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Inhibitory effect of *N,O*-acil-threo-DL-phenylserine derivatives on rat adjuvant arthritis

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Rat adjuvant arthritis (AA), a model of chronic inflammation and an experimental model of rheumatoid arthritis (RA) has been used to evaluate the possible anti-inflammatory action of seventeen *N,O*-acil-threo-DL-phenylserines **1**–**17**. For this purpose experiments on 200 male Wistar rats were performed. The obtained results showed that oral administration of all compounds, except **15**, inhibited the development of AA as compared with the control group. Although the level of arthritic suppression of some preparations was equivalent to aspirin or exceeded it (compounds **2**–**4**, **6**, **10**, **13**), only the preparation **10** in a dose of 100 mg/kg significantly change the paw swelling ($P < 0.02$) which decreased by 27.9–31.7% at the end of experiment. Pronounced inhibition ($P < 0.05$–0.01) of joint swelling and the indices of pathological process activity were revealed in earlier stages of AA under the treatment with compounds such as **6** and **11**. The antiinflammatory effect of some compounds (**9**, **11**, **13**) was observed in the middle of the experiment, although only compound **9** showed significant suppression by 35.9–37.9% at this stage ($P < 0.01$–0.001) which also markedly decreased ESR ($P < 0.05$). The latter index was significantly reduced by the action of preparations **2**–**4**, **6**–**8**. Most of the compounds investigated improved blood indices and prevented the development of polyarthritis.

1. Introduction

The analgesic, anti-inflammatory, and antipyretic drugs are a heterogenous group of compounds belonging to different chemical groups, although the majority are organic acids (Yaksh et al. 1998). Non-steroidal antiinflammatory drugs (NSAIDs) remain the primary pharmacological agents for symptomatic pain relief in patients with arthritis (Botting and Botting 2000; Geis 1999; Hedner and Everts 1998; Smith et al. 1998). However, their use is often restricted by the risk of side effects, particular in the upper gastrointestinal tract. It is known that inhibition of the enzyme cyclooxygenase (COX) is the principal mechanism for both the efficacy and the toxicity of NSAIDs (Crofford 2000; Langenegger and Michel 1999). In the view of the discovery of the two COX isoforms, investigators currently hypothesise that the efficacy of NSAIDs is mediated by their ability to inhibit COX-2 and the toxicity of NSAIDs is mediated by their ability to inhibit COX-1 (Bombardier et al. 2000; FitzGerald and Patrono 2001; McKenna 1999; Vane and Botting 1997). Some authors (Kawai et al.1998; Pairet and Engelhardt 1996; Pairet and van Ryn 1998) have proposed that the different toxicities of the currently available NSAIDs can be explained by each drug's differential inhibition of COX-1 and COX-2. COX-2 is abundantly expressed in the synovium of rheumatoid arthritis (RA) and is relevant to prostaglandin (PG) E_2 biosynthesis in arthritic joints (Siegle et al. 1998; Yamada et al. 2000). Results obtained from studies performed on animal models of arthritis implicate PGE_2 as a major mediator of both local tissue destruction and systemic immunoregulatory changes associated with RA (Williams et al. 1994).

Furthermore, COX-2 messenger RNA (mRNA) and protein are also expressed in the synovial tissues of rat adjuvant-induced arthritis (Egan et al. 2001) that is one of well-established animal models of RA and likely to be connected with the development of the disease (Anderson et al. 1996; Kang et al. 1996). Strong evidence supports the notion that COX-2 is the isoform important for mediating inflammation and pain in animal models of arthritis (Anderson et al. 1996; Crofford et al. 1994; Sano et al. 1992). Continuous infusion of dexamethasone in animals injected with adjuvant suppressed expression of COX (Crofford 2000) and the treatment of arthritic animals with a specific COX-2 inhibitor, SC-58125 (Searle, St. Louis, MO) suppressed paw swelling by 80–85%.

But the role of COX-2 in inflammation may be much more complicated than originally thought (Bakowsky and Hanly 2000). Indeed, recent evidence suggests that COX-2 may have both proinflammatory and antiinflammatory functions (Gilroy et al. 1999; Jones et al. 1999; McCarthy et al. 1999; McCartney et al. 1999; Zarrilli et al. 1999).

Some alterations of renal function are COX-2 related mechanism-based effects. Both COX isoforms are expressed in the human and rat kidneys (Khan et al. 1998; Kömhoff et al. 1997; Nantel et al. 1999; Yang et al. 1998). COX-2 seems to play some role in the renal plasma flow and glomerular filtration rate regulation in the rat kidney (Turul et al. 2001). COX-2 specific inhibitors also do not appear

to have any significant renal sparing effects compared to unselective NSAIDs (Rossat et al. 1999; Swan et al. 2000).

Blocking the action of COX-2 diminished the early inflammatory phase but also increased inflammation at 48 h in carrageenin-induced pleurisy rat model of inflammation (Gilroy et al. 1999). If confirmed, these data suggest that inhibition of COX-2, by either COX-2 selective inhibitors or unselective NSAIDs, could enhance rather than suppress some inflammatory processes (Bakowsky and Hanly 2000).

Another possibility is that compounds exert their effects by a mechanism that is independent of COX activity. This mechanism could include inhibition of proliferation, induction of apoptosis, inhibition of peroxisome proliferator-activated receptor-δ, or increased formation of intracellular ceramide leading to the induction of apoptosis among other effects (He et al. 1999). Interestingly, all of these COX-independent mechanisms have been postulated for NSAIDs such as aspirin and for the new specific COX-2 inhibitors (Masferrer and Needleman 2000).

Thus, some form of NSAID therapy will continue to be necessary for the symptomatic treatment of chronic inflammatory disease, but, no doubt, the search for new NSAIDs with low toxicity and good tolerability remains. A useful antiinflammatory drug might be a compound with at least the same efficacy as existing preparations, but with a better therapeutic index.

Our previous studies (Dirvianskytė et al. 2003 and 2004) have shown a broad spectrum of biological activity of *threo*-phenylserine derivatives. Therefore the aim of our investigation was a search of anti-inflammatory agents among phenylserine derivatives that could possibly suppress the autoimmune process in rats with adjuvant arthritis (AA). AA in rats, a T-cell mediated autoimmune disease, is commonly used as an animal model of chronic inflammation and pain and as one of the most important models of rheumatoid arthritis.

2. Investigations, results and discussion

Acute toxicity tests showed the most compounds to be of low toxicity. For compounds **11**, **16**–**17** LD_{50} was higher than 1500 mg/kg (i.e. less toxic than ASA; $LD_{50} \approx$ 1200 mg/kg), and for preparations **8**–**10** it was 1000 mg/kg. The lowest toxicity was revealed for preparation **5** (3000 mg/kg) and the highest for preparations **2**–**3** (50 mg/kg). The animals were treated by the 1/10 of LD_{50} (Table 1).

The results of our experiments on rats with AA are summarized in Tables 1 and 2. It should be noted that most of the investigated preparations did not significantly change joint swelling, but the inhibition of paw edema was evident under the treatment with all compounds except **15** (Table 1). This preparation in a dose of 130 mg/kg slighly intensified joint swelling during the experiment except day 12 when it decreased by 14.6%. At the end of experiment joint swelling exceeded control by 5.1%.

Significant suppression of joint swelling in comparison with the control group was observed in the early stages of AA for compounds **16** (day 3; $P < 0.02$), **6** (day 5; $P < 0.05$) and **11** (days 5 and 8; $P < 0.01$–0.02). The latter showed the most expres supression (by 53.7% and 35.2%) which exceeded the effect of aspirin (ASA) on the same days of experiment. Compound **9** exerted an analogous effect in the middle of the experiment (days 10 and 12), where inhibition of joint swelling was 35.9%

Table 1: Biological activity of compounds in rats with adjuvant arthritis

Compd.	LD_{50} (mg/kg)	Dose (mg/kg)	Deviation from control (%)										
			Joint swelling (on day of experiment)						Blood indices				
			3	5	8	10	12	15	17	ESR	Leukocytes	Erythrocytes	Hemoglobin
1	500	50	6.9	−18.1	−4.7	−1.6	−15.4	−8.4	−24.6	−10.2	−42.6***	−6.7	−1.4
2	50	5	1.6	2.7	6.2	−4.0	−15.7	−28.2	−26.6	−31.2***	−18.8	6.1	7.9
3	50	5	0.5	−12.2	−7.4	−6.1	−28.0	−39.1	−35.2	−53.9***	−22.1	−13.5**	11.1
4	500	50	0.7	−2.2	−8.2	−10.4	−25.4	−9.9	−23.2	−30.9*	−20.3*	7.6	0.9
5	3000	300	0.5	−11.5	−8.0	−4.4	−0.8	−0.2	−7.1				
6	800	80	−15.1	−26.6^{+}	−2.2	−6.1	−15.5	−28.5	−23.1	−27.6**	−27.1	−2.2	7.3
7	200	20	−15.6	−13.4	4.2	7.4	−26.5	−12.8	−5.0	−13.2*	−38.5^{+}	4.5	2.3
8	1000	100	−7.0	−17.5	−11.5	−21.4	−18.4	−12.8	−13.6	−21.1***	−36.7	0.8	6.4
9	1000	100	−4.8	−14.2	−15.0	−35.9**	37.9***	−12.2	−10.8	−17.2^{+}	−13.8	13.5	−1.6
10	1000	100	0.7	−12.9	−13.7	−15.5	−25.7	−27.9*	−31.7*	−25.4	−15.6	14.9	12.6
11	1600	160	−19.8	−53.7**	−35.2*	−29.7	−23.8	−18.0	−17.5	−51.0	−65.8***	4.7	
12		100	−5.3	−7.1	−39.8	−48.2	−42.1	−10.6	−1.6	−21.6	−1.3	−1.1	4.3
13		140	10.9	15.1	23.0	15.8	16.2	−28.4	−31.2	−10.9			
14		140	2.9	−8.8	−24.0	−19.1	−8.3	−4.5	−10.3	−9.9			
15		130	1.8	5.8	12.3	4.2	−14.6	5.9	5.1				
16	>1500	200	−19.8*	−21.2	−22.5	−32.0	−23.7	−32.9	−18.7	−9.4	−4.5	16.1	3.1
17	≥1500	200	−19.8	−16.7	−13.6	−14.2	−25.9	−17.9	−14.5	−13.9	2.6	11.2	2.6
ASA	≈1200	120	−30.5^{++}	−45.5***	−22.0^{+}	−24.1	−31.4	−14.0	−22.9	−8.2	−35.1***	4.2	13.3

[a] Differences are significant in comparison with control group. $^{+}$ $P < 0.05$, * $P < 0.02$, ** $P < 0.01$, $^{++}$ $P < 0.002$, *** $P < 0.001$

($P < 0.01$) and 37.9% ($P < 0.001$) and exceeded the action of ASA.

Other investigated preparations (**1**–**5**, **7**–**8**, **12**–**15**, **17**) did not exibit a significant suppressive activity on the primary phase of swelling in the adjuvant-injected hind-paw, which is related with an acute inflammation, nor on the onset and development of the second chronic inflammation (between days 10–17), but the tendency of rather potent antiinflammatory activity, especially in the second stage of AA was observed after the treatment with **1**–**4**, **6**, **13**.

At the end of experiment the compounds **1**–**4**, **6**, **10**, **13** inhibited the secondary phase of inflammation, which is depended on the presence of T lymphocytes and diminished joint swelling by 23.1–31.7% and this level of suppression was more expressed than ASA did. But it should be mentioned that only compound **10** (dose 100 mg/kg) significantly suppressed joint swelling by 27.9–31.7% in comparison with control group ($P < 0.02$).

Polyarthritis characterizing the development of an autoimmune process was absent in the groups treated with preparations **1**, **3**, **6**, **10**, **11**. It developed in 10% of rats given **4**, **7**–**9**, **13**, and in 20% of rats given preparations **2**, **5**, and ASA. Treatment with compounds **15** and **16** induced the development of polyarthritis in 30% of animals and with preparation **17** in 40% of rats. The most incidence of polyarthritis was revealed by preparations **12** (80% of animals) and **14** (70% of rats) what corresponded to the development of polyarthritis in control groups (data not presented).

Some compounds tested improved systemic parameters of the disease, such as blood indices and body weight. ESR was significantly lower in groups treated with preparations **2**–**4** and **6**–**9** (Table 1). A significant lowering of leukocyte count was observed in the groups given preparations **1** (by 42.6%), **4** (20.3%), **7** (38.5%), **11** (65.8%) what exceeded the action of ASA (35.1%), except for compound **4**.

There were no essential differences in erythrocyte count and hemoglobin level among the groups, although they were higher in the treated groups than in control with the only exception of the significant lowering of erythrocytes amount by 13.5% under the treatment with preparation **3** ($P < 0.01$) and a slight decrease caused by compounds **1** (erythrocytes and hemoglobin), **6** (erythrocytes) and **9** (hemoglobin) (Table 1).

Rats treated with all compounds showed no significant change in body weight, although it slightly increased in the groups given preparations **1**–**3**, **9**, and **14** and decreased in rats treated with preparations **4**–**5**, **8**, **9**–**13**, **15**–**17** (Table 2).

Many studies have shown that in the course of AA not only joints with the obvious signs of inflammation, but also visceral organs may be affected by the pathological process.

Changes in protein metabolism implicate hepatic involvement in adjuvant disease and the weight of liver increases. It is also known that body and thymus weights are reduced significantly in the arthritic rats compare to nonarthritic animals (Bruot and Clemens 1987). Splenomegaly is also used as a pathological index of AA. Although the mechanism underlying splenomegaly is not investigated in detail, some authors (Tanahashi et al. 1998) consider immunological abnormalities to be an important factor.

It should be noted that no essential differences in the weight of internal organs were observed among the most groups at the end of experiment. Only preparation **9** significantly increased the liver weight ($P < 0.001$). The beneficial therapeutic effect of compound **3** was observed on the thymus, where the absolute weight of this organ increased ($P < 0.01$) (Table 2).

No toxicity from drug administration was noted during the experimental period.

Macroscopic examination of internal organs revealed spleen changes only in 10% of rats treated with preparations **1**, **3**, **7**, and **14**, and in 30% of animals receiving compound **2**. None of the preparations exerted a toxic effect on the liver.

ASA used in our experiment for comparison as known antiinflammatory medicine, did not exert expressed inhibitory effects on joint swelling at the end of the experiment but showed significant inhibition until Day 8. It may depend on very great intensity of AA in rats and once-a day administration of drugs, because ASA, like most drugs, is rapidly metabolised in the body (Haynes et al. 1993).

Thus, our results demonstrate the tendency of most of the phenylserine derivatives investigated to decrease the symptoms of arthritis, inflammatory changes in blood and internal organs and to prevent the development of polyarthritis. Compounds were safe with anti-inflammatory efficacy comparable to aspirin, which inhibits the enzymatic activity of both COX.

It is known that the first wave of joint swelling in AA is related to nonimmune inflammation, while the second wave depends on the immune response, especially the cell-mediated one (i.e the acute phase of arthritis is independent of T lymphocytes, but the chronic phase of adjuvant-induced arthritis is depended on the presence of T lymphocytes) (Crofford et al. 1997).

The results presented here allow to conclude that some phenylserine derivatives, such as **6**, **11**, **16** were in fact rather effective during the first 8 days, i.e. showed a marked antiinflamatory activity only in early stage of AA. That means, that they suppressed nonimmune inflammation and could influence the pathogenetic mechanisms that dominate in early AA. Others, such as **9**, acted in the middle of treatment, and such as **10**, at the end of experiment. So, preparation **9** exerted its action on both stages of the pathological process and **10** on the chronic phase of AA.

Thus, the pharmacological characteristics of the tested compounds largely depend on their structure. The presence of the phenylsulphonyl group in compound **10** results in increased antiinflammatory potency and improved the development of the autoimmune process. Because the toxicity of most of the investigated preparations is low, an increase of their therapeutic dose could improve their action on inflammatory processes.

Summing up, data obtained in this investigation allow to postulate that some phenylserine derivatives can regulate inflammatory and autoimmune processes in the animal model of arthritis and can be useful for the development of drugs that possess antiinflammatory activity.

3. Experimental

3.1. Chemistry

The *threo*-DL-phenylserine derivatives were synthesized by methods reported previously and structures were proved by common spectroscopic and analytical methods.

3.2. Pharmacology

3.2.1. Animals

Male white mice and Wistar rats obtained from Bioreglament (Vilnius, Lithuania), weighing 20–25 g and 170–200 g respectively, were used in

Table 2: Changes in body and organs weight in rats with adjuvant arthritis treated with phenylserine derivatives

Compd.	Dose (mg/kg)	Weight deviation from control (%)				
		Body	Liver	Spleen	Kidney	Thymus
1	50	5.1	2.4	−9.6	−6.4	−12.5
2	5	4.9	−7.2	−18.3	−6.2	10.9
3	5	4.7	−1.3	−2.0	−5.0	61.4**
4	50	−3.7	6.5	−4.1	0.8	−6.2
5	300	−7.8	−1.3	−4.0	−14.2	54.3
6	80	7.0	12.8	5.0	−1.2	−20.1
7	20	13.8	−0.3	−6.6	3.1	16.8
8	100	−8.9	0.4	16.7	−18.7	−12.1
9	100	5.8	38.9***	28.8	6.4	12.5
10	100	−5.8	3.2	8.2	1.6	−6.2
11	160	−4.2	−1.9	−13.7		
12	100	−2.1	−8.8	−8.3		−6.4
13	140	−5.3	8.3	−10.1	0	−22.2
14	140	2.8	−4.4	2.0	−2.7	2.5
15	130	−3.6				
16	200	−11.4	−6.0	−25.6		−0.5
17	200	−1.8	−10.2	−15.8		−2.0
ASA	120	−6.3	−5.2	12.3	−2.3	−13.2

[a] Differences are significant in comparison with control group. ** $P < 0.01$, *** $P < 0.001$

this study. Throughout the study the animals were cared for in accordance with European Convention (1986) and Guide for the Care and Use of Laboratory Animals (1985) and the Lithuanian Laws (Ruksenas and Simkeviciene 2003).

Mice and rats were housed in groups of 5 or 10 respectively in standard plastic cages with chipped hardwood bedding and kept at room temperature and under standard light conditions. Animals received standard chow and water *ad libitum*. They were allowed to acclimate for at least 5 days before testing.

3.2.2. Toxicity tests

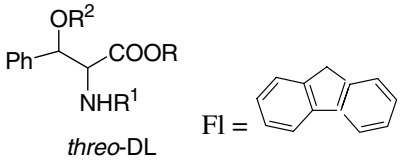
Acute toxicity was determined on mice. The survival of animals (5 in each group) receiving orally the graduated single dose levels of each compound suspended in 1% starch jelly was observed for 8 days (Pizzocheri and Zaninelli 1990). The LD_{50} value was determined by the accepted Litchfield and Wilcoxon (1949) method.

3.2.3. Antiinflammatory activity tests

Antiinflammatory activity was studied in experiments on 200 male Wistar rats divided randomly into treated and control groups.

3.2.4. Induction and evaluation of adjuvant arthritis and other parameters

Adjuvant arthritis (AA) was induced by a single injection of 0.1 ml of complete Freund's adjuvant (Sigma, St. Louis, MO USA) into the left hind paw on day 0. To evaluate the progression of the disease, two parameters were defined: the swelling of the hind paws determined plethysmographically and the development of polyarthritis in three non injected paws. Paw volume and body weights were measured three times a week and the percentage of deviation was determined. The number of rats with developed polyarthritis in treated and control groups was expressed in percentage terms from the general number of animals in the respective groups. At the end of experiment the animals were killed by decapitation under narcosis. The internal organs' weight, ESR and hemoglobin (Hb)



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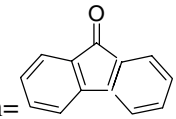
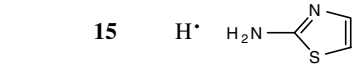
Fln= 

	R	R1	R2	Ref.
1	H	$CO(CH_2)_8CH_3$	H	Straukas et al. (1993)
2	H	$CO(CH_2)_{10}CH_3$	H	Dirvianskyte et al. (2003)
3	H	$CO(CH_2)_{12}CH_3$	H	Dirvianskyte et al. (2003)
4	K	$CO(CH_2)_8CH_3$	H	Dirvianskyte et al. (2003)
5	H	H•HCl	$CO(CH_2)_{10}CH_3$	Dirvianskyte et al. (2003)
6	C_2H_5	H•HCl	$CO(CH_2)_{12}CH_3$	Dirvianskyte et al. (2003)
7	C_2H_5	H•HCl	$COCH_2C_6H_5$	Straukas et al. (1996)
8	C_2H_5	$CO(CH_2)_{12}CH_3$	$CO(CH_2)_{12}CH_3$	Dirvianskyte et al. (2003)
9	$CH_2(CH_2)_{12}CH_3$	$H.CH_3$-C_6H_4−SO_3H	H	Dirvianskyte et al. (2003)
10	$CH_2(CH_2)_{12}CH_3$	$SO_3C_6H_5$	H	Straukas et al. (1996)
11	$CH_2(CH_2)_{12}CH_3$	$COCH_2C_6H_5$	H	Straukas et al. (1996)
12	$CH_2(CH_2)_{12}CH_3$	$SO_2C_{10}H_7$	H	Straukas et al. (1996)
13	$CH_2(CH_2)_{12}CH_3$	O_2S Fl	H	Bulko et al. (1987)
14	$CH_2(CH_2)_6CH_3$	O_2S Fln	H	Bulko et al. (1987)
15	H• 	$COCH_2CH_2CH_3$	H	Straukas et al. (1993)
16	C_2H_5	$COCH_2C_6H_5$	$CO(CH_2)_{12}CH_3$	Dirvianskyte et al. (2004)
17	C_2H_5	$CO(CH_2)_{12}CH_3$	$COCH_2C_6H_5$	Dirvianskyte et al. (2004)

were determined. Leukocytes and erythrocytes were counted with a Picoscale (Hungary) hematological analyzer. Internal organs were examined macroscopically.

3.2.5. Compounds and treatment schedule

Compounds **1**–**17** were used throughout the study. All preparations were prepared *ex tempore* as a fine homogenized suspension in 1 starch jelly and were injected orally (volume 1 ml) in doses shown in Table 2. For comparison the well known drug aspirin (acetylsalicylic acid – ASA) in a dose of 120 mg/kg was used. Control groups received the same volume of starch jelly. Treatment was started since the AA inducing day and continued to day 16. The experiments lasted 17 days.

3.3. Statistical analysis

The results were expressed as mean values ± S.E.M. Differences between control and treated groups were statistically analysed by Student's t test with $P < 0.05$ considered as significant. The percentage of deviation from the control was derived by the following formula: $(T - C)/C \times 100$, where T is the data on the tested group and C is the data on the control.

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