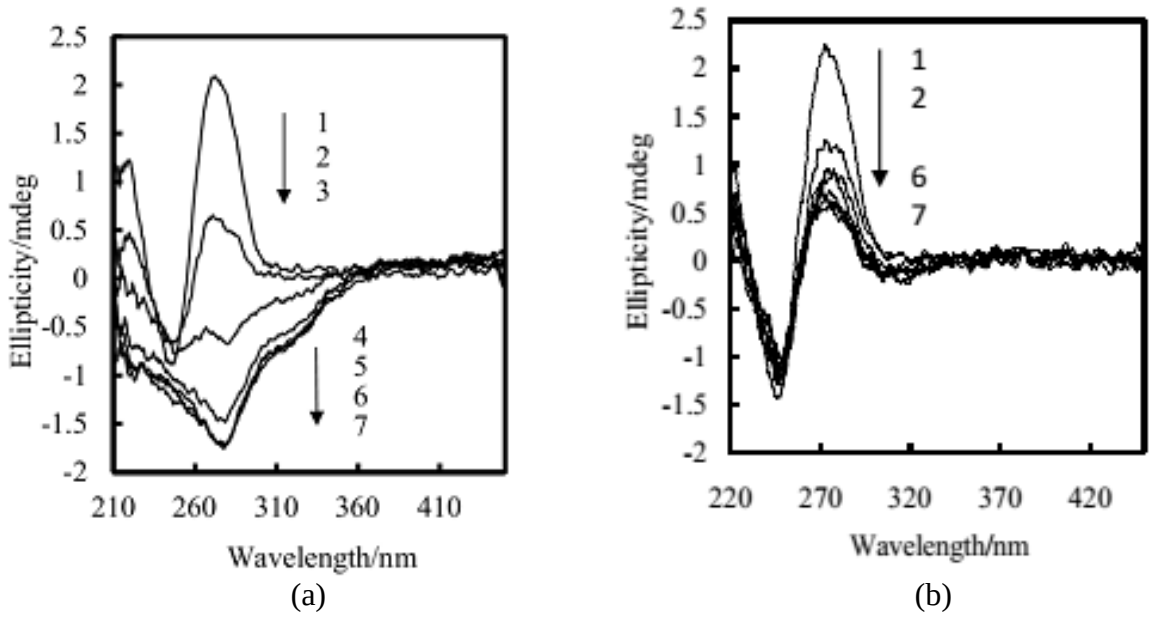


Fig. 4 : CD spectral changes of DNA (1.40×10^{-4} M base pairs) as a function of (a) [Au(III)] and (b) [Cd(II)] in the presence of Alamar Blue (4%, pH 7.20) at I = 0.10 M, NaCl. Arrows indicate spectral decrease as a function of [Au(III)] of (1) 0.00, (2) 1.50, (3) 4.50, (4), 7.00, (5) 10.00, (6) 13.00 and (7) 15.00×10^{-5} M. Cell path length is 10 mm.

Effects of Au(III) and Pd(II) ions and their metalloporphyrins in combination with Alamar Blue on Trypanosoma brucei brucei

Alamar Blue has been used as an indicator to monitor the growth of trypanosomes by changing its color at its reduced form (570 nm) and oxidized form (630 nm) which provided the determination of IC_{50} values. IC_{50} values for aqueous Au(III) and Pd(II) were 3.8 x 10^{-7} and 1.3×10^{-5} M in the presence of Alamar Blue, respectively. On other hand, IC_{50} values for the metalloporphyrins were as follows: $[Au(III)TMPyP]^{5+}$, 8.8×10^{-6}; $[Pd(II)TMPyP]^{4+}$, 4.8×10^{-6} M. The IC_{50} values are comparable each other for metlalloporphyrin, but aqueous Au(III) in the combination with Alamar Blue has shown high toxic effects on the parasites. Toxicity of the mixture of Alamar Blue and Au(III) ion was also tested in *in vivo* with mice which were infected with 4.0×10^4 trypanosomes. The combination of 3.36×10^{-5} M of aqueous Au(III) and 1% Alamar Blue was effective to the infected mice and the combination was non-toxic to uninfected mice.

Alamar Blue is mainly resazurin, a fluorescent dye which is mainly responsible for showing metabolic activities of microorganisms through changing its color [17]. The dye mixture has been used for a long period as an indicator for quantitative measurement of proliferation of various types of cells, for example, human and animal cells, bacteria, mycobacteria, fungi etc. This is because of its non-toxic nature to cells as well as its higher stability in complex biological systems. Alamar Blue can also be used for toxicity test of chemicals in cells. However, as shown by the present study, Alamar Blue test indicated toxicity of Au(III) but Au(III) ion itself was not toxic. The combination of Au(III) ion and Alamar Blue cleaved DNA and killed parasite of *Trypanosoma brucei brucei*. If Alamar Blue test is carried out for screening toxicity of Au(III), we have to be careful when decide whether the chemicals are toxic or not.

Reaction Mechanism of Alamar Blue with Au(III)

Redox reaction

Because of high half-redox potential (0.380 V) of Alamar Blue compared to those of cytochrome oxidase (0.290 to 0.080 V) and NAD (-0.32 V), Alamar Blue is reduced in living cells [39]. However, the redox potentials of Au(III) ion (1.00 V $[AuCl_4]^-$/Au(s)) is higher than that of Alamar Blue [40]. Consequently the reduction of Alamar Blue is suppressed in combination with Au(III) ion, indicating the inhibition of proliferation of cells that suggests toxicity of Au(III). However, as described above Au(III) itself was not toxic.

DNA cleavage mechanism

The mixture of Alamar Blue and Au(III) ion generates ROS in the redox reaction system which are responsible for DNA cleavage. The formation of ROS was confirmed by using different scavengers on DNA cleavage. SOD and catalase inhibited DNA cleavage, suggesting the formation of $O_2^{-\bullet}$ and H_2O_2, respectively from the Alamar Blue-Au(III) redox reaction system. The DNA cleavage was not inhibited by ethanol and DMSO that suggests no formation of hydroxyl free radical ($^{\bullet}OH$). We also observed identical DNA cleavage by using Resazurin since it is main component of the Alamar Blue mixture [17]. Historically resazurin has been used as a chemical indicator to monitor contamination of milk by bacteria and yeast since 1950. The fluorescent dye, resazurin, has also been used for assessing semen quality [41].

Inhibition of Au(III) in vitro study using Alamar Blue

In vitro study was carried out to investigate the inhibition effect of the mixture of Alamar Blue and Au(III) on the growth of the *T. b. brucei* parasite. To carry out this test, the cultured T. b. brucei 427 parasites were grown in HMI-9 medium containing Alamar Blue with and without Au(III) ion and the mixtures were then incubated at 37 °C in 5% CO_2. The results indicated the toxicity of the combination of Alamar Blue and Au(III). It has been reported that mixture of HMI-9 and Au(III) ion generates radical that confirmed by ESR [15]. HEPES, a Good's buffering agent, used in HMI-9 medium to adjust solution pH which generates the radical, nitrogen-centered cationic free radical, in combination with Au(III) ion which confirmed by ESR [12,13]. Consequently, it is thought that the present Alamar Blue test by using parasites in HMI-9 medium similarly was inhibited by two kinds of radicals: one is nitrogen-centered cationic-free radical formed from HEPES involved in HMI-9 medium and Au(III) and the other is ROS formed from Alamar Blue and Au(III).

Conclusions

Alamar Blue test has been used for cell proliferation, however the test is interrupted inhibited in combination with the Au(III) ion. The interruption inhibition effect was ascribed by high redox potential of Au(III) and it was concluded that Au(III) was toxic. However, no toxicity effect in DNA cleavage was observed by only the Au(III) ion. The mixture of Alamar Blue and Au(III) ion cleaved DNA through the formation of reactive oxygen species (ROS). The mixture showed significant trypanocidal effect on trypanosomes in HMI-9 medium. In *in vivo* test, combination of 1% Alamar Blue and 3.36×10^{-5} M Au(III) was effective to the infected mice and was not toxic to uninfected mice.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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References

1. S.D. Aust, L.A. Morehouse and C.E. Thomas, Role of metals in oxygen radical reaction. *J. Free Radic. Biol. Med*. **1**, 3 (1985).
2. H. Bartsch and L. Tomatis, Comparison between carcinogenicity and mutagenicity based on chemicals evaluated in the IARC monographs**.** *Environ Health Perspect*. **47**, 305 (1983).
3. S. Ahmad and K.T. Kitchin, Plasmid DNA damage caused by methylated arsenicals, ascorbic acid and human liver ferritin**.** W.R. *Cullen, Toxicol. Lett*. **133**, 47 (2002).
4. IARC Working Group, In *Tobacco habits other smoking; betelquid and areca nut chewing; and some related nitrosamines*. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man. IARC, Lyon, **38**, p 392 (1985).
5. K. Sakano, M. Mizutani, M. Murata, S. Oikawa, Y. Hiraku and S. Kawanishi, Procyanidin B2 has anti- and pro-oxidant effects on metal-mediated DNA damage. *Free Radic. Biol. Med*. **39**, 1041 (2005).
6. M. Valko, H. Morris and M.T.D. Cronin, Metals toxicity and oxidative stress**.** *Curr. Med. Chem*. **12**, 1161 (2005).
7. M. Murata, T. Tezuka, S. Ohnishi, T. Takamura-Enya and Y. S. Hisamatsu, Carcinogenic 3-nitrobenzanthrone induces oxidative damage to isolated and cellular DNA. *Free Radic. Biol. Med*. **40**, 765 (2006).
8. U. Giri, M. Iqbal and M. Athar, Porphyrin-mediated photosensitization has a weak tumor-promoting activity in mouse skin: possible role of in situ– generated reactive oxygen species. *Carcinogenesis* **17**, 2023 (1996).

9. T. Kensler, K. Guyton, P. Egner, T. McCarthy, S. Lesko and S. Akman, Role of reactive intermediates in tumor promotion and progression. *Prog. Clin. Biol. Res*. **391**, 103 (1995).
10. N. E. Good, G. D. Winget, W. Winter, T. N. Connolly, S. Izawa and R. M. M. Singh, Hydrogen Ion Buffers for Biological Research, *Biochemistry*, **5**, 467 (1966).
11. J. K. Grady, N. D. Chasteen and D. C. Harris, Radicals from "Good's" buffers, *Anal. Biochem*., **173**, 111 (1988).
12. A. Habib and M. Tabata, Oxidative DNA damage induced by HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer in the presence of Au(III). *J. Inorg. Biochem*. **98**, 1696 (2004).
13. M. Tabata, A. Habib and K. Watanabe, DNA Cleavage by Good's Buffers in the Presence of Au(III)**.** *Bull. Chem. Soc. Jpn*. **78**, 1263 (2005).
14. E. Nyarko, T. Hara, D.J. Grab, M. Tabata and T. Fukuma, Toxic effects of mercury(II), cadmium(II) and lead(II) porphyrins on *Trypanosoma brucei brucei* growth. *Chemico-Biological Interact*. **139**, 177 (2002) 177-185.
15. E. Nyarko, T. Hara, D.J. Grab, A. Habib, Y. Kim, O. Nikolskaia, T. Fukuma and M. Tabata, In vitro toxicity of palladium(II) and gold(III) porphyrins and their aqueous metal ion counterparts on *Trypanosoma brucei brucei* growth. *Chemico-Biological Interact*. **148**, 19 (2004).
16. T.A. Salzman, A.M. Stella, E.A. Wider de Xifra, A.M. Batlle, R. Docampo and A.O. Stoppani, Porphyrin biosynthesis in parasitic hemoflagellates: Functional and defective enzymes in *Trypanosoma cruzi*. *Comparative BiochemPhysiol B: Comparative Biochem*. **72**, 663 (1982).
17. J. O'Brien, I. Wilson, T. Orton and F. Pognan, Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem*. **267**, 5421 (2000).
18. A.B. Stephen, K. Ruhi, G. Xiadong, A.R. Paul and J. Joseph, A new Alamar Blue viability assay to rapidly quantify oligodendrocyte death. *J Neurosci Methods* **91**, 47 (1999).
19. H. Gloeckner, T. Jonuleit and H.D. Lemke, Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue™. *J. Immunol. Methods* **252**, 131 (2001).
20. G.R. Nakayama, M.C. Caton, M.P. Nova and Z. Parandoosh, Assessment of the Alamar Blue assay for cellular growth and viability in vitro. *J. Immunol. Methods* **204**, 205 (1997).
21. S.L. Voytik-Harbin, A.O. Brightman, B. Waisner, C.H. Lamar and S.F. Badylak, Application and evaluation of the Alamar Blue assay for cell growth and survival of fibroblasts. *In Vitro Cell Dev. Biol. Anim*. **34**, 239 (1998).
22. M.L. Sykes and V.M. Avery, Development of an Alamar Blue viability assay in 384-well format for high throughput whole cell screening of *Trypanosoma brucei brucei* bloodstream form strain 427. *Am. J. Trop. Med. Hyg*. **81**, 665 (2009).
23. T.M. Buttke and J.A. Mccubrey, Use of an aqueous soluble tetrazolium/formazan assay to measure viability and proliferation of lymphokine-dependent cell lines. T.C. Owen, *J. Immunol. Methods* **157**, 233 (1993).
24. M.B. Hansen, S.E. Nielsen and K. Berg, Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* **119**, 203 (1989).
25. D. Barnes and G. Sato, Serum-free cell culture: a unifying approach. *Cell* **22**, 649 (1980).
26. R.I. Freshney, In *culture of animal cells, A manual of basic technique*, New York, (1983).
27. P.F. Kruse, In *tissue culture, methods and applications*, Academic Press, New York (1973).
28. A. Habenicht, In *Growth factors, differentiation factors and cytokines*, Springer, Berlin (1990).
29. F.R. Balkwill and F. Balkwill, In *Cytokine molecular biology: A practical approach*, Oxford University Press, London (2000).
30. J.S. Heilig, In *Current Protocols in Molecular Biology, Large-Scale Preparation of Plasmid DNA*: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl (Ed), Greene Publishing Associates and Wiley-Interscience, New York, **1**, p. 1.7.1. (1987).
31. E.J. Gibbs, M.C. Maurer, J.H. Zhang, W.M. Reiff, D.T. Hill, M. Malicka-Blaszkiewicz, R.E. McKinnie, H.Q. Liu and R.F. Pasternack, Interactions of porphyrins with purified DNA and more highly organized structures. *J. Inorg. Biochem*. **32**, 39 (1988).
32. S. Igarashi, H. Suzuki and T. Yotsuyanagi, The equilibrium constants of cadmium(II), lead(II), magnesium(II), zinc(II)-α,β,γ,δ-tetrakis(1-methylpyridium-4-yl)porphinecomplxes. *Talanta* **42**, 1171 (1995).
33. P. Hambright, Chemistry of water soluble porphyrins. In: K.M. Kadish, K.M. Smith, R. Guilard (ed), The Porphyrin Handbook, Academic Press, San Diego, CA, vol. 3, pp. 129-210 (2000).

34. Z. Abou-Gamra, A. Harriman and P. Neta, Redox chemistry of gold(III)porphyrins in water. *J. Chem. Soc. Farady Trans* **82**, 2337 (1986).
35. A. Harriman, M.C. Richoux and P. Neta, Redox chemistry of metalloporphyrins in aqueous solution. *J. Phys. Chem*. **87**, 4957 (1983).
36. H. Hirumi and K. Hirumi, Axenic culture of African trypanosome blood stream forms. *Parasitol. Today* **10**, 80 (1994).
37. R. Kaminsky and R. Brun, In vitro assays to determine drug sensitivities of African trypanosomes: a review. *Acta Tropica* **54**, 279 (1993).
38. M. Hills, C. Hudson and P.G. Smith, In *Global monitoring of the resistance of malarial parasites to drug: statistical treatment of micro-test data, WHO Working Paper No. 2.8.5. for the informal consultation on the epidemiology of drug resistance of malaria parasites*, Geneva (1986).
39. S.N. Rampersad, Multiple applications Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassay. *Sensors* **12**, 12347 (2012).
40. A.J. Bard, R. Parson and J. Jordan, In *Standard Potentials in Aqueous Solution, International Union of Pure and Applied Chemistry*, Marcel Dekker Inc, New York (1985).
41. R.E. Erb and M.H. Ehlers, Resazurin reducing time as an indicator of bovine semen capacity, *J. Dairy Sci*. **33**, 853 (1950).