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Research Article

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

Halima A Gbaj[1], Inass A Sadawe[1], Nisreen H Meiqal[1], Salah M Bensaber[1], Abdulathim A A Alshoushan[2], Massaud Salem Maamar[3], Anton Hermann[4] and Abdul M Gbaj[1]*

[1]*Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, Libya*

[2]*Food and Drug Control Centre (LFDA), Tripoli, Libya*

[3]*Zoology Department, Faculty of Science, Tripoli University, Libya*

[4]*Department of Biosciences, University of Salzburg, Salzburg, Austria*

***Corresponding Author**: Abdul M Gbaj, Associate Professor of Genetics and Biochemistry, Department of Medicinal Chemistry, Faculty of Pharmacy, University of Tripoli, Libya.

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Abstract

Snake bites can cause severe medical emergency. The venom injected into the victim after a bite can initiate grave debilitating and life-threatening effects and eventually eliciting morbidity or mortality. Snake venoms containing phospholipases A_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. We performed molecular docking and fluorescence studies in order to determine the potential modes of interaction of 6-amino-5-bromouracil with phospholipase A_2. 6-Amino-5-bromouracil forms hydrogen bonds with an active site of the enzyme and changes the fluorescent spectrum of phospholipases A_2. Our results indicate that 6-amino-5-bromouracil increases the mean survival time by six times protection fold, but could not protect mice from death when pretreated with the 6-amino-5-bromouracil alone. The uses of 6-amino-5-bromouracil could to some extent delay the snake venom toxicity. Further chemical synthetic analogues and *in vivo* studies would be necessary to substantiate the obtained results.

Keywords: *Snake Bite; Cerastes cerastes; 6-Amino-5-bromouracil; Fluorescence; Phospholipase A2; Molecular Docking*

Introduction

Snakebites are a foremost public health problem particular in India, Australia and African countries including Libya, Algeria, Egypt and Tunisia [1,2]. In some parts of Africa, more than 400 snake species are present, of which about 30 are venomous. The highly venomous species belong to four snake families namely: *Colubridae, Viperidae, Elapidae,* and *Atractaspididae*, to cause human deaths up to approximate 140,000 per year, 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 contains numerous enzymes exhibiting proteolytic activity and leads to multiple kinds of intoxications [3]. The lethal cause of snake venom mainly results from its active components PLA_2. *Cerastes cerastes* snake venoms cause proteolysis, shock, blood clotting, release of bioactive substances such as bradykinin, histamine eliciting necrosis, hemorrhage and numerous other effects [4-6]. Necrosis might result from a direct action of myotoxins, such as myotoxic PLA_2s on plasma membranes

of muscle cells, or indirectly, as a consequence of blood vessel degeneration and ischemia caused by hemorrhage. PLA_2s interacts with membrane phospholipid components which promotes the release of intracellular creatine kinase, which might be used as a biomarker for myotoxic activity assessment [7,8]. The snake venom has physiopathological processes encouraged researchers to obtain natural or chemically synthesized inhibitors.

Some PLA_2 inhibitors are 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 terpenoid compounds have irreversible inhibitory effects on different PLA_2s obtained from snakes [9,10]. Phospholipase A_2 (PLA_2) activity can be inhibited by some inhibitors as reported by Lewin., *et al.* (2016) who 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 this paper was to study the inhibitory effect of 6-amino-5-bromouracilon phospholipase A_2 enzyme to treat snake envenomation using molecular docking and fluorescence studies.

Materials and Methods

Preparation of aqueous 6-amino-5-bromouracil solution

19 mg of 6-amino-5-bromouracil (Matrix Scientific, Columbia, # 078889) were dissolved in 100 µl DMSO. The solution was filled up 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 of the animals and obtained in liquid form from the Department of Zoology, Faculty of Science, University of Tripoli (Libya) and stored at -20°C till use. An aliquot of 7.5µl venom was added to 800 µl of normal saline (0.9% sodium chloride). A dose of 100µl (100 ng) was intraperitoneally injected into male Swiss Albino mice weighing 18 ± 2 g.

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. 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 (62Å X 62Å X 62Å) with a grid-point spacing of 0.376Å and the grid boxes were centered. The number of genetic algorithms 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 2.0Å, which was dependent on the binding free energy. Lastly, the dominating configuration of the binding complex of 6-amino-5-bromouracil and phospholipase A_2 enzyme fragments with a minimum energy of binding were determined which relied strongly on the information of 3D structures of the phospholipaseA_2binding site and ultimately generated a series of phospholipase binding complexes.

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 venom samples was determined by the spectrophotometric method of Markwell., *et al* [13]. Bovine serum albumin (BSA, Sigma) was used for standard assays.

Fluorescence spectra

Fluorescence emission and excitation spectra were measured using a 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 ± 2g) 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 one hour 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 (12h light/dark cycle, light on 07:00h) 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.

Calculation of LD99 of *Cerastes cerastes* venom

The median lethal dose (LD99) of *Cerastes cerastes* venom was determined according to previously developed methods [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 hour 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.

Detoxification of venom by 6-amino-5-bromouracil

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 5µg/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 (anti-snake venom). The numbers of mortalities were recorded within 24h.

Statistical analysis

The difference among various treated groups and control group were analyzed using one-way-ANOVA followed by 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) as well as 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) were 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 are *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, TYR63and 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.

Fluorescence spectra and 6-Amino-5-bromouracil

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 caused by addition of 6-Amino-5-bromouracil (105 μM) 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 by any shift which may indicate that tryptophan (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 not accompanied by any shift and this may indicate that binding of 6-amino-5-bromouracil may have accomplished a conformational change that moves TRP into a relatively more hydrophobic region. This explanation is consistent with Gorbenko., *et al.* (2007) who found that TRP fluorescence is quenched by interaction with polar ligands. Binding of proteins to lipid membranes decreases the ease of access to these polar ligands and consequently decreases the obtained quenching effects [22].

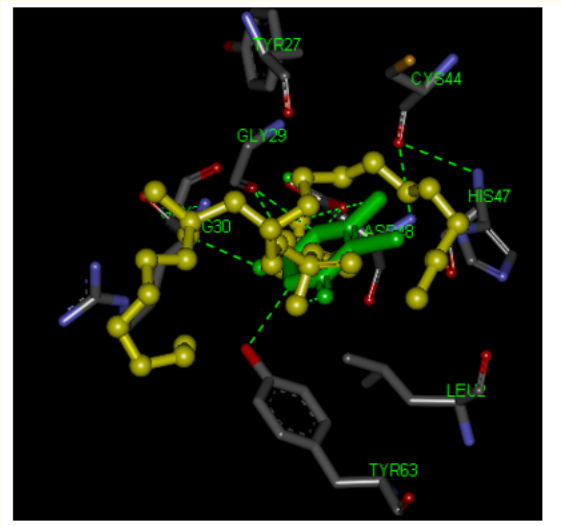


Figure 1: *Interaction model between 6-amino-5-bromouracil (green) and Gel (yellow) with cobra-venom phospholipase A2 (1pob) active site. Hydrogen bonds are represented by green broken lines.*

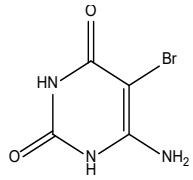
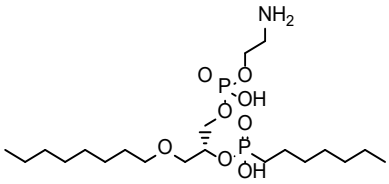
Compounds	Hydrogen bond formation between Cobra-venom phospholipase A_2 (1pob) with 6A5BU and Gel	Hydrogen bond distance Å	Donor atom	Acceptor atom	Binding energy (ΔG) kcal/mol.
6-amino-5-bromopyrimidine-2,4(1H,3H)-dione or 6-amino-5-bromouracil (6A5BU) 	GLY31:HN of 1pob and O10 of 6A5BU H2 of 6A5BU and OH of pob 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

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.*

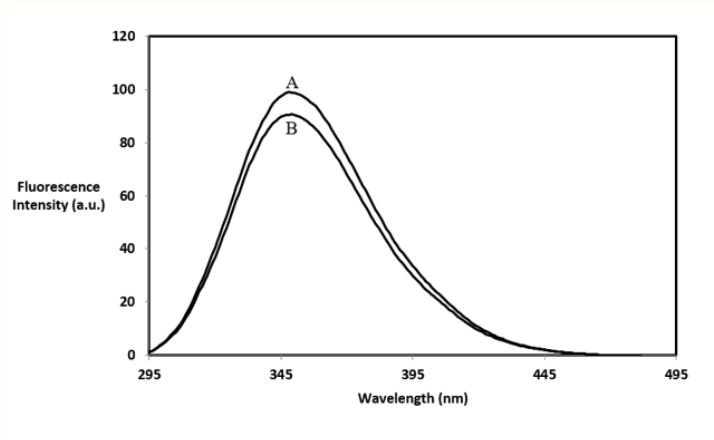


Figure 2: *(A) Plot of fluorescence emission of snake venom (Cerastes cerastes) (24.6 µg/ml) vs wavelength from 288 - 540 nm using excitation of λ_{280} nm in 0.01 M Tris, 0.1M NaCl at pH 7.4. (B) Fluorescence perturbation of snake venom by addition of 105 µM 6-amino-5-bromouracil. Spectra were corrected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

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 intrinsic fluorescence, which 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 the tryptophan residue located within 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 with 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 tryptophan. In addition, the quenching reaction obtained can be used not only to probe topological features of 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 [25,26].

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 antivenom

The *Cerastes cerastes* venom at a dose of 5 µg/kg (LD99) produces 100% mortality in mice. 6-Amino-5-bromouracil significantly increased the mean survival time up to 6.3 ± 0.23 hours. The protection fold could not protect animals from death when *Cerastes cerastes* venom was used alone. 6-Amino-5-bromouracil when used at a dose of 100 µl (stock 18.5 mM) solution was found to be more effective against *Cerastes cerastes* venom (4.8 hours survival time) when compared with 6.2 hours by using 300 µl (stock 18.5 mM) 6-amino-5-bromouracil solution. ASV [polyvalent anti-snake venom by Haffkine Bio-Pharmaceuticals Company (India)] was found to be more effective as compared to 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 in snakebite victims. PLA_2s is able to operate on pre- or post-synaptic junctions as antagonist of ion channels and muscarinic or nicotinic receptors causing neurotoxic effects such as paralysis and respiratory failure [29,30]. In addition, PLA2s can cause local tissue damage resulting in blistering, swelling, necrosis and bruising. Furthermore, it has systemic effects such as hypovolemic shock, aggravate hemostatic and cardiovascular effects as coagulopathy, hemorrhage and hypotension. It is also reported that PLA2s is able to trigger severe pain [31,32].

Because PLA_2 activity is a significant part of venom toxins, it appears an interesting candidate for investigation of PLA_2 inhibitors by directly testing drugs. It was observed that 6-amino-5-bromouracil when given to the mice after they received *Cerastes cerastes* snake venom significantly increased mean survival time and the results were found to be even better when 6-amino-5-bromouracil was used at a higher dose (300 µl, stock 18.5 mM). This could be possibly due to interaction of active venom components mainly PLA_2 with6-amino-5-bromouracil which is consistent with the result obtained by molecular docking.

In the literature we found a few small molecules which were able to inhibit snake venom PLA_2 and delay its toxicity [33,34] which supports our findings. It has been reported that Varespladib and methyl-varespladib (its orally bioavailable prodrug) were able to delay the effects of twenty eight medically important snake venoms from six continents- but not for a long time [35]. Varespladib and methyl-varespladib were also able to suppress host response safely and are more effective against snake venom PLA_2 (picomolar concentrations) than against mammalian $sPLA_2$ [36] and were able to perform protection against the injurious effects of hemorrhage, hemolysis and other tissue obliteration [35].

Our results are in agreement with some previous studies [37,39], as pyrimidine-2,4,6-trione has been found as a promising efficient and selective inhibitor of cell matrix metalloproteases [38,39]. Other studies report on the efficiency of pyrimidine-2,4,6-trione derivatives, mainly one named RO 28-2653 developed by a Hoffman-La Roche research group, in anticancer therapy [40,41]adamalysins, serralysins, and matrixins (collagenases. However, 6-amino-5-bromouracil has not been mentioned as PLA_2inhibitor or has been used for 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 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 snake bite envenomation and makes it a reasonable candidate for clinical trials. 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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