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6-Amino-5-Bromouracil Delay the Onset of Toxicity of *Cerastes cerastes* Venom in Albino Mice

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ABSTRACT

Snake bite is a severe medical emergency in North Africa region and other parts of the world. When venom injected into the victim after the bite causes grave debilitating and life-threatening effects eventually resulting in mortality and morbidity. Snake venom phospholipases A_2 (PLA_2) have local and systemic actions that induce pathophysiological effects in the victim. 6-Amino-5-bromo-1H-pyrimidine-2,4-dione (6-amino-5-bromouracil) is a derivative of uracil and has an inhibitory effect on many enzymes. Molecular docking and fluorescence studies were performed in order to determine the potential modes of action of 6-amino-5-bromouracil with phospholipases A_2. 6-Amino-5-bromouracil showed hydrogen bonds with an active site of enzyme and changes in the fluorescent spectrum of PLA_2. The achieved results revealed that 6-amino-5-bromouracil treated animals have shown to increase the mean survival time and the protection fold, but could not protect mice from death when used alone. The use of 6-amino-5-bromouracil could lead to prevent the snake venom toxicity and further chemical synthesis analogues and *in vivo* studies would be necessary to substantiate the obtained results.

Keywords: Snake bite, *Cerastes cerastes*, 6-amino-5-bromouracil, Fluorescence, Phospholipase A_2, Molecular docking

Abbreviations: PLA_2: Phospholipases A_2; 6A5B: 6-Amino-5-Bromouracil

INTRODUCTION

Snakebite is a foremost public health problem in numerous African countries including Libya, Algeria Egypt and Tunisia [1,2]. It is a meticulous confront, even though, in some parts of Africa, which is home for more than 400 snake species, of which about 30 venomous species, belonging to four snake families namely: Colubridae, Viperidae, Elapidae, Atractaspididae and they are appeared to cause human deaths, as reported by the World Health Organization [2]. *Cerastes cerastes* is one of the snakes commonly threatening human life in Libya. The *Cerastes cerastes* venom is containing numerous enzymes that have proteolytic activity and leads to multiple kinds of intoxications [3]. The lethal cause of snake venom mainly results from its active components mainly PLA_2. *Cerastes cerastes* snake venoms cause proteolysis, shock, blood clotting, release of bioactive substances such as bradykinin and histamine, necrosis, hemorrhage and numerous other effects [4-6]. Necrosis might result from a direct action of myotoxins or myotoxic PLA_2s on plasma membranes of muscle cell or indirectly, as a consequence of blood vessel degeneration and ischemia caused by hemorrhaging. PLA_2s interact with membrane phospholipid components which lead to promote release of intracellular creatine kinase, which might be used as a biomarker for myotoxic activity assessment [7,8]. The snake venom physiopathological processes encouraged researchers to obtain natural or chemically synthesised inhibitors.

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Some PLA_2 inhibitors may be found in different organisms. Manoalide (A) is a non-steroidal sesquiterpenoid from the marine sponge *Luffariella variabilis*, while manoalide (B) was synthetically synthesized based on its natural analogue. These terpenoids compounds have irreversible inhibitory effects on numerous PLA_2s obtained from snakes [9,10]. The phospholipase A_2 (PLA_2) activity can be inhibited by some inhibitors as reported in the literature who have found that a chemical compound called varespladib and its orally bioavailable prodrug, methyl-varespladib had an inhibitory effect on PLA_2 ($sPLA_2$) at nanomolar and picomolar concentrations against 28 medically *vital* snake venoms from six continents [11]. The aim of the paper was to study the inhibitory effect of 6-amino-5-bromouracil on phospholipase A_2 enzyme to treat snake envenomation using molecular docking and fluorescence studies.

MATERIALS AND METHOD

Preparation of aqueous 6-amino-5-bromouracil solution

A weight of 19 mg from 6-amino-5-bromouracil (Matrix Scientific, Columbia, and Catalog Number 078889) was dissolved in 100 µl DMSO and the solution was completed to 5 ml with 0.9% sodium chloride to give a final stock solution of 18.5 mM and stored at –20°C until use.

Venoms

Snake (*Cerastes cerastes*,) venom was extracted by manual stimulation and were obtained in liquid forms, from the Department of Zoology, Faculty of Science, University of Tripoli (Libya) and stored at –20°C until use. An aliquot of 7.5 µl venom from the venoms was added to 800 µl of normal saline. A dose of 100 µl (100 ng) was intraperitoneally injected in 18 ± 2 g male Swiss Albino mice.

Molecular docking

The starting geometry of the 6-amino-5-bromouracil was constructed using chem3D Ultra (version 8.0, Cambridge soft Com., USA). The optimized geometry of 6-amino-5-bromouracil with the lowest energy was used for molecular docking. Crystal structures of the crystal structure of cobra-venom phospholipase A_2 in a complex with a transition-state analogue (1POB) was downloaded from the Protein Data Bank https://www.rcsb.org/structure/1POB. Molecular dockings of 6-amino-5-bromouracil with 1POB was accomplished by Auto Dock 4.2 software from the Scripps Research Institute (TSRI) (http://autodock.scripps.edu/). Firstly, polar hydrogen atoms were added into protein molecules. Then, partial atomic charges of the phospholipase enzymes and 6-amino-5-bromouracil molecules were calculated using Kollman methods [12]. In the process of molecular docking, the grid maps of dimensions (62 Å × 62 Å × 62 Å) with a grid-point spacing of 0.376 Å and the grid boxes are centered. The number of genetic algorithm runs and the number of evaluations were set to 100. All other parameters were default settings. Cluster analysis was performed on the results of docking by using a root mean square (RMS) tolerance of 2.0 Å, which was dependent on the binding free energy. Lastly, the dominating configuration of the binding complex of 6-amino-5-bromouracil and phopholipase A_2 enzyme fragments with minimum energy of binding were determined which relied strongly on the information of 3D structures of the phopholipase A_2 binding site and ultimately generated a series of phopholipase binding complex.

Absorbance spectra

Absorbance spectra were measured on a Jenway UV-visible spectrophotometer, model 6505 (London, UK) using quartz cells of 1.00 cm path length. The UV-Vis absorbance spectra were recorded in the 200-500 nm range and spectral bandwidth of 3.0 nm. For the final spectrum of each solution analyzed baseline subtraction of the buffer solution was performed. The protein content of venoms samples was determined by the spectrophotometric method as described [13]. Bovine serum albumin (BSA, Sigma) was used for standard assays.

Fluorescence spectra

Fluorescence emission and excitation spectra were measured using Jasco FP-6200 spectrofluorometer (Tokyo, Japan) using fluorescence 4-sided quartz cuvettes of 1.00 cm path length. The automatic shutter-on function was used to minimize photo bleaching of the sample. The selected wavelength chosen provided aggregate excitation of tryptophan and tyrosine residues. The emission spectrum was corrected for background fluorescence of the buffer. The changes of fluorescence emission intensity and fluorescence shifts were monitored in which the formation of the system was formed by sequential addition of aliquots of Tris buffer, *Cerastes cerastes* venom and finally 6-amino-5-bromouracil.

Experimental animals

Swiss Albino male mice (18 ± 2 g) were used for the experiments. In order to reduce the contact caused by environmental alterations and handling during behavioral studies, mice were acclimatized to the Laboratory Animal Holding Center and laboratory surroundings for three days and at least 1 h before the experiments, respectively. Mice were kept under standard conditions with food (low protein diet) and water available *ad libitum*. The animals were housed six per cage in a light-controlled room (12 h light/dark cycle, light on 07:00 h) at 27°C and 65% relative humidity. All experiments were carried out between 11:30 and 14:00 h. Each test group consisted of at least six mice and each mouse was used only once. All animal experiments were conducted according to guidelines set by Institutional Animal Ethics Committee of University of Tripoli. Five groups of mice were used in this study. The first group of six mice received only 100 µl (100 ng of total protein) of the *Cerastes cerastes* venom (LD99 5 µg/kg). Groups 2-4 of six mice each (serving as treatment groups) were given an

equivalent amount of the *Cerastes cerastes* venom with 100 µl, 200 µl and 300 µl of 6-amino-5-bromouracil (stock 18.5 mM) solution, respectively. Group 5 of six mice received 100 µl of the *Cerastes cerastes* venom and ASV. The number of mortality was recorded within 24 h.

Calculation of LD99 of *Cerastes cerastes* venom

The median lethal dose (LD99) of *Cerastes cerastes* venom was determined according to the previously developed method [14,15]. A range of doses of venom in 800 µl of physiological saline was injected intraperitoneally using groups of six mice for each venom dose. The LD99 was calculated with the confidence limit at 99% probability by the analysis of mortality occurring within 24 h of venom injection. The anti-lethal potentials of 6-amino-5-bromouracil (stock 18.5 mM) solution were determined against LD99 of *Cerastes cerastes* venom.

STATISTICAL ANALYSIS

The difference among various treated groups and control group were analysed using one-way-ANOVA followed using unpaired Student's t test. The results were expressed as the mean ± SEM of the number of experiments done, with $P<0.05$ indicating significant difference between groups.

RESULTS AND DISCUSSION

Molecular docking analysis

Table 1 shows the binding energies of 6-amino-5-bromouracil, Gel and cobra-venom phospholipase A_2 (1POB) obtained by the molecular docking strategy. In this study, molecular dockings of the 6-amino-5-bromouracil and Gel with cobra-venom phospholipase A_2 (1POB) were performed using Auto Dock 4.2 to investigate the binding mode of 6-amino-5-bromouracil and Gel with cobra-venom phospholipase A_2 (1POB) and to obtain information about interaction forces between 6-amino-5-bromouracil and cobra-venom phospholipase A_2 (1POB). 6-Amino-5-bromouracil, gel and cobra-venom phospholipase A_2 (1POB) was kept as flexible molecules and were docked into seven forms of rigid phospholipase A_2 to obtain the preferential binding site to 6-amino-5-bromouracil and gel on phospholipase A_2. The molecular docking results are shown in **Table 1**. The modeling studies showed that there is *van der Waals*, hydrogen bonding and electrostatic interactions between 6-amino-5-bromouracil and gel with phospholipase A_2. The contribution of *van der Waals* and hydrogen bonding interaction is much greater than that of the electrostatic interaction because the sum of *van der Waals* energy, hydrogen bonding energy and desolvation free energy is larger than the electrostatic energy, which is consistent with the literature [16,17]. The 6-amino-5-bromouracil, gel and cobra-venom phospholipase A_2 (1POB) interactions are shown in **Figure 1**. 6-Amino-5-bromouracil showed a good binding energy (-5.52 kcal/mol) when compared to standard gel (-4.26 kcal/mol) as mentioned in **Table 1**. **Figure 1** shows four hydrogen bonds between 6-amino-5-bromouracil and cobra-venom phospholipase A_2 while gel shows six hydrogen bonds with cobra-venom phospholipase A_2. In addition, 6-amino-5-bromouracil showed good docking interaction with the cobra-venom phospholipase A_2 binding site (GLY31, TYR63 and ASP48) **(Figure 1)** and similarly Gel showed good docking interaction with the cobra-venom phospholipase A_2 binding site (ARG30, GLY29 and ASP48). The interaction of both ligands (6-amino-5-bromouracil and Gel) with the cobra-venom phospholipase A_2 binding site of the enzyme is essential for effective inhibition as previously reported for gel [18,19]. Therefore, 6-amino-5-bromouracil and gel may be considered as the effective phospholipase A_2 inhibitor.

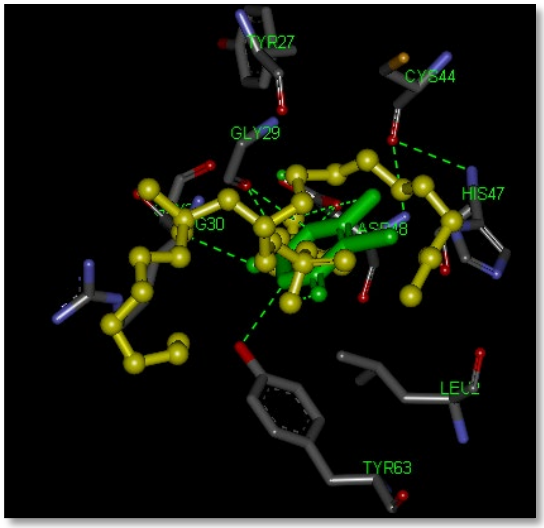
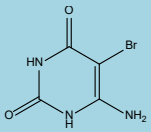
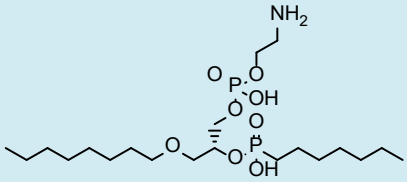


Figure 1. Shows the interaction model between 6-amino-5-bromouracil and gel with cobra-venom phospholipase A_2 (1POB) active site. 6-Amino-5-bromouracil molecule was shown in green color while the gel molecule in yellow color. The hydrogen bonds are represented using green broken line.

Table 1. Various energies in the binding process of 6-amino-5-bromouracil and gel and cobra-venom phospholipase A_2 (1POB) obtained from molecular docking. The unit of all energies (ΔG) is kcal/mol.

Compounds	**Hydrogen bond formation between Cobra-venom phospholipase A_2 (1POB) with 6A5Bu and gel**	**Hydrogen bond distance Å**	**Donor atom**	**Acceptor atom**	**Binding energy (ΔG) kcal/mol**
6-amino-5-bromopyrimidine-2,4(1H,3H)-dione or 6-amino-5-bromouracil (6A5BU) 	GLY31:HN of **1pob** and O10 of 6A5BU H2 of 6A5BU and OH of **1pob** TYR63 H5 of 6A5BU and OH of **1pob** ASP48 H3 of 6A5BU and OH of **1pob** ASP48	1.75938 1.76047 1.64556 2.29695	HN H2 H5 H3	O10 OH OH1 OH1	-5.52
[(2R)-1-[2-aminoethoxy(hydroxy)phosphoryl]oxy-3-octoxypropan-2-yl]oxy-heptylphosphinic acid (Gel) 	ARG30:HN of **1pob** and O15 of gel ARG30:HE of **1pob** and O15 of gel ARG30:HH of **1pob** and O14 of gel ARG30:HH21 of **1pob** and O15 of gel H32 of gel and O of **1pob** GLY29 H33 of gel and OH1 of **1pob** ASP48	2.03948 2.25903 2.39906 2.49538 2.25109 2.11707	HN H HH21 HH21 H32 H33	O15 O15 O14 O15 O OH1	-4.26

Fluorescence spectra and 6-amino-5-bromouracil

Fluorescence spectroscopy has proved to be helpful in studies on ligand binding. Steady-state fluorescence quenching and fluorescence polarization are the primary techniques for studying structure and function of proteins [20]. The fluorescence spectrum shows a decrease of fluorescence intensity **(Figure 2)** of the snake venom due to addition of 105 µM of 6-amino-5-bromouracil which could be related to various processes. It is well known that a decrease in fluorescence intensity can be caused by a range of molecular interactions such as molecular rearrangements, excited-state reactions, ground state complex formation, collisional quenching or energy transfer. The decrease in fluorescence emission intensity as shown in **Figure 2** was not accompanied any shift which may indicate that Trp residues buried in a hydrophobic environment have moved into a relatively polar environment consistent with earlier reports [21]. The decrease in fluorescence emission intensity **(Figure 2)** was accompanied was not accompanied by any shift and this may indicate that binding of 6-amino-5-bromouracil may have accomplished a conformational change that moves tryptophan into a relatively more

hydrophobic region. This explanation is consistent with Gorbenko et al. [22] who found tryptophan fluorescence is quenched by interactions with polar ligands. Binding of proteins to lipid membranes decreases ease of access to these polar ligands and consequently decreases the obtained quenching effects.

The obtained fluorescence quenching with 6-amino-5-bromouracil refers to the process that decreases the fluorescence intensity of the snake venom. Snake venom show fluorescence and the intrinsic protein fluorescence is due to aromatic amino acids, mainly tryptophan, considering that phenylalanine has a very low quantum yield and emission by tyrosine in native proteins is often quenched. The fluorescence of the snake venom is mainly due to tryptophan of phospholipase, which can be selectively measured by exciting at 295 nm, because there is no absorption by tyrosine at this wavelength. Tryptophan fluorescence is extremely responsive to the environment polarity and shifts in its emission spectrum toward slower wavelengths (blue shift) can be observed as the increased hydrophobicity [23,24]. Changes in emission spectra from tryptophan can be seen in response to snake venom phospholipase conformational transitions, subunit association, 6-amino-5-bromouracil binding or denaturation, which affect the environment surrounding the indole ring of tryptophane. In addition, the quenching reaction obtained can be used not only to probe topological features of the phospholipase structures, but also to follow protein conformation changes that affect accessibility to tryptophan. It is reported that any treatment of the native protein that involves a change in the tryptophan environment can be followed by fluorescence quenching **(Figure 2)** [25,26].

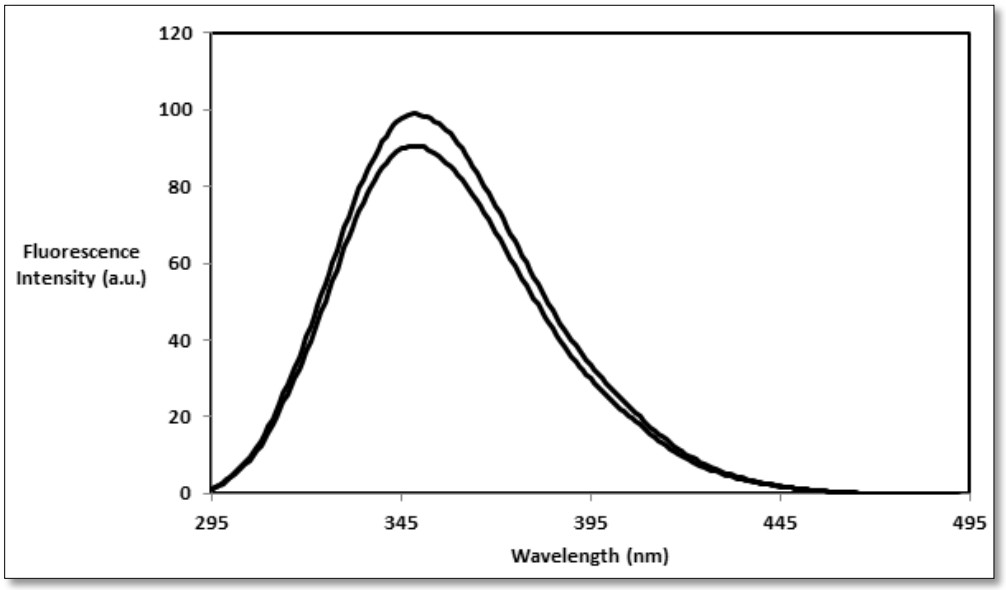


Figure 2. (A) Plot of fluorescence emission of snake venom (*Cerastes cerastes*) (24.6 µg/ml) *vs.* wavelength from 288-540 nm using excitation of λ_{280} nm in 0.01 M Tris, 0.1 M NaCl at pH 7.4. (B) Fluorescence perturbation of snake venom by addition of 105 µM 6-amino-5-bromouracil. Spectra were corrected for small background fluorescence contributions from the buffer solution and were scaled to visualize the shift.

Calculation LD99 of *Cerastes cerastes* venom

Lethality data of *Cerastes cerastes* venom was calculated. The LD99 of *Cerastes cerastes* venom from this study was 5 µg/kg as reported previously [14].

Acute toxicity of *Cerastes cerastes* venom and its neutralization by 6-amino-5-bromouracil and anti-venom

The *Cerastes cerastes* venom at the dose 5 µg/kg (LD99) produces 100% mortality in mice. The 6-amino-5-bromouracil significantly increases the mean survival time up to 6.3 ± 0.23 h. The 6-amino-5-bromouracil when used at the dose of 100 µl (stock 18.5 mM) solution was found to be more effective against *Cerastes cerastes* venom (4.8 h) when compared with 6.2 h produced by 300 µl. ASV (polyvalent anti-snake venom by Haffkine Bio-Pharmaceuticals Company (India)) was found to be more effective as compared with the aqueous 6-amino-5-bromouracil showing mean survival of two days for five mice and complete survival of one mouse and was consistent to our previously published work [14].

The pharmacological effects of *Cerastes cerastes* venoms could be classified into three main types, neurotoxic, hemotoxic [27-29] and cytotoxic. The main toxin related to these effects is PLA_2s, which is responsible for many pharmacological effects happening in snakebite victims. The PLA_2s is able to operate on pre- or post-synaptic junctions as antagonist of ion channels and muscarinic or nicotinic

receptors to persuade cruel neurotoxicity such as paralysis and respiratory failure [29,30]. In addition PLA_2s can cause local tissue damage resulting in blistering, swelling, necrosis, and bruising. In addition it has systemic effects such as hypovolemic shock; encourage hemostatic and cardiovascular effects as coagulopathy, hemorrhage and hypotension. It is also reported that PLA_2s are able of triggering severe pain [31,32].

Because phospholipase A_2 (PLA_2) activity is a significant part of venom toxicity, it has been required candidate PLA_2 inhibitors by directly testing drugs. It was observed that 6-amino-5-bromouracil when given to the mice after they received snake venom of *Cerastes cerastes* venom significantly increased mean survival time and the results were found to be better when it was used at higher dose 300 µl (stock 18.5 mM) of 6-amino-5-bromouracil solution. This could be possible due to interaction of active venom components mainly PLA_2 with 6-amino-5-bromouracil which is consistent with the result obtained by molecular docking.

In the literature there are few small molecules can inhibit PLA_2 of the snake venom and delay the toxicity of the snake venom which can support the obtained results in this paper [33,34]. It has been reported that Varespladib and methyl-varespladib (it's orally bioavailable prodrug) were able to delay the effects of twenty eight medically important snake venoms from six continents but not for long time. Also, it has been reported that varespladib and methyl-varespladib were able to suppress host response safely and are more effective against snake venom PLA_2 (picomolar concentrations) than against mammalian sPLA2 [11]. They were able to perform protection against the injurious effects of hemorrhage, hemolysis and other tissue obliteration [35].

Results obtained by using 6-amino-5-bromouracil are not very astonishing and are in agreement with some previous studies [36-38], as one pyrimidine-2,4,6-trione has been published as a promising efficient and selective inhibitor of cell matrix metalloproteases [37,38]. Other studies have also revealed the efficiency of pyrimidine-2,4,6-trione derivatives, mainly one named RO 28-2653 developed by Hoffman-La Roche research group, in anticancer therapy [39,40]. However, 6-amino-5-bromouracil has not ever been pointed as PLA_2 inhibitor or been used in the neutralization of snake venom enzymes.

CONCLUSION

Rapid development and use of a broad-spectrum PLA_2 inhibitor alone or in combination with other small molecule inhibitors of snake toxins might fill the dangerous therapeutic gap spanning pre-referral and hospital setting. There is an urgent need for economical, stable and effective snakebite treatments that can be used in places where medical access is limited. 6-Amino-5-bromouracil has ability to delay the snakebite envenomation make it reasonable candidates for consideration of clinical trials and warrant further examination by skilled practitioners and basic researchers in the field of snakebite. Further elaborative work is necessary for the better understanding of the mechanism of venom inhibition. We expect that the results presented herein can motivate future efforts in finding potent pyrimidine-2,4(1H,3H)-dione derivatives that can be used for snake venom phospholipases A_2 inhibition *in vivo*.

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