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Research Article

Screening of anti-inflammatory activity of 4.5-dihydroisoxazol-5-carboxamide (PAR-2 inhibitors) based on formaldehyde oedema model among white lab rats

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Abstract

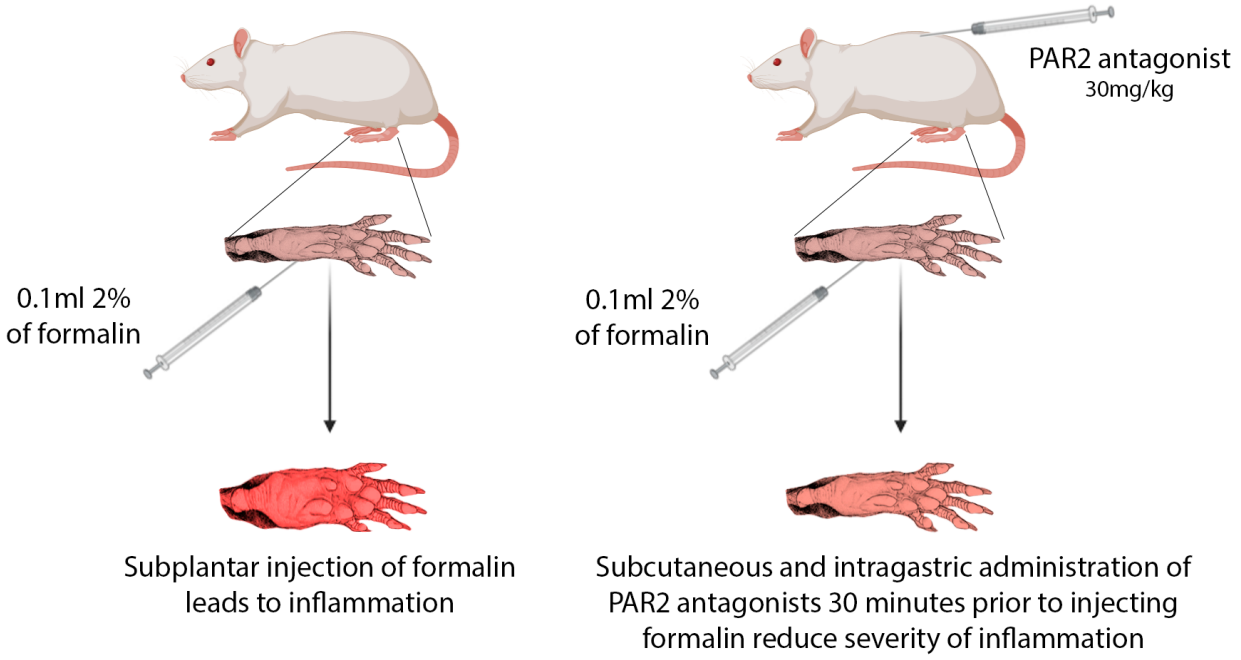
Introduction: Rheumatoid arthritis (RA) is an immune-inflammatory disease intrinsic to up to 1.0% of the world's population. Standard drugs for RA therapy are basic medications, glucocorticoids and non-steroid anti-inflammatory drugs, which often only ease or hinder the course of disease, not curing the patient completely. Also, on the average 20-50 % of patients are unresponsive to treatment, allergic to the prescribed drugs or find them ineffective. That is why medications with a different mechanism of action are being widely researched, some of them being antagonists of proteinase-activated receptors (PAR-2).

Materials and Methods: The inflammatory process was reproduced by injecting a 2% solution of neutral formalin in a volume of 0.1 mL under the aponeurosis of the posterior left foot. A total of 84 white rats were used in the experiment. Diclofenac sodium was administered as a reference drug.

Results and Discussion: An experiment on researching the impact of 5 samples of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development among rats showed that the compound R001, compared with other substances studied, differs in the late onset of the therapeutic effect when ingested; the restoration of the foot volume to the initial level occurs only after the introduction of R005, R004 and R002. R005 to a greater extent than other compounds prevents the development of edema and has greater therapeutic efficacy than diclofenac sodium both with intragastric administration and subcutaneous injection.

Conclusion: All five compounds in question showed anti-inflammatory activity, with the spectrum not so unequivocal both in qualitative and quantitative values.

Graphical Abstract



Keywords

4.5-dihydroisoxazol-5-carboxamide derivatives, formaldehyde oedema, PAR-2 inhibitors, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is an incurable-as-of-today immune-inflammatory disease with multi-factor aetiology intrinsic to up to 1.0 % of people worldwide (GBD 2021 Rheumatoid Arthritis Collaborators 2023), increasing with age. The reason for RA development is still unknown, although the risk of contracting the disease is considered to be 60% due to genetic factors (susceptibility genes HLA-DRB1, TNFRSF14 и PTPN22 are closely related to developing RA) (Ding et al. 2023). The influence of malfunctioning immune system is also great; therefore, the disease pathogenesis activates mechanisms of both congenital and adaptative immune response (Scherer et al. 2020).

Nowadays, the “treat to target” (T2T) strategy adopted in 2010, is dominant in treating RA. It does not recommend any particular drugs, but only gives general recommendations for treatment. The main goal of pharmacotherapy of RA is to achieve remission (or low activity) of the disease (Calabro et al. 2016). The widely adopted standards of RA drug therapy with basic anti-inflammatory medications, glucocorticoids and non-steroid anti-inflammatory drugs have remained unchanged for a long time now, although they often only ease or hinder the course of disease, not curing the patient completely (Prasad et al. 2023). Besides, clinical experience proves that drugs used in clinical treatment of RA are not perfect. On the average, 20-50% of patients are unresponsive to treatment, allergic to the prescribed drugs or find them ineffective, so they have to stop treatment due to serious side effects (Calabro et al. 2016). In view of this, new pharmacological therapy targets are being researched. Presently, clinical and pre-clinical research to discover and work out new anti-rheumatic drugs based on Janus kinases inhibitors, NF-kB transcription factor, mitogen-activated kinase p38, histone deacetylase, low molecular antagonists of proteinase-activated receptors PAR-2, anti-PAR-2-specific monoclonal antibodies is underway.

Among the promising therapeutic targets are receptors activated by proteinase or proteinase-activated receptors, PARs. They belong to the class of receptors connected to G-protein (GPCR). The definitive peculiarity of these receptors is their irreversible activation by proteinase. Discovered in the 1990s, the 4 forms of PARs: PAR-1, PAR-2, PAR-3 and PAR-4 – are expressed on the cell membrane of practically all organs and systems and regulate many physiological functions, including contraction of smooth muscle cells, sensitivity to pain, release of lipid mediators, cytokines and neuropeptides. Activation of PAR is related to such clinical manifestations

as inflammation, oedema and pain (Bao et al. 2013).

The factor of PAR activation is coagulation proteinase FXa – key factor of blood coagulation system (leads to thrombin formation from prothrombin). FXa is also a key modulator of system inflammatory response through PAR-1 and PAR-2 activation of NF-kB transcription. PARs activation induces degradation of I-κB, protein-inhibitor, which prevents translocation of NF-kB to nucleus in order to activate promotor parts of many proinflammatory genes. Therefore, PAR-2 activation promotes inflammation, fibrosis and proliferation of dense connective tissue, while PAR-2/ FXa system is a key modulator of system inflammatory response. FXa inhibition prevents launching pathology development mechanism (Russo et al. 2023).

As for drugs for PA therapy, the use of low molecular PAR-2 antagonists is preferable, since they, unlike peptide ones, are more stable *in vivo* and have better pharmacokinetic profile and availability (Cho et al. 2016).

Aim of the study: to search for new anti-inflammatory medications – PAR-2 antagonists, the derivatives of 4.5-dihydroisoxazol-5-carboxamide.

Materials and Methods

Methods

Synthesis of PAR-2 antagonists, the derivatives of 4.5-dihydroisoxazol-5-carboxamide, was based on the preliminary mathematical forecasting the pharmacologically important properties of the whole number of multinuclear derivatives of imidazole, isoxazole and oxazole with a wide structural variety. The agonistic activity of the compounds towards PAR-2 was evaluated in vitro CHO cell line with high expression of human PAR-2 (Abassi et al. 2009). Five most active PAR-2 antagonists – derivatives of 4.5-dihydroisoxazol-5-carboxamide, the structure of which is shown in Figure 1, were chosen for further in vivo research of their pharmacological properties.

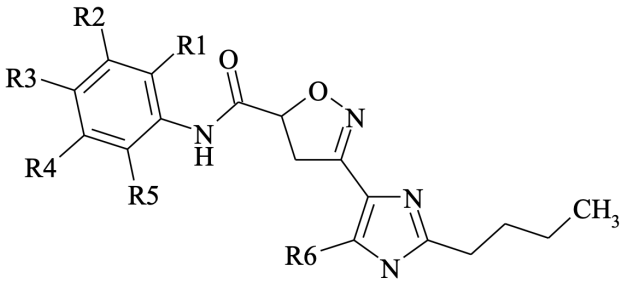


Figure 1. General formula of synthesised derivatives of 4.5-dihydroisoxazol-5-carboxamide.

Animals

Experiments aimed at studying the influence of five selective PAR-2 antagonists on inflammatory process involved 82 white outbred lab rats with body weight of 180-220 g, kept at the temperature of 22±2 C^0, 55±5% humidity and 12/12-hour light cycle, with unlimited access to food and water. Research on animals was approved by the independent Ethics Committee of the federal state budget educational institution of higher education "Yaroslavl State Medical University" under Ministry of Health of the Russian Federation, Minutes of Meeting No.6 on September 14, 2023.

Modeling inflammation process

Inflammation process was reproduced by injecting 0.1 mL of 2% neutral formalin (Mironov 2012; Kong et al. 2015). All animals were divided into 7 groups, with 12 rats in each: the first – control group (rats were injected with saline); rats in the second group were administered with 25 mg/kg diclofenac sodium as the reference anti-inflammatory medication; rats in groups 3 to 7 were given 30 mg/kg of 1% suspension of the compounds under research. Each group of animals was subdivided into two sub-groups with medication given enterally or parenterally. All medications were administered 30 minutes prior to injecting formalin. Derivatives of 4.5-dihydroisoxazol-5-carboxamide were used under laboratory codes R001 through R005. Duration of the experiment was 24 hours. 90, 180, 300, 420 minutes and 24 hours after administering formalin; the fixed area of the affected paw of every rat was examined with a digital anhydrous plethysmometer PH1901 (Russia) and local temperature was measured with a thermometer with a remote probe (HANNA-HI 98509 Checktemp 1).

Statistical analysis

The obtained results were statistically processed with Biostat programme. The number of determinations of each reading in different experiments was 6-10. For inter-group comparison, Student's *t*-test was applied (in case of normal distribution) or non-parametric Wilcoxon criterion (in case of no normal distribution), for multiple comparisons Student's criterion with Bonferroni correction was applied. Accuracy of inter-group differences was defined with Student's paired t-criterion. Differences were considered significant at $p<0.05$ (Glantz 1998).

Results and Discussion

After subplantar injection of formalin, animals in control group developed inflammatory oedema: within the first 90 minutes of the experiment, size of paw was authenticated as growing by 35.0±6.0% (Δ34.3±5.0), with local temperature increasing by 23.7±3.2% (Δ5.6±0.7 C^0). Pathology was increasing for 5 hours: paw size grew practically 1.5 times (Δ45.3±4.0), the temperature – by 1/3; the process stabilised by the 7th hour of observation. Though within the next 24 hours the inflammation dynamics was reversed, the size of paw was still authenticated as increased by 15%.

Diclofenac sodium, like all medications in the experiment was administered in two ways: oral (enteral) or parenteral (subcutaneous). It should be mentioned that in both cases the reference medication did not affect either oedema formation or a local temperature increase at the first stage of the experiment (90 minutes after administering formalin), and, 24 hours later, the research parameters were still authenticated as being higher than the original ones. With diclofenac sodium administered enterally, inflammatory process dynamics was mostly the same as with that of control group (Tables 1 and 2).

Table 1. Impact of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development (affected paw size in mm³) with enteral administering

Medication	n	Original value	After 90 min	After 180 min	After 300 min	After 420 min	After 24 hours
Control	10	99.8±2.4	133.4±3.7*	142.6±3.2*	145.9±3.6*	141.8±4.8*	114.0±4.7*
Diclofenac 25 mg/kg	6	107.8±3.8	146.6±6.4*	142.8±3.6*	150.6±3.2*	142.8±3.0*	119.4±4.0*
R001 30 mg/kg	6	107.0±7.4	147.0±6.0*	153.2±7.0*	153.8±8.6*	142.2±8.0*	123.8±7.8*
R002 30 mg/kg	6	109.6±2.4	144.4±9.6*	142.4±9.8*	144.2±10.8*	137.6±8.8*	117.0±6.0
R003 30 mg/kg	6	106.2±3.6	152.4±5.4*/**	149.0±4.6*	150.0±7.4*	144.2±6.2*	123.8±40*
R004 30 mg/kg	6	110.0±3.8	148.2±7.6*	142.4±8.6*	138.6±8.4*	136.2±8.2*	115.6±2.4
R005 30 mg/kg	6	100.1±4.7	133.4±4.2*	130.2±3.0*/**/***	129.8±2.8*/**/***	122.2±5.6*/**/***	109.0±3.4

Note: * – authenticated difference from original value; ** – authenticated difference from control; *** – authenticated difference from diclofenac group.

Table 2. Impact of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development (local temperature of affected paw in C⁰) with

Medication	n	Original value	After 90 min	After 180 min	After 300 min	After 420 min	After 24 hours
Control	10	24.8±0.4	30.4±0.6*	31.8±0.3*	32.7±0.4*	32.4±0.3*	29.2±0.6*
Diclofenac 25 mg/kg	6	23.1±0.3	32.7±0.6*/**	32.5±0.5*	32.6±0.5*	31.3±0.5*	26.9±1.6*
R001 30 mg/kg	6	23.0±0.2	32.8±3.0*	33.1±0.3*/**	32.7±0.4*	31.9±0.3*	26.6±0.7*
R002 30 mg/kg	6	22.7±0.2	32.8±3.0*	32.9±0.4*/**	33.2±0.3*	33.3±0.2*	28.4±1.2*
R003 30 mg/kg	6	23.1±0.3	32.9±0.2*/**	32.5±0.6*	33.5±0.4*	32.4±0.7*	27.7±1.2*
R004 30 mg/kg	6	22.9±0.2	33.4±0.4*/**	32.8±0.3*/**	33.5±0.3*	33.1±0.2*	25.5±0.8*
R005 30 mg/kg	6	22.8±0.1	33.0±1.3*	33.2±0.7*	32.8±0.3*	31.9±1.0*	27.1±1.6*

Note: * – authenticated difference from original value; ** – authenticated difference from control; *** – authenticated difference from diclofenac group.

The only valid differences were the following: after 180 and 420 minutes of the experiment, the oedema development rate as compared to that in the control group decreased from 43.7±4.4% to 32.0±2.6% and from 43.2±4.7% to 31.0±2.2% respectively. After the parenteral administeration of diclofenac, its therapeutic effect was more evident (Tables 3 and 4). In particular, the medication authentically decreased the rate of local temperature increase in rats' paws throughout the experiment by 27-41% (from 5.6±0.7 C⁰ in control group to 3.9±0.3 C⁰ in diclofenac group after 90 minutes and from 7.6±0.6 C⁰ to 4.9±0.9 C⁰ after 420 minutes), and decreased the rate of local temperature increase after 180 minutes (between the 180th and 420th minutes after administering formalin, paws size in control group increased by 43.2-47.2%, and with diclofenac administered – only by 29.6-32.2%).

With R001 enteral administering, anti-inflammatory effect was growing slowly (Tables 1 and 2): affected paw size was growing throughout 5 hours of the experiment (Δ of original paw size by the 90th minute was 39.8±2.4 mm³, and by 300th minute – already 46.8±4.4 mm³) and only after that it started to decrease, although at a greater rate than in control group. Thus, by the 7th hour, the rat's paw size with formalin oedema was already 11% smaller than after 90 minutes. At the same time, in control group, this value was higher by even 23%. There was authenticated higher local temperature of the affected paws than that in control group from the 90th to 420th minutes of the experiment.

Table 3. Impact of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development (affected paw size in mm^3) with subcutaneous administering

Medication	n	Original value	After 90 min	After 180 min	After 300 min	After 420 min	After 24 hours
Control	10	99.8±2.4	133.4±3.7*	142.6±3.2*	145.9±3.6*	141.8±4.8*	114.0±4.7*
Diclofenac 25 mg/kg	6	96.2±4.4	129.4±4.4*	**126.8±3.4***/**	**126.0±2.4***/**	**124.2±3.2***/**	113.2±3.6*
R001 30 mg/kg	6	91.0±3.0	127.8±6.2*	**128.8±3.2***/**	**122.2±2.6***/**	**118.0±2.4***/**	107.6±1.8*
R002 30 mg/kg	6	101.0±5.6	133.6±7.0*	135.6±8.6*	136.4±11.0*	127.0±7.0*	114.4±6.0*
R003 30 mg/kg	6	97.0±2.6	127.4±6.6*	**126.8±3.8***/**	**125.6±4.4***/**	**116.8±1.6***/**	112.4±3.6*
R004 30 mg/kg	6	91.6±4.6	131.8±5.2*	129.6±6.8*	128.6±7.2*	**121.6±7.0***/**	104.6±3.8*
R005 30 mg/kg	6	90.7±3.0	123.0±5.6*	**127.4±3.4***/**	**118.6±4.8***/**	**112.0±4.0***/**/***	**104.6±3.6***/***

***Note*:** * – authenticated difference from original value; ** – authenticated difference from control; *** – authenticated difference from diclofenac group.

Table 4. Impact of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development (local temperature of affected paw in C^0) with subcutaneous administering

Medication	n	Original value	After 90 min	After 180 min	After 300 min	After 420 min	After 24 hours
Control	10	24.8±0.4	30.4±0.6*	30.4±0.6*	32.7±0.4*	32.4±0.3*	29.2±0.8*
Diclofenac 25 mg/kg	6	27.8±1.2	31.2±0.5*	31.2±0.5*	31.9±0.5*	32.1±0.4*	31.4±0.9*
R001 30 mg/kg	6	26.8±0.6	32.4±0.6*/**	32.4±0.6*/**	31.9±1.1*	31.1±1.4*	29.6±1.1*
R002 30 mg/kg	6	26.6±0.6	32.3±0.6*/**	32.3±0.6*/**	32.5±0.4*	31.3±1.3*	29.0±1.5*
R003 30 mg/kg	6	27.8±1.1	32.7±0.5*/**	32.7±0.5*/**	32.9±0.3*	31.8±0.3*	28.4±1.5
R004 30 mg/kg	6	26.7±0.6	33.3±0.4*/**/***	33.3±0.4*/**/***	32.3±0.4*	32.8±0.4*	28.6±0.9*
R005 30 mg/kg	6	27.7±1.0	32.3±0.8*	32.3±0.8*	32.9±0.2*	32.2±0.4*	31.5±0.8*

Note: * – authenticated difference from original value; ** – authenticated difference from control; *** – authenticated difference from diclofenac group.

To some extent, it was characteristic of all the other research compounds (R002, R003, R004 and R005), administered enterally. With subcutaneous administering (Tables 3 and 4), anti-inflammatory activity of R001 compound increased: between 90-180 minutes of the experiment, authenticated increased paw size stabilisation was observed (Δ36.8-37.8 mm^3), and then, starting with the 5th hour of the experiment, there was an authenticated oedema level decrease by 17%. The oedema level decreased by 27% compared to control.

After enteral administering R002, the size of the paw was decreasing quicker than in the control throughout the whole experiment. The decrease started from the 3rd hour after administering formalin, and, by 7th hour, the oedema level dropped by 37%. By the 24th hour of the experiment, the paw size returned to original. Parenteral administering of medication did not lead to any increase in its anti-inflammatory activity. In particular, the substance effect was observed within the 3rd hour of the experiment, and an authenticated oedema decrease after 420 minutes was down by 38% compared to control. However, after parenteral administering, there was an authenticated local temperature drop by 1.6 times compared to control group values.

After enteral administering R003 on the 90th minute of experiment, the paw size tended to increase as compared to control group. The oedema level decrease started from the 180th minute and by 420th minute it was 26% lower than original (maximum) value. On the whole, within 180-420th minutes, the rate of oedema level decrease was 1.5-1.8 times higher than that in control group. Like in case of R002, parenteral administering R003 did not lead to any increase units anti-inflammatory activity; medication effect was observed from the third hour of the experiment, and an authenticated oedema decrease by he 420th minute of the experiment was 30% compared to control and 10-18% of its maximum value at the beginning of the experiment. Also an authenticated drop in the local temperature increase by 1.7 times was noted compared to control group values in the 300th and 420th

minutes of experiment.

Enteral administering R004 for therapy of formalin oedema no later than epy 3rd hour of epy experiment caused an authenticated 1.8-time decrease in the affected paw size as compared to the untreated rats; by the 7th hour, the oedema level dropped by 36%. By the end of the experiment, the paw size restored to original. Parenteral administration of the medication did not increase its anti-inflammatory activity but contributed to normalisation of local temperature on the affected paw by the 24th hour of the experiment.

With enteral administration of R005, in the 90th minute of the experiment, the smallest oedema level of the affected paw was authenticated as compared with other experiment groups, and, compared to original value, the rate of oedema development was 10% lower than in control. R005 was the only one of the research compounds, which between the 180th and 420th minutes of the experiment showed an authenticated oedema level decrease not only when compared to control, but also to diclofenac group: by the 180th minute – by 10% and 10%; after 300 minutes – by 12% and 16%; and after 420 minutes – by 16% and 17%, respectively. Complete recovery of the paw to original size occurred by the 24th hour. With subcutaneous administering R005, the paw size started to decrease as compared to control also from the 180th minute: after 180 minutes – by 11%; after 300 minutes – by 19%; and after 420 minutes – by 21%, respectively. R005 is more active than diclofenac, as it decreased the affected paw size within the 7-hour and 24-hour intervals by 10% and 8%, respectively. Local temperature (by Δ) of the paw decreased as compared to control group with R005 subcutaneous administering between the 180th and 420th minutes by 34-43%.

Conclusion

Thus, the experiment on researching impact of 5 samples of derivatives of 4.5-dihydroisoxazol-5-carboxamide on formalin oedema development among rats proved the anti-inflammatory activity of all the research compounds. However, the spectrum of this activity was not unequivocal considering both qualitative and quantitative values:

•R001 compound compared to other research substances has a later start of therapeutic effect if administered enterally (latent period of more than 5 hours).

•Administering by injection increases the anti-inflammatory effect only for R001 and reference preparation diclofenac sodium.

•Paw returns to its original size only after administering R002, R004 and R005.

•R005, more than others, prevents oedema development, decreasing it already by the 90th minute of the experiment.

•With researched oedema type, R005 has higher therapeutic efficiency than diclofenac sodium, both with enteral administering and subcutaneous injection.

Furthermore, the research showed lack of definite parallelism between local temperature and paw size (oedema intensity). Inversion of temperature reaction was discovered depending on the way of administering medications: with enteral administering, local temperature was higher in experimental groups, with parenteral administering – in control group.

Summing up the results obtained, we can line up the following compounds activity series: R005>>R002 = R004> diclofenac sodium = R003>R001.

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Conflict of interests

The authors declare no conflict of interests.

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