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FURTHER EVALUATION OF NEWLY SYNTHESIZED ANTI-INFLAMMATORY PYRROLE DERIVATES: ROS FORMATION IN LIPOSOME MODEL SYSTEM IN VITRO

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Summary. Antioxidants are important components of cocktail therapy of both acute and chronic inflammation. The oxidative activity of eight pyrrole derivatives, previously designed, synthesized and evaluated as potential anti-inflammatory agents, was studied using TBARS test for in vitro registration of Fe-induced lipid peroxidation in liposome model system. In the current study, significant antioxidant effects (up to 78%) were observed in comparison with control (liposomal suspension with induced lipid peroxidation without any additives). All tested pyrrole derivatives exhibited dose-dependent activity whereat the highest values were observed predominantly at 10^{-4} M. The compounds being identified to possess the highest anti-inflammatory activity in our previous study (**2e, 2f, 3b and 4d**) were found again to be the most active ones in the antioxidant assay. The highest antioxidant effects were comparable with that of the reference Metamizol being about 90% of the activity of Paracetamol tested in the same system.

Key words: inflammation, NSAID, oxidative stress, pyrroles, TBARS test

ПОНАТАТЪШНИ ИЗПИТАНИЯ НА НОВОСИНТЕЗИРАНИ ПРОТИВОВЪЗПАЛИТЕЛНИ ПИРОЛОВИ ПРОИЗВОДНИ: ROS ОБРАЗУВАНЕ В ЛИПОЗОМНА МОДЕЛНА СИСТЕМА IN VITRO

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Резюме. Антиоксидантите са важни компоненти на коктейлната терапия както на острите, така и на хроничните възпаления. Оксидативната активност на осем пиролови производни, проектирани, синтезирани и изпитани преди като потенциални противовъзпалителни агенти, бе изследвана чрез TBARS за in vitro регистриране на Fe-индуцирано пероксидиране на липиди в липозомна моделна система. В настоящото изследване бяха наблюдавани значителни антиоксидантни ефекти (до 78%) в сравнение с контролата (липозомна суспензия с индуцирано пероксидиране на липиди без никакви добавки). Всички тествани пиролови производни показаха дозозависима активност, при което най-високите стойности бяха наблюдавани предимно при 10^{-4} М. Съединенията, които в нашето предишно изследване бяха идентифицирани с най-висока противовъзпалителна активност (**2e, 2f, 3b и 4d**), се оказаха отново най-активните при антиоксидантните изпитания. Най-високите антиоксидантни ефекти бяха съпоставими с тези на референтната субстанция метамизол с около 90% от активността на парацетамол, тестван в същата система.

Ключови думи: възпаление, НСПВС, оксидативен стрес, пироли, TBARS тест

Introduction

Oxidative damage of the tissues takes important place in the pathogenesis of chronic inflammatory diseases, for instance all kinds of rheumatoid arthritis [11]. Light inflammation may not require specific therapy while severe inflammatory processes could have lethal outcome; chronic inflammation increases the risk of cancer. Anti-oxidative cocktail therapy includes application of antioxidants, super-oxide dismutase (SOD) mimetics, and cooling and nitric-oxide synthase (NOS) inhibitors.

The infiltration of damaged tissue by phagocytes is a typical process for the inflammatory reactions. The latter initiate oxidative stress in living organism: the leukocyte activations induce “respiratory burst” with release of reactive oxygen species (ROS)

and hypochloric acid is synthesized by enzyme myeloperoxidase (MPO). Increased phospholipase A2 activity leads to production of arahidonic acid by membrane lipids followed by enzymatic oxidation (by lipoxygenases up to leucotriens and by cyclooxygenases (COX) up to prostaglandins and tromboxans). Metabolism of arahidonic acid is well known as a second major phagocytic source of ROS (as lipid hydroperoxides and hydroxyl radicals) [10].

Free radical processes in living organism could be impact by exogenic way as follows:

- direct reducing of abnormal production of ROS by means of antioxidant molecules;
- by inactivation of already formed prooxidants;
- by means of phagocyte inhibitors or by COX inhibitors.

Pyrrole derivatives gained popularity as a recognized template in the search for new nonsteroidal anti-inflammatory drugs (NSAIDs) including specific COX-2 inhibitors [4, 12]. On the other hand, contemporary evaluations of heterocyclic compounds pointed out pyrroles as prospective antioxidants as well [6, 9, 14]. Since a number of related biochemical pathways interfere in both oxidative and inflammatory reactions as noticed above, the present study aims at *in vitro* evaluation of the antioxidative activity of eight recently synthesized pyrrole derivatives whose *in vivo* screening showed significant anti-inflammatory effects [3].

Material and Methods

Preparation of the target compounds

The pyrrole derivatives being considered were synthesized by Paal–Knorr cyclization between relevantly substituted 1,4-dicarbonyl compounds and primary amines according to the procedure reported in our previous paper [3]. Their design was based on the architecture of the tricyclic COX-2 inhibitors with Celecoxib (CAS 169590-42-5) used as a prototype whereas the desired structural diversity was achieved by introduction of structural elements typical for a number of active molecules, such as salicylic, isonicotinamide and pyrazolinone moieties as shown in Fig. 1. For the sake of convenience the original compound codes [3] were retained.

Antioxidative activity

TBARS-test (Thiobarbituric Acid Reactive Substances) was applied for *in vitro* registration of Fe-induced lipid peroxidation in liposome model system in comparison with control (liposomal suspension with induced lipid peroxidation without any additives). The influence on the ROS formation in liposome suspensions was investigated by measurement of malondialdehyde (MDA), which is the final product of lipid peroxidation in the same model systems. The sensitivity of TBARS has made this assay the method of choice for screening and monitoring lipid peroxidation, a major indicator of oxidative stress [1, 13]. Despite of some controversy regarding the specificity of TBARS toward compounds other than MDA, the test remains the most widely employed assay used to determine lipid peroxidation [1].

According to TBARS-test, MDA is captured by thiobarbituric acid in a colored product comprising 532 nm chromophore by means of condensation presented in Figure 2.

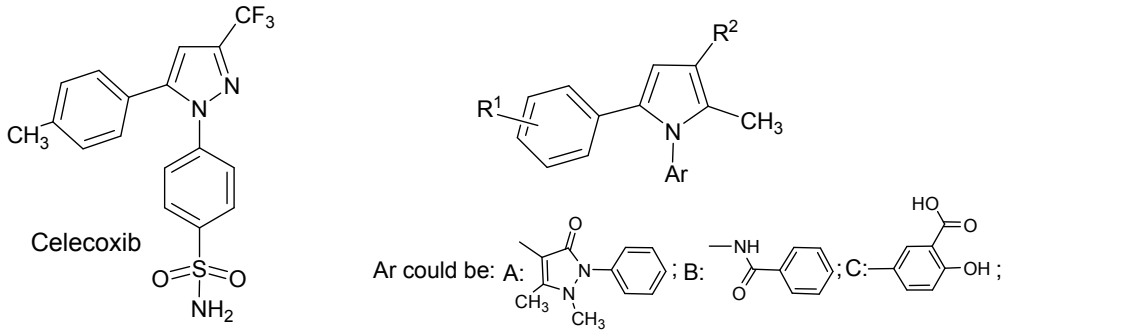

Fig. 1. *Eight pyrrole derivatives designed as potential NSAIDs with Celecoxib used as a prototype*

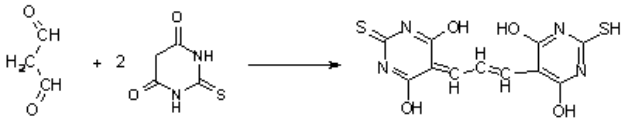

Fig. 2. *Condensation of TBA with MDA and creation of 532 nm chromophore*

The concentration of MDA as a product of lipid peroxidation is calculated from the molar extinction coefficient (E) as specified below. Lecithin liposomes are isolated by the method of Folch [8].

1 ml 20% solution of trichloroacetic acid was added for 30 min at 37°C after the incubation of Lecitine liposomes in PBS pH 7.4 in the presence of 2.5 x 10^{-3} M/l $FeCl_2$. The samples were centrofuged at 3000 rpm for 10 min, the supernatant was taken off and 1.8 ml of 0.5% thiobarbituric acid was added to 2 ml of supernatant. The resulted solution was incubated at 90°C for 40 min to obtain pink color with λ_{max} = 532 nm [2]. The molar extinction coefficient (E) was measured at 532 nm with Ultra Visible Spectrophotometer (Cecil 3000 series, Cambridge, England) with polystyrene quvettes, and the antioxidant activity (AOA%) was calculated for each of the tested compounds according to equation (1):

$$AOA_{\%} = \frac{E_k - E_1}{E_k} * 100 \qquad (1)$$

where: $E_к$ presents the extinction of the control at λ =532 nm;

E_1 is the extinction of the investigated sample at λ = 532 nm.

ANOVA statistical method for evaluation of significance of the present results was applied, Bonferoni test for statistical significance in the range P > 0.05 was applied and results are presented as ± S.D.

Results and discussion

Antioxidative activity

The relevant antioxidant activities registered at 10^{-4}M, 4 x 10^{-5}M, 10^{-5}M are presented in Figure 3.

Although the highest antioxidative activity was observed at the referent drug Paracetamol (near 90%), statistical significant effects were registered within all three subcategories (A, B and C) of the tested series. All tested pyrroles derivatives exhibited dose-dependent activity with highest values observed predominantly at 10^{-4} M.

The compounds being identified to possess the highest anti-inflammatory activity in our previous study (such as **2e**, **2f**, **3b** and **4d** presented in Fig. 3) were found again to be the most active ones in the current antioxidant assay with activities comparable to that of the referent drug Metamizol and about 90% of that of Paracetamol. The results justified the main conception of the applied structure design confirming that all three types of structural moieties (salicylic, isonicotinamide and pyrazolinone) may contribute to the total antioxidative activity of the pyrrole derivatives.

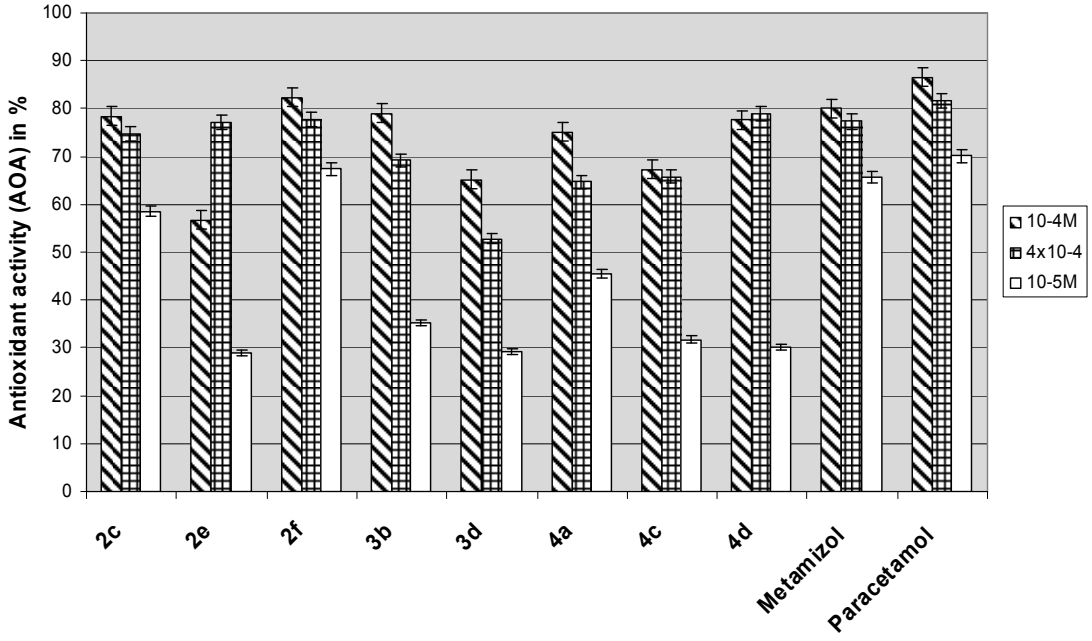

Fig. 3. *Antioxidative activity of the compounds evaluated at three different concentrations according to TBARS test*

According to previous studies [5, 7], electron-donating substituents to the pyrrole ring should increase electrondensity at carbon atoms in the heterocycle, which subsequently increases radical scavenging activity. In contrast, the presence of electron-withdrawing substituents should decrease the ability to scavenge free radicals. Due to the presence of both types of substituents in the tested compounds (such as electron-donating methyl- and electron-withdrawing acetyl-, respectively ethoxy-carbonyl group), their individual contributions were not distinguishable and further investigations should clarify these particular effects. Regarding the influence of the substituents R^1 in the phenyl residue, it is interesting to notice that the introduction of a second methoxy group was proved to be unfavorable, since it changed the antioxidative activity of **3b** to almost pro-oxidative, observed at the 3,4-di(CH_3O)-derivative **3d**.

The total findings encourage the search for prospective antioxidants in the group of anti-inflammatory pyrrole derivatives, including future evaluations of antioxidative activity in model systems in vivo.

Conclusion

The complex nature of inflammatory processes in living organisms is related to variety of biological targets and requires complex properties of the adequate drugs. The evident parallel between the anti-inflammatory effects and the antioxidant activities of the evaluated pyrrole derivatives could serve as reliable background and starting point for future design of prospective structures capable for multiple interactions.

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