https://doi.org/10.31925/farmacia.2019.4.18 **ORIGINAL ARTICLE**

IN VITRO EFFECTS OF SOME SYNTHESIZED AMINOACETANILIDE N'-SUBSTITUTED ON HUMAN LEUKOCYTES SEPARATED FROM PERIPHERAL BLOOD

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Abstract

Local anaesthetics are frequently used in several medical areas like surgical interventions, stomatology or preparing the patient for imagistic techniques like endoscopies. In most cases, the local anaesthetics are administrated in inflammatory processes and several studies demonstrated that these drugs may influence the response of local immune system cells. The adverse effects reported are correlated with the route of administration and the interaction with cellular components at the site of administration. Some lidocaine analogues with aminoacetanilide N'-substituted structure were previously synthesized, characterized and pharmacologically tested in preclinical studies regarding the local anaesthetic effects and were selected for the present research. The study aims to determine the effects of the synthetized aminoacetanilide N'-substituted on the release of oxygen metabolites by polymorfonuclear cells and on the cellular viability of human leukocytes isolated from the peripheral blood.

Rezumat

Anestezicele locale sunt utilizate frecvent în multe domenii medicale, cum ar fi intervențiile chirurgicale, stomatologia sau pregătirea pacientului pentru tehnici imagistice, de exemplu endoscopiile. În majoritatea cazurilor, anestezicele locale sunt administrate în procese inflamatorii și mai multe studii au demonstrat că aceste medicamente pot influența răspunsul celulelor sistemului imunitar local. Efectele adverse raportate sunt corelate cu calea de administrare și interacțiunea cu componentele celulare la locul de administrare. Anumiți analogi de lidocaină cu structura aminoacetanilidă N'-substituită au fost anterior sintetizați, caracterizați și testați farmacologic în studii preclinice, privind efectele anestezice locale și au fost selectați pentru prezenta cercetare. Studiul își propune să determine efectele aminoacetanilidei N'-substituite asupra eliberării metaboliților oxigenului de către celulele polimorfonucleare și asupra viabilității celulare a leucocitelor umane izolate din sângele periferic.

Keywords: aminoacetanilide N'-substituted, reactive oxygen species, polymorphonuclear cells

Introduction

Local anaesthetics are frequently used in several medical areas like surgical interventions, stomatology or preparing the patient for imagistic techniques like endoscopies [14]. The adverse effects reported are correlated with the route of administration and the interaction with cellular components at the site of administration [22]. In most cases, the local anaesthetics are administrated in different inflammatory states.

The response of the cells involved in the inflammation process are essential for tissue repair mechanisms at the site of administration. An increased and prolonged signalling of pro-inflammatory signals may aggravate the affected area and delay the healing process. The most important cells with an essential role in cell signalling are polymorphonuclear cells (PMN) which interact with endothelial cells and favour tissue recovery [9, 21].

Previous studies sustain the effect of local anaesthetics belonging to the lidocaine class on modulating the inflammatory responses at the site of administration especially on the PMN signalling, like modulating the level of some cytokines and the reactive oxygen species release [5, 13, 15-17].

Some lidocaine analogues with aminoacetanilide N'-substituted structure were previously synthesized, characterized and pharmacologically tested in pre-clinical studies regarding the local anaesthetic effects and were selected for the present research [12].

The study aims to determine the effects of the synthetized aminoacetanilide N'-substituted on the release of

oxygen metabolites by the human polymorphonuclear cells (PMN) and on the cellular viability of this type of cells isolated from the peripheral blood.

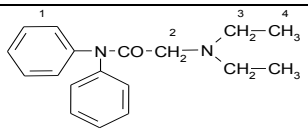
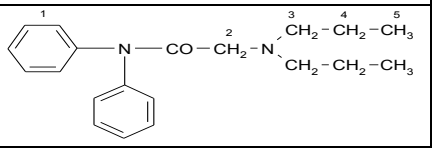
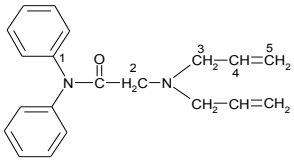
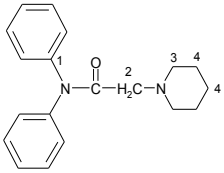
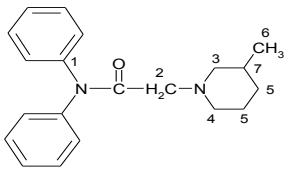
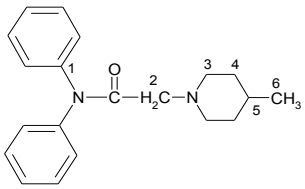
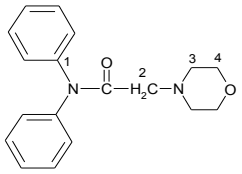
Materials and Methods

The analogues of lidocaine with aminoacetanilide N'-substituted structures were synthetized within the Faculty of Pharmacy, University of Medicine and Pharmacy "Carol Davila", Bucharest, Romania.

The synthesis method presumed the changing of the lipophilic aromatic group of lidocaine, constituted by a benzene ring, with a more lipophilic group. The anaesthetic acetamide chain was maintained and the hydrophilic group was changed by replacing the radicals linked to the nitrogen contained in the aminic group of lidocaine. The seven compounds included in the study are presented in Table I.

Table I

Aminoacetanilide N'-substituted derivatives

S1	2-diethylaminoacetyldiphenylamine	
S2	2-di-(n)-propylaminoacetyldiphenylamine	
S3	2-diallylaminoacetyldiphenylamine	
S4	2-(1'-piperidyl)acetyldiphenylamine	
S5	2-(3'-methyl-1'-piperidyl)acetyldiphenylamine	
S6	2-(4'-methyl-1'-piperidyl)acetyldiphenylamine	
S7	2-(N-morpholino)acetyldiphenylamine	

Separation of the populations from the human peripheral blood

Cells were separated from human peripheral blood collected in our laboratory from volunteer healthy donors. The subjects' written consent was obtained in each case and the study was approved by the institutional ethics committee. The whole blood was centrifuged in Ficoll-Paque density gradient media (GE Healthcare, purchased from Sigma-Aldrich Chemie Gmbh, Munich, Germany). Leucocytes area was washed three times with culture medium IC_{65} (purchased from the "Cantacuzino" National Institute), and was then resuspended in culture medium $RPMI_{1640}$ (Sigma-Aldrich Chemie Gmbh, Munich, Germany) supplemented with foetal bovine serum (FBS) (EuroClone, Pero, Italy) and sodium

bicarbonate (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for the *in vitro* cultivation.

Cell sediments, formed by polymorphonuclears (PMN) and red blood cells, was incubated 30 minutes at 4ºC in NH_4Cl 0.84 % solution in TRIS buffer ([2-amino-2-(hydroxy methyl)-aminomethane])-HCl (Sigma-Aldrich Chemie Gmbh, Munich, Germany) for lysing the red blood cells.

Cells suspension was washed three times in phosphate buffered saline (PBS) (Sigma-Aldrich Chemie Gmbh, Munich, Germany), adjusted to 2 x 10^6 cells/mL and retaken in buffer HBSS (Hank's Balanced Salt Solution) (Gibco, Grand Island, New York, USA) [4, 7, 8].

Cells viability determined by MTT test for human peripheral PMN cells cultivated in vitro after the treatment with aminoacetanilide N'-substituted derivatives (S1-S7)

PMN cells were separated as described above and cultivated *in vitro* at a density of 1 x 10^6 cells/mL and 5% CO_2 atmosphere incubator. The control group of cells was cultivated for 24 or 48 hours without any treatment. All treated groups were cultivated in the presence of 5 µg of substance/1 x 10^6 cells. All substances were previously dissolved in ultrapure water. Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay in respect to the manufacturer's instructions (Thermo Fischer Scientific). Cells were incubated with 1 mg/mL MTT (3-(4,5-dimethylthiazol-yl-2)-2,5-diphenyl tetrazolium bromide) solution at 37ºC for 2 h. The yellow dye [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) was reduced to insoluble purple formazan granules by metabolically active cells. The precipitated formazan was dissolved in dimethyl sulfoxide, and after 10 min of slow shaking, the absorbance was recorded at 550 nm. [1, 6, 11, 19, 20]

The effect of the treatment with aminoacetanilide N'-substituted derivatives on in vitro Reactive Oxygen Species (ROS) release by the human polymorphonuclear cells (PMN) using a chemiluminiscence method

Polymorphonuclear cells are releasing ROS in the presence of extracellular stimulus both *in vivo* and *in vitro*. This physiological process can be measured *in vitro* by a chemiluminescence technique.

Reagents. Cultivation medium TC_{199} (Gibco, Grand Island, New York, USA), IC_{65} ("Cantacuzino" National Institute), HBSS solution (Gibco, Grand Island, New York, USA), PBS (Sigma-Aldrich Chemie Gmbh, Munich, Germany), materials for leucocytes separation, luminol (Sigma-Aldrich Chemie Gmbh, Munich, Germany), dimethyl sulfoxide (DMSO)) (Sigma-Aldrich Chemie Gmbh, Munich, Germany), luminometer or a liquid scintillation counter (Beckman) for reading the β radiations; zymosan (Sigma-Aldrich Chemie Gmbh, Munich, Germany), dead bacteria, concanavalin A (ConA) (Sigma-Aldrich Chemie Gmbh, Munich, Germany), phytohemagglutinin (PHA) (Sigma-Aldrich Chemie Gmbh, Munich, Germany).

Luminol solution, PMN cells suspension, and the substances used for cells stimulation, were kept in separate containers at room temperature, in dark. In special luminometer containers were added 0.5 mL of opsonised zymosan (OZ) prepared as it follows: 5 mg of zymosan adapted to dark, 20 µL of luminol solution 0.1 M; and adding HBSS to the final volume of 5.5 mL. It was added 0.5 mL suspension of PMN cells (time 0), followed by homogenisation. The chemi-luminescence values were registered every 3 minutes for an interval of 30 minutes for human PMN cells stimulated with opsonized zymosan. Blank tests are represented by containers in which, instead of opsonized zymosan, there were introduced 0.5 mL HBSS [2, 3, 10, 18, 23].

The groups considered for the study were: PMN (inactive PMN cells), PMN + OZ (activated PMN cells) and groups PMN + OZ + Si (activated PMN cells treated with synthesized aminoacetanilide in a dose of 5 µmol, where i = 1 - 7 corresponding to the substances presented in Table I).

Statistical analysis

Biostatistical analysis was implemented using the R statistical software (R version 3.5.3). The data set contains a large number of numeric variables, which implies a correlation study between them. Thus, based on Pearson type correlation matrices, the numerical variable denoted with PMN + OZ + Si is very dependent on the linear type with respect to the MINUTE variable and the second polynomial type relative to PMN + OZ variable, which leads to the construction of a regression multiple model. The p and R-squared values are extremely convincing in choosing these regression models. The second statistical approach is based on comparing the averages of PMN, PMN + OZ and PMN + OZ + Si numerical variables both globally and relative to each substance considered separately by Kruskal Wallis nonparametric tests and post-hoc Pairwise comparisons using Wilcoxon rank.

Results and Discussion

The cell viability test was performed on human polymorphonuclear cells (PMN) separated from the peripheral blood and was determined by MTT test at 24 h and 48 h (Figure 1).

After 24 h, a significant decrease of the PMN viability ($p < 0.05$ *vs*. control group) was observed in case of the treatment with aminoacetanilides: S3, S4 and S5. No significant effects were observed in case of the treatment with S1, S2, S6 and S7 comparing to the control group.

After 48 h, a significant decrease of the cell viability ($p < 0.05$) was noticed in case of the groups treated with S1, S6 and a high significant decrease was

observed in case of the treatment with aminoacetanilides S3, S4 and S5. The treatment with S2 and S7 had no significant influence on the cell viability 48 h after the administration, compared to the control group.

The chemiluminescence method was used to determine the effect of the treatment with aminoacetanilide N'-substituted derivatives on *in vitro* reactive oxygen species (ROS) release by the human polymorphonuclear cells. The regression curves corresponding to the chemiluminescence results are presented in Figure 2

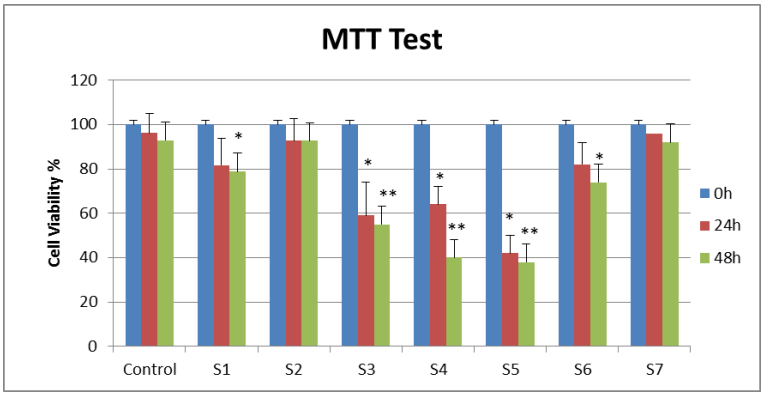


Figure 1.

Determination of the cell viability by MTT Test on human peripheral PMN cells after the treatment with aminoacetanilide N'-substituted derivatives. Error standards are represented by bars in the chart; Statistical significance *t*-test vs. control group *p < 0.05, **p < 0.01

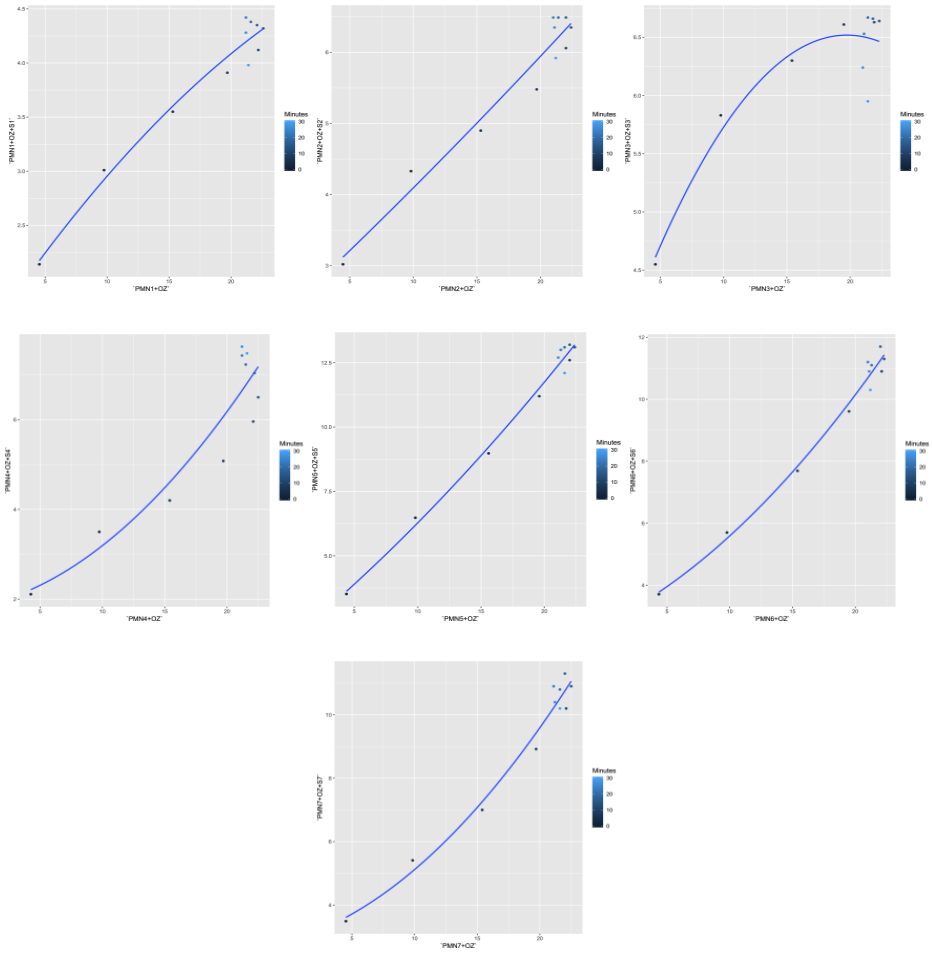


Figure 2.

The multiple regression curves where PMN + OZ + Si (activated PMN cells treated with synthesized aminoacetanilides, where i = 1 - 7) are modelled by PMN + OZ (activated PMN cells) and MINUTES (time variable)

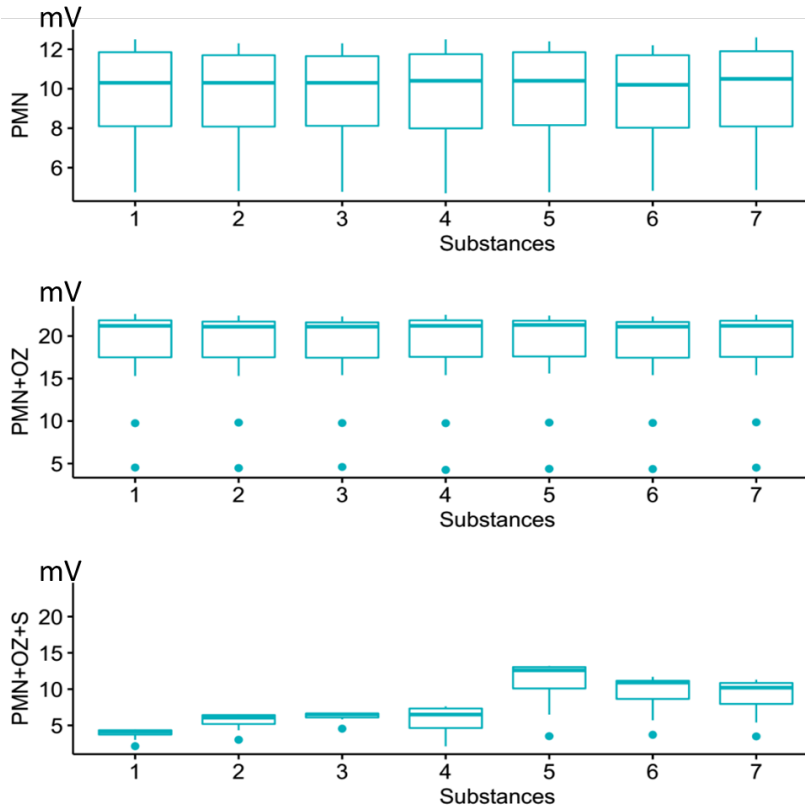


Figure 3.
Viscosity curves and the statistical mean of differences expressed by the regression lines

A decrease of ROS level was noticed in case of all treatment applied corresponding to the PMN + OZ + Si (i = 1 - 7) groups comparing to the non-treated activated PMN group (PMN+OZ) (Figure 3)

For the statistical interpretation of the results obtained within the chemiluminescence study, two types of statistical analyses were performed.

The first type of analysis was based on studying the behaviour of the dependent variable denoted PMN + OZ + S relative to time factor denoted with MINUTES and variable denoted with PMN + OZ, considered in this context independent variable. Because the studied variables had similar distributions, we determined the Pearson correlation matrix and we observed very high values, close to 1, which showed a very strong association between these variables, the only exception being S3 with medium correlation value. Thus, relative to all the seven substances considered for the study, there is a multiple interaction, namely linear type in respect to MINUTES and polynomial of second order in relation to the variable PMN + OZ. Multiple R-squared values for all these regression models are greater than 0.95 for all substances except, also, the third substance, where the value of Multiple R-squared is 0.86. The values of these regression models are extremely significant: $p < 0.001$ (Figure 2).

The second type of statistical analysis is based on the evaluation of the differences between the numerical vector averages for these variables denoted with PMN, PMN + OZ and PMN + OZ + S both global and relative to each substance considered separately. Since the samples number is not very large, n = 11, and the Shapiro Wilk test to testing normality has dropped with an extremely significant p-value, $p < 0.001$, then the nonparametric test best suited to this situation is Kruskal-Wallis H test. For the PMN and PMN + OZ variables, we obtained $p = 0.99$, which implies no significant differences between the average values compared.

We have a completely different situation when the seven substances are involved because the p- values are extremely significant, $p < 0.0001$, which implies significant differences between the averages of the seven samples denoted with PMN + OZ + Si (i = 1 - 7). For this last variable PMN + OZ + Si (i = 1 - 7), it is necessary to continue the statistical analysis with post-hoc tests detailing how large are the differences and what pairs are targeted. Since the statistical assumptions have not changed, a nonparametric test, namely a signed-rank test for paired data, is applied in the post-hoc analysis. The matrix of the p values for the evaluation of the differences is presented below and can be evaluated visually in the Figure 4.

	S1	S2	S3	S4	S5	S6	S7
S2	0.0050	-	-	-	-	-	-
S3	4e-05	0.0735	-	-	-	-	-
S4	0.0245	0.3489	0.8470	-	-	-	-
S5	0.0028	0.0056	0.0083	0.0073	-	-	-
S6	0.0028	0.0083	0.0146	0.0077	0.0613	-	-
S7	0.0028	0.0090	0.0146	0.0167	0.0366	0.3520	-

Figure 4.
The matrix of the p-values for the evaluation of the differences obtained within the chemiluminescence determination of the synthesized aminoacetanilide N'-substituted

With the exception of pairs S6-S7, S2-S3-S4, whose differences are not significant, the effects of substances are significantly different or even extremely significant in many situations.

The ROS level decreased in all treated groups and considering the results after the statistic interpretation of data, the treatment with S1 induced the most significant effect on the PMN cells (Figure 2 and Figure 4). Considering the results obtained from the MTT test (Figure 1), the aminoacetanilide S1 we observed a less toxic effect on human leucocyte cultivated *in vitro*.

The decrease of ROS levels is correlated with an inhibitory activity on human leucocytes and an anti-inflammatory effect of the local anaesthetics [5, 12]. The effect could be a beneficial one in case of topical application on short term administrations by contributing to a longer pain relief effect [12]. Though, depending on the dose and period of time of administration the anti-inflammatory effect could favour a secondary infection of topic lesions and further preclinical studies are required to determine this potential effect.

Conclusions

Local anaesthetics are usually administered in inflammatory states and their possible interference with local cell signalling processes may influence the tissue repair mechanisms within at the affected area.

The viability of the human polymorphonuclear cells (PMN) separated from human peripheral blood determined by MTT test was not significantly influenced by the previously synthesized aminoacetanilide N'-substituted derivatives (S1-S7) after 24 h treatment with S1, S2, S6 and S7, and after 48 h of treatment with S2 and S7.

The *in vitro* treatment with all synthesized amino-acetanilides decreased the ROS level released by the PMN. Significant responses were obtained after the treatment with aminoacetanilide S1 and the low toxicity on cell viability recommend this substance for future preclinical studies regarding the anti-inflammatory effect of this local anaesthetic.

Acknowledgement

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