THE NEW ARMENIAN MEDICAL JOURNAL
Vol.13 (2019), No 3, p.65-71

IN VITRO AND *IN VIVO* TOXICITY STUDY OF α-AMINO-ARYLPROPANOIC ACID DERIVATIVES

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Received 15.05.2019; accepted for printing 27.02.2019

Abstract

The anti-inflammatory and antinociceptive activity of S(+)-2-amino-2-(benzylamino) propanoic acid (NPAA 34), R(-)-2-amino-2-(benzylamino) propanoic acid (NPAA 35), S(-)-2-amino-2-methyl-3-phenyl propanoic acid (NPAA 36), S(-)-2-amino-3-(4'-fluoro) phenylpropanoic acid (NPAA 38) and their non-selective COX inhibiting properties demonstrated in our previous studies approved that synthesized α-amino-aryl propanoic acid derivatives could serve as a potential source for discover and development of new non-steroidal anti-inflammatory drugs. In view of this the main purpose of presented study was investigation of testified compounds toxicity as an important drugs development step – safety testing. In vitro cytotoxicity by flow cytometry and in vivo toxicity study for determination on certain toxicometry parameters and hazard class assessment were carried out. Obtained results indicated that investigated compound NPAA 38 can be classified to the Category 3 (LD_{50} > 300 mg/kg) and NPAA 34 to the Category 4 (LD_{50} > 2000 mg/kg) by Globally harmonized system of classification and labeling of chemicals and third class of the moderately toxic chemicals according WHO classification (LD_{50} value of NPAA 38 for rat administrated per os was 1300±102 mg/kg and for NPAA 34 was more than 2000 mg/kg). In a single application of the compounds to the intact rats' skin irritating effect was not revealed while they have a moderate irritant effect on the mucous membranes of rabbit's eyes. According to flow cytometry data, the S(+)-2-amino-2-(benzylamino) propanoic acid (NPAA 34) and S(-)-2-amino-2-methyl-3-phenyl propanoic acid (NPAA 36) do not display cytotoxicity. It was demonstrated, that in 500 μM concentrations the total number of peripheral blood mononuclear cells (PBMC) and monocytes was not changed significantly in presence of investigated non protein amino acids.

Keywords: *α-amino-arylpropanoic acid, derivates, toxicity, hazard class, cytotoxicity.*

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs), especially propionic acid derivatives such as ibuprofen, ketoprofen etc. are commonly used medicines in worldwide to treat pain, reduce fever and inflammation. Some of them also have ability to decrease platelet aggregation [*Dhall H et al., 2016*]. However, their long-term use derives serious side effects including gastrointestinal, cardiovascular, and renal, which limits their prescriptions [*Park K et al., 2012; Harirforoosh S et al., 2013*]. That's why development of new agents among NSAIDs with high efficacy and as possible less adverse effects remains an important direction in modern medicine.

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Based on structure – activity relationship (SAR) statements of arylpropionic acid derivatives of anti-inflammatory drugs, as a new source for NSAIDs it was proposed α-amino-arylpropionic acid derivatives of non-protein amino acids (NPAA). The synthesis of compound was carried out by Sagiyan A. and coworker in Armbiotechnology Center of RA [*Saghyan A et al., 2014; Saghyan A, Langer P, 2016*]. The new synthesized α-amino-arylpropionic acid derivatives contain necessary for cyclooxygenase (COX) inhibition pharmacophore functional groups: carboxyl- and aryl-groups, connected by 2-3- carbon atoms chain.

Based on above, in our previous study it was investigated anti-inflammatory, antinociceptive activity of 4 new structures of α-amino-arylpropanoic acid NPAA derivatives (Fig. 1) in comparison with mentioned activity of already known anti-inflammatory drug of 2-arylpropionic acid derivative – ketoprofen [*Zhamharyan A et al., 2013*]. Obtained data indicated that new synthesized α-amino-arylpropanoic acid derivatives appear significant antinociceptive and anti-inflammatory activities due to COX non selective inhibition. The COX1 and COX2 inhibitor activity of investigated compounds were verified by both in vitro screening test and by docking analysis of structures [*Zhamharyan A et al., 2014*]. Thus, the results obtained in our previous study prove, that new synthesized non-protein amino acids could serve as a promised source for development of new anti-inflammatory and analgesic drugs.

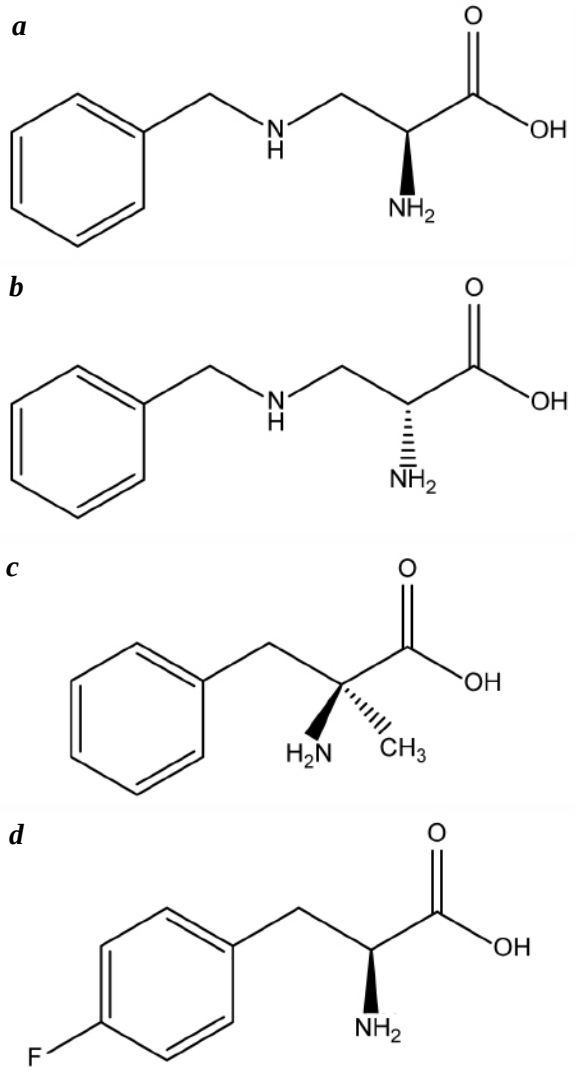

FIGURE 1. *Chemical structures of investigated non-protein amino acids -* ***(a)*** *S(+)-2-amino-3-(benzylamino)propanoic acid,* ***(b)*** *R(-)-2-amino-3-(benzylamino)propanoic acid,* ***(c)*** *S(-)-2-amino-2-methyl-3-phenylpropanoic acid,* ***(d)*** *S(-)-2-amino-3-(4'-fluoro)phenylpropanoic acid*

Taking in account significance of toxicity investigation as a most important issue in drug development in view of their safety testing, the aim of presented study was investigation of acute toxicity of S(+)-2-amino-3-(benzylamino)propanoic acid (NPAA 34), S(-)-2-amino-2-methyl-3-phenylpropanoic acid (NPAA 36) and S(-)-2-amino-3-(4`-fluoro)phenylpropanoic acid (NPAA 38) and in vitro cytotoxicity as well.

MATERIALS AND METHODS

Studies on certain toxicometry parameters and hazard class assessment of 3-(4`-fluoro) phenylpropanoic acid (NPAA 38) and S (+)-2-amino-3-(benzylamino)propanoic acid (NPAA 34) were done by different ways of introducing (per os, cutaneous) at different times of experiment. As evaluation criteria served manifestation of intoxication signs, lethality of experimental animals. The integral indicators of toxic effect such as the behavior feed and water consumption by animals, body weights etc. were observed in dynamics within 14 *days*. At the end of research, a macroscopic examination of the internal organs (blood filling, hemorrhage, changes in appearance and the presence of visual macroscopic changes) was done.

The toxicological research of formulation was done in line with the following tasks:

√ assessment of a mean LD_{50} (acute oral and acute dermal values), [*Guidelines, 1988*]

√ study of cutaneous-irritating study [*Guidelines, 1980*]
√ study of irritating effect on mucous membranes [*Guidelines, 1988*].

The toxicological studies were done on the basis of the Laboratory of Environmental Hygiene and Toxicology of the Research Center of Yerevan State Medical University after M. Heratsi and Institute of Molecular Biology RA. All procedures both in vivo and in vitro were carried out in accordance with the GLP standards and EPA guidelines.

Animals

The experiments were carried out on white nonlinear adult rats, rabbits of both sexes. The animals were accommodated in a vivarium of the Yerevan State Medical University. The background conditions were corresponding to relevant requirements: the air temperature for rats was at 20-24°C, rabbits – 15-21°C; relative humidity – 45-65%, background data of ultrasound <20 *Hz* [*Inter-Governmental Council, 2014*]. Food and tap water were available *ad libitum*. Experimental animals on passed the adaptive period for 7-14 days before the starting the research. All experimental procedures were conducted in accordance to the rules of ethical recommendations approved by YSMU Ethic Committee [*European Convention 1991; EASC, 2014*].

In vivo toxicological studies

Acute oral toxicity:

Laboratory rats (male, female) were divided into 4 groups (10 animals in each group), a mean body weight of each group made 0.150-0.200 *kg*. After preliminary sighting experiments NPAA 38 was tested in doses of 1000 *mg/kg*, 1300 *mg/kg*, 1600 *mg/kg* and NPAA 34 in doses of 1000 *mg/kg*, 1500 *mg/kg*, 2000 *mg/kg* were administered by using a metal non-traumatic oral gavage (*per os*), once on an empty stomach in the form of freshly prepared aqueous solution. The group of the same mean body weight rats (10 animals in group) served as a control. Distilled water in the volume adequate to experimental group was administered to control animals in the same way. The observation period was 14 days. During this period the appearance and behavior of animals, pelage status, attitude to food, reactions to tactile and pain stimuli, rate, number of lethal cases and date were observed. In dynamics the body weight was controlled as indicator of possible toxic effect. At the end of experiment rats were sacrificed and a macroscopic examination of the internal organs was done [*Guidelines, 1988*].

The cutaneous-irritating effect

The cutaneous-irritating effect -local irritating effect was studied on white rats group with mean body weight 0.20-0.25 *kg*. On one side of animals (the right one) clipped in advance (2x2 *cm*) a single application of 0.5 *ml* solution of NPAA 38 (1600*mg/kg*) and NPAA34 (2000*mg/kg*) were done. The opposite side served as a control. Exposure time made 4 hours with observation period of 14 days. To avoid licking, erasing the drugs animals were fixed in special devices [*Guidelines, 1980*]. The observation was started upon the end of exposure and continued during 14 days. The condition of pelage status, its thickness, hyperemia, erythema and edema were taken into account.

The irritating effect on mucous membranes

The study was conducted in the group rabbits (n=6) with mean weight 3-3.5 *kg*. A single administration of drugs in native form (0.1 *mg*) was done gently into the conjunctivas sac of the right eye by soft pulling down of the lower eyelid from the eyeball. The opposite eye (the left one) served as a control. Daily observation was conducted for 14 days to monitor cornea and the mucous membrane status. The effect was assessed by the manifestation and severity of hyperemia, increased vascular pattern of the eyeball, tearing, moisturizing and discharge from the eyes, swelling and partial reversal of the eyelids, blepharospasm, and corneal opacities [*Guidelines, 1988*].

In vitro cytotoxicity study

Cytotoxicity of investigated non protein amino acids was done by FLOW Cytometry method [*Crowley L et al., 2016*]. The method based on interaction of damaged cell's DNA with propionium iodide (PI) (BD Biosciences), causing creation of fluorescence product. The resulting complex associates with cell damage, as PI can penetrate the cell only in case of membrane damage. Peripheral blood mononuclear cells (PBMCs) were isolated from freshly obtained EDTA-treated samples by gradient centrifugation on a discontinuous density gradient (Histopaque 1077, Sigma). The blood and gradient component were mixed in equal quantities. Centrifugation was carried out for 40 *min* at a

speed of 1500 *rpm* at 20°C. After centrifugation, the fraction of mononuclear cells was collected and washed several times with phosphate buffer (PBS). Collected cells were divided into 3 groups, the first group served as a control, NPAA 34 with a concentration of 500 μM was added to the second group, and NPAA 36 with a concentration of 500 μM was added to the third group and all groups were incubated for 24 *hours*. One day later, the supernatant was discarded from the cells culture by centrifugation, 10 *min* before measurement in the cells culture were added to 30 μM PI and placed in cytometer The damaged cell's DNA+PI complex was measured by BD FACS Calibur flow cytometer according to the manufacturer's instructions (BD Biosciences).

Statistical analysis: Acute oral LD_{50}, standard errors and other toxicity variables were calculated by using the Litchfield-Wilcoxon probity analysis method in Prozorovky modification.

Data was proceeded and analyzed using standard software (Microsoft Excel). The difference in mean values was estimated by using a two-tailed Student's test for independent samples (t-test). The null hypothesis of equality of averages was rejected at a significance level of $p<0.05$, in all cases of statistical data processing [*Glantz S, 1999*]. The hazard class by oral and dermal toxicity was assessed according to the WHO classification [*WHO, 2009*].

RESULTS

In vivo toxicity study

The maximum tolerated dose (DL_0) of NPAA 38 administrated per os was 1000 *mg/kg*, absolutely lethal dose (DL_{100}) – 1600 *mg/kg*. Acute oral dose: LD_{50} value (DL_{50} per os) for rats was made 1300 ± 102 *mg/kg*. The maximum tolerated dose (DL_0) of NPAA 34 administrated per os was 1000 *mg/kg*, 1500 *mg/kg*, 2000 *mg/kg*. Acute oral dose: LD_{50} value (DL_{50} per os) for rats was more than 2000 *mg/kg* (Table 1).

Species sensitivity of the funds is not pronounced. Acute intoxication NPAA 38 was manifested in a certain disorder. The dirtiness of pelage status, short-term impairment of coordination, as well as indifferences to food and water, to pain and tactile stimuli, tremor of extremities were observed. Depending on the dose administered lethal cases were registered within the first two days after exposure to drugs. Intoxication signs were gone on 1-2 days.

The clinical picture of acute intoxication of NPAA 34 poisoning is not expressed. When drug administered at a dose of 2000 *mg/kg* after a brief disturbance, the experimental rats subsequently did not differ in their behavior and appearance from the control group. The impact of the drug in a dose of 2000 *mg/kg* did not register the death of animals.

Analysis of the dynamics of mean body weight of experimental rats after a single oral administration of the NPAA38 in doses 1000 *mg/kg*, 1300 *mg/kg* body weight did not reveal significant differences in comparison with the control group, $p>0.05$ (Table 2).

A macroscopic examination of the internal organs of experimental animals after exposure NPAA38 showed hyperemia and hemorrhages of the internal organs, necrotic areas in the stomach.

In macroscopic examination of the internal organs of experimental animals after exposure NPAA34 no changes were registered.

In a single application of the drugs to the intact rats' skin irritating effect wasn't revealed, namely visible disorders such as erythema, edema, cracks, symptoms, necrosis, dryness, desquamation etc. we're not registered.

TABLE 1.

Acute toxicity indicators of the studied substances

Substances	Inactive dose (*mg/kg*)	lethal doze (*mg/kg*)				Hazard class, WHO
	DL_0	DL_{16}	DL_{50} Median	DL_{84}	DL_{100} Absolute	
3-(4`-fluoro) phenylpropanoic acid (NPAA 38)	1000	1130	1300±102	1130	1600	3
S(+)-2-amino-3-(benzyl amino) propanoic acid (NPAA 34)	1000	-	>2000	-	-	3
	1500	-		-		
	2000	-		-		

Table 2.

Dynamics of experimental rats body weight changes after administration of 3-(4`-fluoro) phenylpropanoic acid per oral.

Substances	Dose, *mg/kg* body weight		
	control group	1000	1300
Body weight, *kg* (prior to experiment)	0.149±0.013	0.148±0.015	0.146±0.13
after 14 days	0.159±0.002	0.157±0.014	0.156±0.012
Weight gain, *%*	6.2	5.7	5.1

In testing the irritating effects of substances on mucous membranes by introducing the formulation into the conjunctivas sac hyperemia and profuse tearing with a gradual decrease were observed on the second day of experiment. These signs disappeared completely on the third day that proved a slightly irritating effect of NPAA 38 and NPAA 34 on the mucous membranes of the rabbit's eyes.

In vitro cytotoxicity study

Cytotoxicity was assessed at the level of monocytes and PBMC. Thus, cytotoxicity study results of S(-)-2-amino-2-methyl-3-phenylpropanoic acid (NPAA 36) and S(+)-2-amino-2-(benzylamino) propanoic acid (NPAA 34) show that in a control group total number of PBMC and monocytes was 89.8±1.82% and 13.92±0.64% accordingly (Fig.2).

After incubation of blood samples with NPAA 34 and NPAA 36 solution in 500 μM concentrations the total number of PBMC and monocytes didn't decrease compare with control level and even was a little bit increased, indicate on absence of cytotoxicity of investigated compounds (Table 3).

Discussion

Administration of NPAA 34 compound in doses 1000, 1500, 2000*mg/kg* and NPAA 38 compound in dose 1000*mg/kg* didn't lead to the death of animal during 15 *days* and didn't reveal any statistically significant differences in the dynamics of the body mass and the condition of inner organs compare with control group of animals. Administration of NPAA 38 compound in doses 1600 *mg/kg* (LD_{100}) leads to the death of all animals of the experimental group. The acute oral LD_{50} value of NPAA 38 for the rats made 1300 ±102 *mg/kg* body weight. In these studies, there were no differences in LD_{50} values between male and female rats. A macroscopic examination of the internal organs of experimental animals after exposure NPAA 38 showed hyperemia and hemorrhages of the internal organs, some necrotic areas in the stomach. The substances NPAA

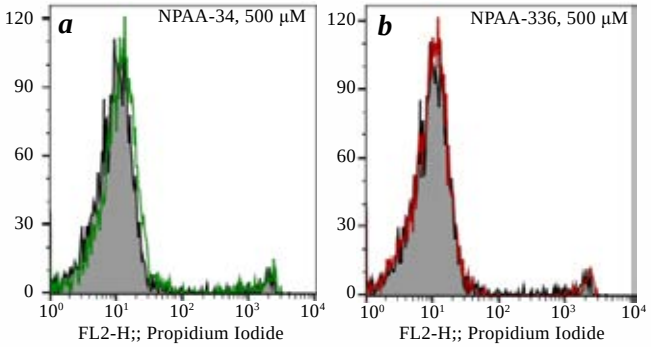

Figure 2. *The cytotoxicity histograms of NPAA-34 and NPAA-36 on monocytes. Note: Grey filled histogram is for control (untreated sample) (**a**), Colored not filled histogram is for NPAA- treated sample (**b**)*

Table 3.

Cytotoxicity of investigated non protein amino acids (M±SE, n=6).

	Concentration (μM)	Peripheral blood mononuclear cells		Monocytes	
		Total %	Dead %	Total %	Dead%
Control	0	89.8 ± 1.82	1.25 ± 0.17	13.92 ± 0.64	3.42 ± 0.51
S(+)-2-amino-2-(benzylamino) propanoic acid (NPAA 34)	500	90.8 ± 0.99	1.12 ± 0.19**	13.58 ± 0.86	2.7 ± 0.49**
S(-)-2-amino-2-methyl-3-phenylpropanoic acid (NPAA 36)	500	90.8 ± 1.03	0.98 ± 0.13**	13.65 ± 0.92	2.56 ± 0.35**

**p>0.05, in comparison with control group

38 and NPAA 34 do not have a cutaneous-irritating effect. They have a moderate irritant effect on the mucous membranes of rabbit's eyes.

Obtained data indicate that S(-)-2-amino-3-(4`-fluoro)phenylpropanoic acid (NPAA 38) more toxic than S(+)-2-amino-2-(benzylamino)propanoic acid (NPAA 34). This result approved by data about more toxicity of fluorinated derivatives compare to parent amino acids [*Kortagere S et al., 2008; Taryn L et al., 2012*]. The more toxicity of fluoro derivative in contrary of benzylamino derivative also may be explained by insertion of fluorine atom to the aromatic ring which leads to bleeding of the rat's stomach in experimental group by increasing acidic properties of compound and local irritating effect in gastrointestinal tract.

On the other hand according to flow cytometry data, the S(+)-2-amino-2-(benzylamino)propanoic acid (NPAA 34) and S(-)-2-amino-2-methyl-3-phenylpropanoic acid (NPAA 36) aren't cytotoxic. This indicates the fact that digital indicators, which were obtained after counting of lymphocytes and monocytes and their determination with PI, do not practically differ when the same process is carried out in the presence of the investigated non protein amino acids. So investigated compounds didn't damage cell membrane and don't appear cytotoxicity.

CONCLUSION

Thus, according the results of *in vivo* acute toxicity and *in vitro* cytotoxicity study:

1. Testifying compounds can be classified to the third class of the moderately toxic chemicals according to WHO classification [*WHO, 2009*] and investigated compound NPAA 38 can be classified to the Category 3 (LD_{50} > 300 *mg/kg*) and NPAA 34 to the Category 4 (LD_{50} > 2000 *mg/kg*) by GHS.

2. Synthesized new **α**-amino-arylpropanoic acid derivatives don't appear cytotoxicity on monocytes and PBMC in 500μM concentration.

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