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TOXICITY OF HEMOGLOBIN DERIVATIZED WITH OXIDIZED ADENOSINE TRIPHOSPHATE AGAINST TUMORAL HUMAN CELLS

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ABSTRACT. Adenosine triphosphate (ATP) has a high affinity for the organic phosphate binding site of hemoglobin (Hb), affecting the affinity for oxygen. Periodate-oxidized-ATP (oATP) behaves as an affinity tag for several enzymes that use ATP as a substrate and has the ability to form intermolecular aldimine bonds by reaction with free amino groups on proteins. Due to its unique structure, oATP has been used to produce structural and functional modifications of Hb to obtain a compound with a low affinity for oxygen that could be used as a blood substitute. However, the oATP-Hb derivative was shown to present a notable increase in pro-oxidative reactivity compared to Hb *in vitro*, and accordingly exhibited toxicity in *in vivo* studies. This pro-oxidant reactivity was alleviated by crosslinking Hb with bovine serum albumin (BSA) in an oATP-Hb-BSA copolymer. We now show that oATP and oATP-Hb-BSA display high-affinity toxicity towards cancer cells, and may hence deserve further investigation as adjuvants in anti-cancer therapy.

Keywords: *hemoglobin, cells, oATP, blood substitute, cancer*

INTRODUCTION

Red blood cells (erythrocytes) are uniquely suited to the task of oxygen transport for several important reasons. Firstly, these cells contain a high concentration of hemoglobin (35 g/dL), capable of transporting 47 mL O_2 /100 mL

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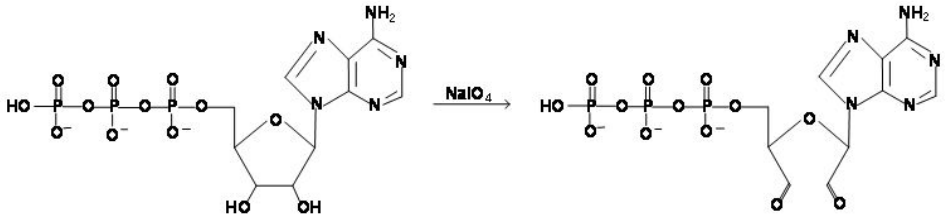
of red cells [1]. The resulting overall oxygen binding capacity of the erythrocytes is about 18 mL/dL [2]. Secondly, erythrocytes are deformable; they can therefore efficiently diffuse through capillary vessels, thus providing a reasonable diffusion distance of oxygen from the alveolar space of the lung to hemoglobin, or from hemoglobin to cells. Thirdly, hemoglobin binds oxygen cooperatively and is under the control of the Bohr effect: local pH conditions and carbon dioxide affect the oxygen-binding behavior of hemoglobin in ways favorable for oxygen transport [3].

Hemoglobin (Hb) is a tetrameric protein with a diameter of 5.5 nm containing four prosthetic heme groups. Adult hemoglobin contains two types of subunits - two α-chains (consisting of 141 amino acids each) and two β-chains (consisting of 146 amino acids each) [4]. The three-dimensional structures of the two subunits are very similar, and the quaternary structure shows strong interactions between the subunits [2]. Hemoglobin must efficiently bind oxygen in the lungs, where pO_2 is around 13.3 kPa, and release oxygen to the tissues, where pO_2 is 4 kPa. Oxygen binding to the heme Fe in human Hb is modulated by the effector BPG (2,3-bisphosphoglycerate). In addition to BPG, the ability of Hb to release O_2 is also determined by other metabolites or anions in the intercellular compartment: H^+ , NO, adenosine triphosphate (ATP), Cl^- , CO_2 [5].

The development of a synthetic or semi-synthetic blood substitute has for decades been an active research area, aiming to reduce the shortage of blood needed for transfusions. Blood substitutes would have advantages over human blood such as accessibility, validity and are free of risk of infection. There are currently two main categories of blood substitutes under development: protein (especially hemoglobin, but also hemerythrin)-based oxygen carrier molecules (HBOCs) and fluorocarbonate-based carrier molecules [6].

Free hemoglobin is lethal because of redox activity, filtration in the kidneys, affinity for oxygen or NO dioxygenase reactivity; if reactivity of this type is controlled or reduced, Hb becomes the ideal candidate for a blood substitute. In order to achieve this function Hb undergoes a series of modifications/transformations such as: intra- and intermolecular cross- linking, polyethylene glycol derivatization, genetic modification, or encapsulation [7]. The first generation of polymers (Hb polymerized with glutaraldehyde, conjugated Hb or Hb cross-linked with oxidized adenosine triphosphate, oATP) exhibit renal toxicity, as well as other side-effects. Clinical studies have shown toxicity to the body, and these substitutes are no longer used - except for the glutaraldehyde-polycondensed Hb currently approved for human use in South Africa and Russia. The second generation of products (substitutes obtained by recombination and genetic modification) are based on a better understanding of vasoconstriction and have lower toxicity [8,9].

One of the crosslinking agents previously employed for Hb derivatization is the dialdehyde obtained by oxidation of adenosine triphosphate (ATP) with periodate, cf. Scheme 1. The two aldehyde groups allow condensation with amino groups on the surface of Hb.



Scheme 1. Oxidation of adenosine triphosphate (ATP) by treatment with periodate.

The oATP-Hb derivative of hemoglobin was previously shown to present a notable increase in pro-oxidative reactivity compared to Hb *in vitro*, and accordingly exhibited toxicity in *in vivo* studies. This pro-oxidant reactivity was alleviated by crosslinking Hb with bovine serum albumin (BSA) in an oATP-Hb-BSA copolymer. Better performance *in vivo* was seen for the Hb-BSA copolymer when glutaraldehyde was employed as crosslinker instead of oATP. As a result, neither oATP-Hb nor oATP-Hb-BSA are currently pursued as viable blood substitute candidates [10–12]. We now report that oATP and oATP-Hb-BSA in fact display high-affinity toxicity towards cancer cells, and may hence deserve further investigation as adjuvants in anti-cancer therapy.

RESULTS AND DISCUSSION

The pro-oxidant reactivity of Hb has been argued to be a key factor responsible not only for a number of physio-pathological conditions, but also for the biological side-effects of Hb-based blood substitute candidates [4,12,21–27,13–20]. Such reactivity is enhanced under low-oxygen conditions [28–30]. Such hypoxic conditions are typical for tumors; the question may then be asked, whether the inherent pro-oxidative capacity of Hb-based blood substitute candidates may be turned into an advantage by deploying such molecules against tumors. To this, end, Hb derivatized with oATP (Hb-oATP) and the corresponding copolymer with BSA (Hb-BSA-oATP) are tested here against two lines of tumoral cells.

The cells used for these tests are DLD-1 and HT-29 - human colon tumour cells. As shown in Figure 1, he IC_{50} (half inhibitory concentration) for DLD-1 cells treated with Hb-oATP is 9.5 μM, while that for Hb-BSA-oATP is

26.0 μM. These values reflect a clear inhibitory activity on DLD-1 tumor cell growth.

As shown in Figure 2, the IC_{50} on the HT-29 cell line for Hb-oATP is 11.8 μM, while that of Hb-BSA-oATP is 16.7 μM. These values may be interpreted to reflect an efficient inhibitory activity on the growth of HT-29 tumor cells.

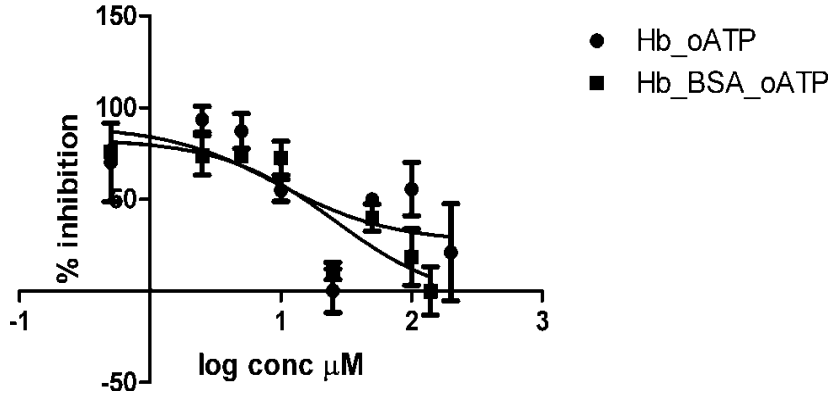


Figure 1. Sigmoidal dose-response curves representing DLD-1 tumor cell viability 24 hours after treatment with Hb-oATP and Hb-BSA-oATP.

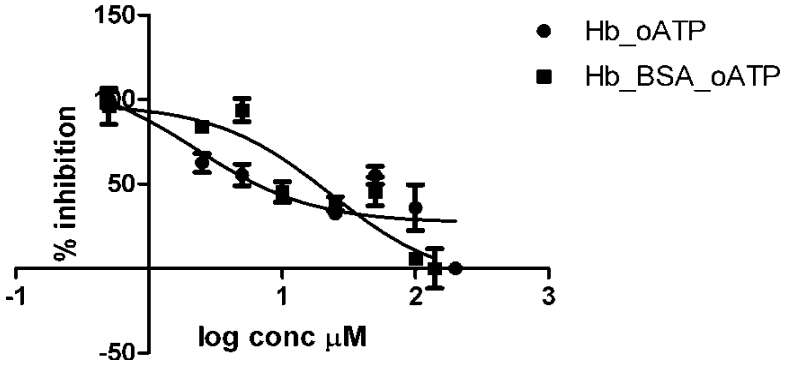


Figure 2. Sigmoidal dose-response curves representing the viability of HT-29 tumor cells 24 h after treatment with Hb-oATP and Hb-BSA-oATP.

In both of the tested tumoral cell lines the IC_{50} values suggest that Hb-oATP has a slightly better cytotoxicity. Concentrations close to the IC_{50}, or even ten times higher, can easily be achieved in a patient by injecting relatively small amounts of oATP-derived hemoglobin - i.e. concentrations that

would not invoke the side reactions otherwise known in some blood substitute candidates [4,12,21–27,13–20]. This suggests a possible utility of Hb-oATP as an active component for anti-tumor therapy. Although at 100 μM both Hb derivatives display an almost 100 % degree of inhibition, it is not expected that they would be efficient as anti-tumoral agents on their own / alone.

Of the two candidates examined here, further tests were performed using the BSA copolymer on normal / healthy human cells, since (as detailed above) this substance was previously shown to elicit less side-effects during *in vivo* experiments. These additional tests were performed on healthy isolated peripheral human blood lymphocytes using Ficoll-Paque tubes. Several cell types were used for testing: CD4 positive and CD8 positive T lymphocytes, CD19 positive B lymphocytes and CD14 positive monocytes. These cells were treated with Hb-BSA-oATP at a concentration of 100 μM in cell medium and cell survival was assessed 24 h after treatment using the MTT viability assay. As shown in Figure 3, after treatment with Hb-BSA-oATP, lymphocyte cell viability was slightly altered, in CD4+ and CD14+ cells cell viability decreased, while in CD19+ B cells it was observed that treated cells had better viability than untreated ones (Anova test, Bonferroni post-test, $p<0.05$). No influence was detected on the CD8+ population. Notably, overall these effects were minor and far smaller than the degrees of magnitude seen in Figures 1 and 2 for tumoral cells.

Measurements on cytokine signaling molecules in cells shown in Figure 3 after exposure to Hb-BSA-oATP treatment did reveal measurable changes, with pro- as well as anti-inflammatory potential. This suggests a possible practical utility in further exploring the molecular immunology responses behind the interaction of oATP-derived Hb with tumoral as well as with healthy cells.

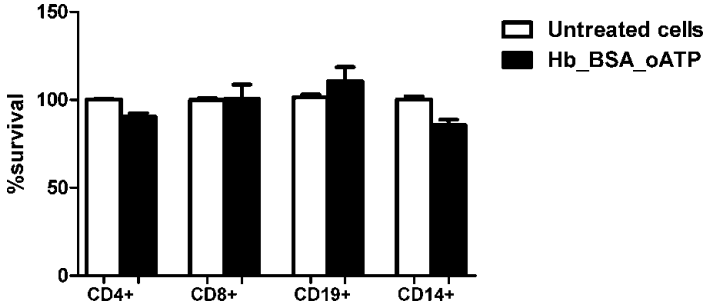


Figure 3. Influence of Hb-BSA-oATP on human cells *in vitro* after 24 h post exposure: survival of CD4 positive and CD8 positive T lymphocytes, CD19 positive B lymphocytes and CD14 positive monocytes monitored 24 h post treatment was followed.

CONCLUSIONS

Following tests on two colon tumor cell lines, it can be stated that Hb-oATP as well as Hb-BSA-oATP display cytotoxicity (slightly stronger for the former than for the latter) against tumoral cell lines. Following treatment of peripheral mononuclear cell subpopulations with oATP-derived hemoglobin in the presence of antioxidant (BSA), cell viability was followed with the MTT assay. In T lymphocytes and monocytes viability was maintained, the reduction being statistically insignificant, and in B lymphocytes the treatment induced a slight increase in viability. These findings may warrant further investigations on possible therapeutic perspectives of oATP-derivatives of Hb in antitumor therapy, as well as further investigation of the cellular response mechanisms towards these and other Hb derivatives previously proposed as blood substitute candidates.

EXPERIMENTAL SECTION

The following substances were used to conduct the experiments: bovine hemoglobin (Hb), purified as previously described [1], adenosine triphosphate (ATP), sodium periodate, glutaraldehyde, sodium borohydride were obtained from Sigma-Aldrich (Munich, Germany) and used as received. The human cell lines were manipulated and treated as previously described (including the respective ethical visas) [18,22,27,31–33]. The Hb-oATP polymer and Hb-BSA-oATP copolymer stock solutions were produced as previously described [16,34]. A Hanna pH 212 pH meter (Hanna Instruments, Italy) was used to monitor and correct the pH value of the solutions, generally adjusted with 20% hydrochloric acid or sodium hydroxide. The concentrations of Hb solutions were determined using a Cary 50 UV-vis spectrophotometer (Varian, Inc., Foster City, CA, USA).

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