ORIGINAL ARTICLE

NEW POTENTIAL NONSTEROIDAL ANTI-INFLAMMATORY DRUGS WITH ANTILEUKOTRIENIC EFFECTS: INFLUENCE ON MODEL PROTEINS WITH CATALYTIC ACTIVITY

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Summary: Unspecific and side effects caused by interaction with proteins belong to common problems of many structures synthesized as potential medicaments. Possible *in vitro* interactions with proteins of a group of phenylsulfonyl benzoic acid derivatives (VÚFB 19363, 19369, 19370, 19371, and 19760) as new potential anti-inflammatory compounds with antileukotrienic activities were studied in the present work. Three purified enzymes were used as model proteins with catalytic activities: Pig heart aspartate aminotransferase (AST, EC 2.6.1.1), alanine aminotransferase (ALT, EC 2.6.1.2), and glutamate decarboxylase (GAD, EC 4.1.1.15) from *E. coli.* Catalytic activities during incubation of individual compounds (6×10^{-5} M solution to 5×10^{-2} M suspension) at 37°C with enzymes served as criteria of stability and function of the proteins. No immediate influence of any compound studied on enzyme activities was found. Aminotransferase activities were not affected even during incubation up to 20 d. In the case of GAD, the compounds VÚFB 19369, 19370, 19371, and 19760 had stabilizing influence on GAD activity during incubation at enzyme concentrations of 11.25 and 5.62 mg prot/l. The lack of an immediate effect of compounds and the stability of enzymes during incubation them are favorable and support the prospective of the compounds as potential drugs.

Key words: *Alanine aminotransferase; Aspartate aminotransferase; Glutamate decarboxylase; Antileukotrienic drugs; Phenylsulfanylbenzoic acid derivatives; Potential drugs; NSAIDs; Influence of drugs; Protein stability*

Introduction

Unspecific and side effects of drugs caused by their interaction with various functional proteins, which may appear as enzyme inhibition or inactivation, belong to common problems of many structures synthesized as potential medicaments. We found such effects in studies of several groups of compounds prepared or already used in human therapy (5,6,8).

Enzymes are often used in drug-protein interaction studies, because their advantage is the fact that their function may be expressed as catalytic activity, which makes it possible to investigate the influence of various compounds on these proteins easily by enzyme assay.

In the present study, a group of phenylsulfanylbenzoic acid derivatives (Fig. 1) with multiple antileukotrienic activities was checked for interaction with three proteins with catalytic activity – aspartate aminotransferase (AST, EC 2.6.1.1, pig heart), alanine aminotransferase (ALT, EC 2.6.1.2, pig heart), and glutamate decarboxylase (GAD, EC 4.1.1.15, *E. coli*).

Material and Methods

1. Compounds studied

Phenylsulfanylbenzoic acid derivatives with potential antileukotrienic effects (VÚFB 19363, 19369, 19370, 19371, and 19760) were synthesized as described in (7). Their structural formulae are given in Fig. 1.

2. Enzymes

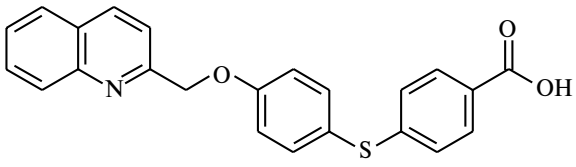
Alanine aminotransferase (ALT), the cytosolic enzyme from the pig heart, lyophilized (SERVA), or as suspension in saturated ammonium sulfate (Boehringer Mannheim).

Aspartate aminotransferase (AST), the cytosolic enzyme from the pig heart (SERVA), suspension in saturated ammonium sulfate.

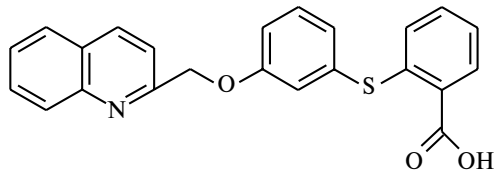
Both aminotransferases were reconstituted as solutions in 0.1 M phosphate buffer pH 7.4 with 0.05 % sodium azide.

Glutamate decarboxylase from *E. coli* (suspension in 4.2 M ammonium sulfate containing 0.1 mM pyridoxal phosphate and 0.1 mM DTT, Sigma) was used because of

its higher stability in comparison with the mammalian enzyme. The enzyme was reconstituted in 0.2 M citrate-phosphate buffer, pH 5.0 (= pH optimum of GAD from *E. coli*) with 0.05 % sodium azide.



VÚFB 19363



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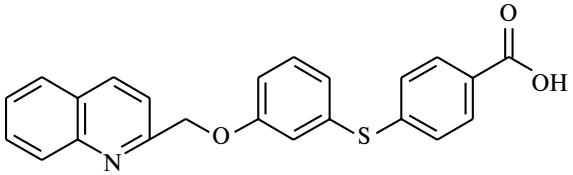
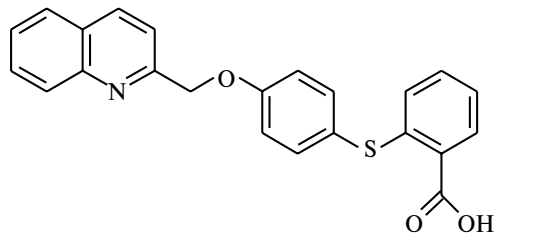
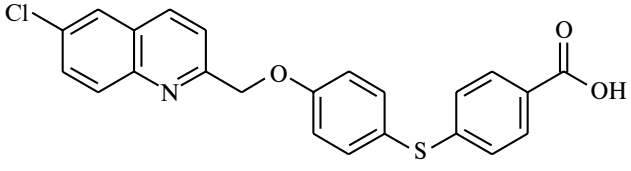


VÚFB 19370



VÚFB 19371



VÚFB 19760

Fig. 1: Structural formulae of phenylsulfanylbenzoic acid derivatives studied.

3. Arrangement of experiments

1) Immediate in vitro inhibition of enzymes (ALT, AST, GAD) by the individual compounds under study was measured.
2) Incubation of enzymes with individual compounds:
   Aminotransferases: About 10^{-5} M AST and about 2.2 x 10^{-5} M ALT were incubated at 37°C for 20 or 28 days with
   a) 5 x 10^{-2} M suspension of the compound VÚFB 19363
   b) 6 x 10^{-5} M solution of the compound VÚFB 19363 or an 8 x 10^{-5} M solution of compound VÚFB 19369, 19370, 19371, and 19760.

   GAD: The enzyme solution (concentrations 56.25, 11.25, and 5.62 mg prot/l in different experiments) was incubated with 1 x 10^{-3} M suspensions of individual compounds (representing saturated solutions during the whole time of incubation) for 14 days at room temperature (25°C). Time profiles of enzyme activities were monitored.

4. Enzyme assays

Aminotransferase activities were determined by the kinetic UV method with addition of pyridoxal-5'-phosphate to the incubation mixture (Roche Diagnostics, Mannheim, Germany, transaminating catalytic activity combined with lactate or malate dehydrogenase reaction) common in clinical chemistry laboratories (1, 2). Sample aliquots (100 μl) were diluted (ALT) or (AST) with 0.05 M phosphate buffer, pH 7.4, in order to obtain catalytic activities within the analytical range of the employed method. The enzyme activities were assayed on a Hitachi 917 analyzer. All experiments were performed in triplicates. ALT and AST catalytic activities in Precinorm U and Precipath U (both from Roche Diagnostics) were measured before and after each assay. The between-run coefficients of variation of the both assays for Precinorm U and Precipath U were lower than 2.0 %.

GAD activity was assayed by the radiometric method with 1-^{14}C-glutamate as the substrate (4). The incubation mixture contained the enzyme, substrate L-glutamate-1-^{14}C, coenzyme pyridoxal-5'-phosphate, and the compounds tested. The mixture was incubated at 37°C for 30 min and the radioactivity of $^{14}CO_2$ liberated from the mixture and trapped by means of 0.1 ml of 30 % KOH was measured in a scintillation cocktail using a 1219 Rackbeta scintillation counter LKB Wallac (Radioisotope Laboratory, Faculty of Pharmacy, Charles University, Hradec Králové).

Results and Discussion

1. Aspartate and alanine aminotransferase

Results are summarized in Table 1 and Table 2.

No immediate inhibition of aminotransferases ALT and AST by any potential NSAID compound studied was found.

No significant changes in aminotransferase activities were found during incubation of the enzyme with individual

members of a group of phenylsulfanylbenzoic acid derivatives: VÚFB 19363 in 6 x 10^{-5} M concentration, VÚFB 19369, 19370, 19371, and 19760 in 8 x 10^{-5}M concentration. Some gradual decrease in AST activity was found with 5 x 10^{-2} M suspension of the compound VÚFB 19363. We suggest that some partial inactivation of the enzyme protein takes place when a high concentration of the compound is available for reaction with the enzyme protein. This effect is comparable with that found in our study of a group of natural compounds under similar conditions (3).

Tab. 1: Influence of potential NAISDs of VÚFB on ALT activity *in vitro.*

Day	0	4	8	13	20
Control	5.16±0.06	5.06±0.06	4.97±0.01	4.41±0.09	4.46±0.13
19760	4.86±0.07	5.20±0.04	4.95±0.02	4.63±0.10	4.70±0.04
19371	5.03±0.09	5.25±0.06	5.15±0.06	4.77±0.07	4.79±0.08
19370	5.06±0.02	5.06±0.11	4.86±0.06	4.71±0.10	4.73±0.05
19369	5.06±0.04	5.23±0.06	5.02±0.04	4.80±0.10	4.87±0.10
19363	4.73±0.09	5.05±0.07	4.99±0.08	4.66±0.08	4.68±0.05

Enzyme activity in μkat/l (the mean ± SD of 3 samples)

Tab. 2: Influence of potential NAISDs of VÚFB on AST activity *in vitro.*

Day	0	4	8	13	20
Control	4.81±0.05	4.58±0.08	4.12±0.20	3.38±0.05	3.48±0.08
19760	4.71±0.05	4.72±0.06	4.50±0.15	3.35±0.08	4.14±0.08
19371	4.65±0.10	4.34±0.01	4.20±0.13	3.28±0.13	3.96±0.05
19370	4.46±0.08	4.52±0.04	4.06±0.06	3.45±0.08	3.70±0.01
19369	4.73±0.02	4.49±0.02	3.85±0.20	3.69±0.04	3.65±0.03
19363	4.73±0.12	4.51±0.08	4.74±0.05	3.66±0.05	3.96±0.06

Enzyme activity in μkat/l (the mean ± SD of 3 samples)

2.Glutamate decarboxylase

Various conditions were used in order to investigate the influence of compounds under study on GAD *in vitro*: No immediate inhibition of GAD by any potential NSAID compound studied was found at any enzyme protein concentration used.

In spite of the fact that the enzyme was reconstituted in the same way for each experiment, final specific activities of GAD expressed as the activity per declared protein concentration in samples were not identical (see Table 3, in which the results are summarized).

Some changes in GAD activity during a longer incubation with 1 x 10^{-3} M suspensions of individual compounds (including "activation" of GAD) were found. Striking is an increasing effect of compounds studied accompanying a decreasing concentration of the enzyme protein (compare Fig. 2 to Fig. 4), which suggests that more probably some irreversible interferences of the compounds studied with enzyme molecules take part than the "classical" reversible inhibition or activation of enzymes.

Because of differences in the control activities of GAD found in the individual experiments, we tried to recalculate the results of investigations as percentage of control activi-

Tab. 3: Influence of potential NAISDs of VÚFB on GAD activity *in vitro*. A summary of results.

	1st experiment		2nd experiment		3rd experiment		
GAD protein concentration	5.62 mg prot/l	56.25 mg prot/l	11.25 mg prot/l	5.62 mg prot/l	56.25 mg prot/l	11.25 mg prot/l	5.62 mg prot/l
Control activity[a]) in dpm (0 d)	2754	23519	4610	1665	50997	9573	3271
Time profile of control activity	Signif. decrease, marked at 1d + 2d, then independent of incubation	Constant during incubation except some increase at 1 d	Signif. *increase* at 1d + 2 d, independent of incubation	Signif. decrease since 1d	*Increase*, signif. since 2d, independent of incubation	Signif. decrease since 2d, then small change only (min. 7d)	Signif. decrease since 1d
19363	Inhibition, increasing during incubation	Inhibition, increasing during incubation	Inhibition, increasing during incubation	0	0	Inhibition at 7d + 14d	0
19369	0	0	Inhibition, independent of time	Activation	-	-	-
19370	0	0	Inhibition, independent of time	Activation	0	Activation since 2d	Activation
19371	0	0	Inhibition, independent of time	Activation	0	Inhibition, independent of time	Activation
19760	Inhibition, independent of time	Inhibition, independent of time	Inhibition, independent of time	Activation	0	0	Activation

0 = no effect; - = not studied

[a]) The mean of 4 parallel samples

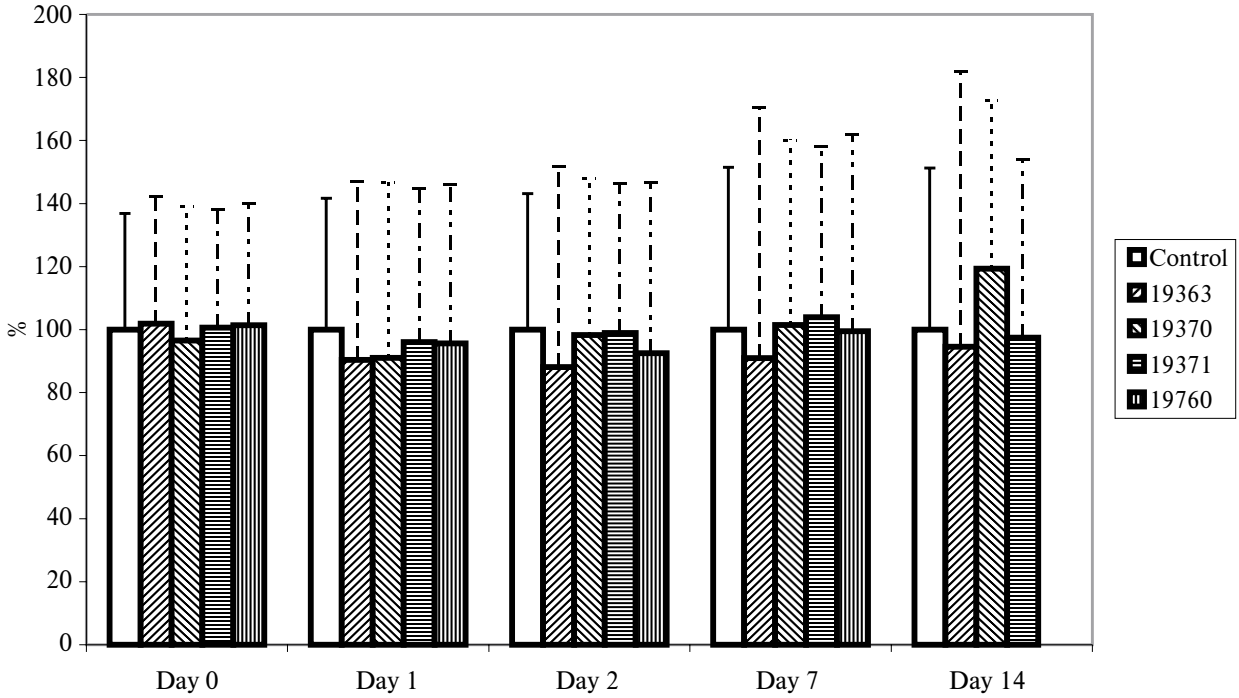


Fig. 2: Time profile of GAD activity in the presence of the VÚFB compounds tested.
GAD concentration in a sample: 56.25 mg prot/l. The enzyme activities are presented in % of the respective control values. Each point represents the mean of two independent measurements of four parallel samples. Vertical bars express SD in % of the respective mean.

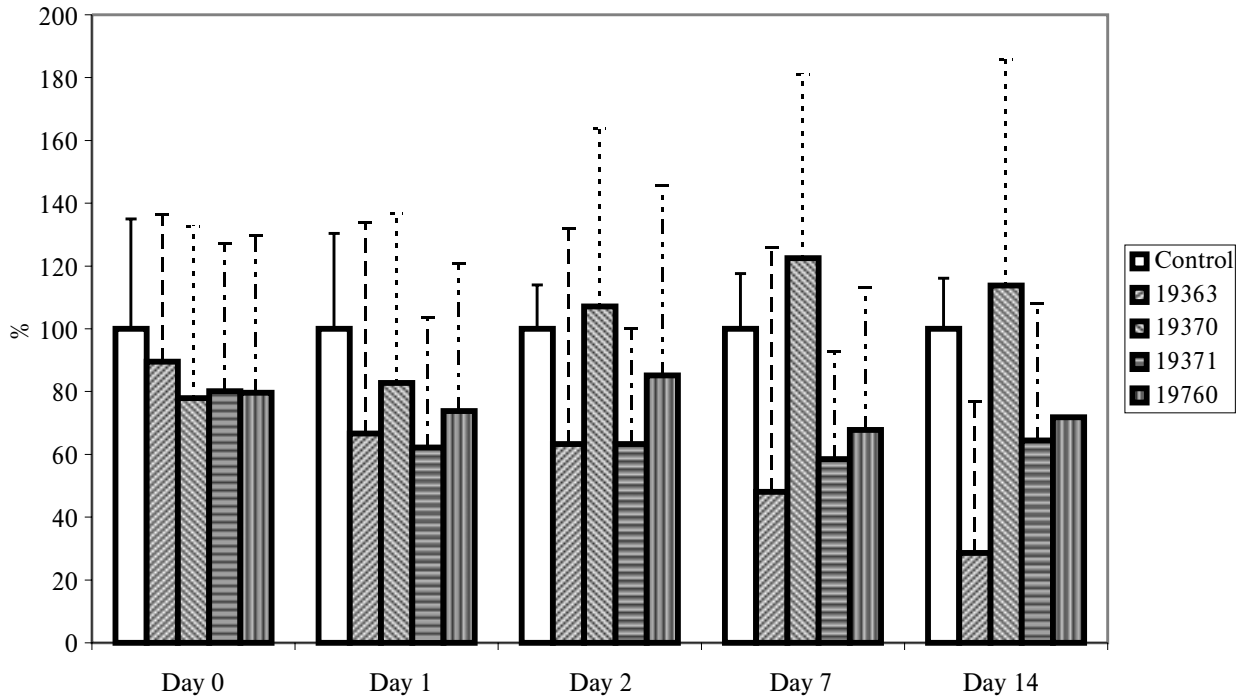


Fig. 3: Time profile of GAD activity in the presence of the VÚFB compounds tested.
GAD concentration, 11.25 mg prot/l. For details see Fig 1.

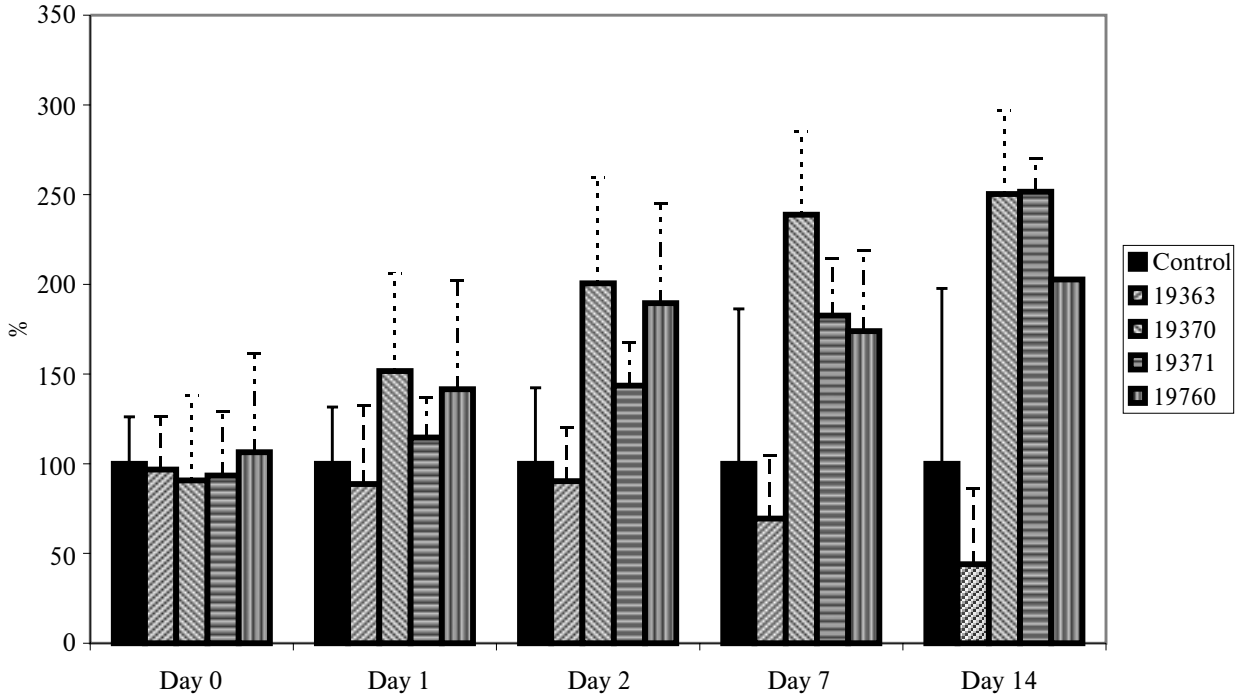


Fig. 4: Time profile of GAD activity in the presence of the VÚFB compounds tested.
GAD concentration, 5.62 mg prot/l. For details see Fig 1.

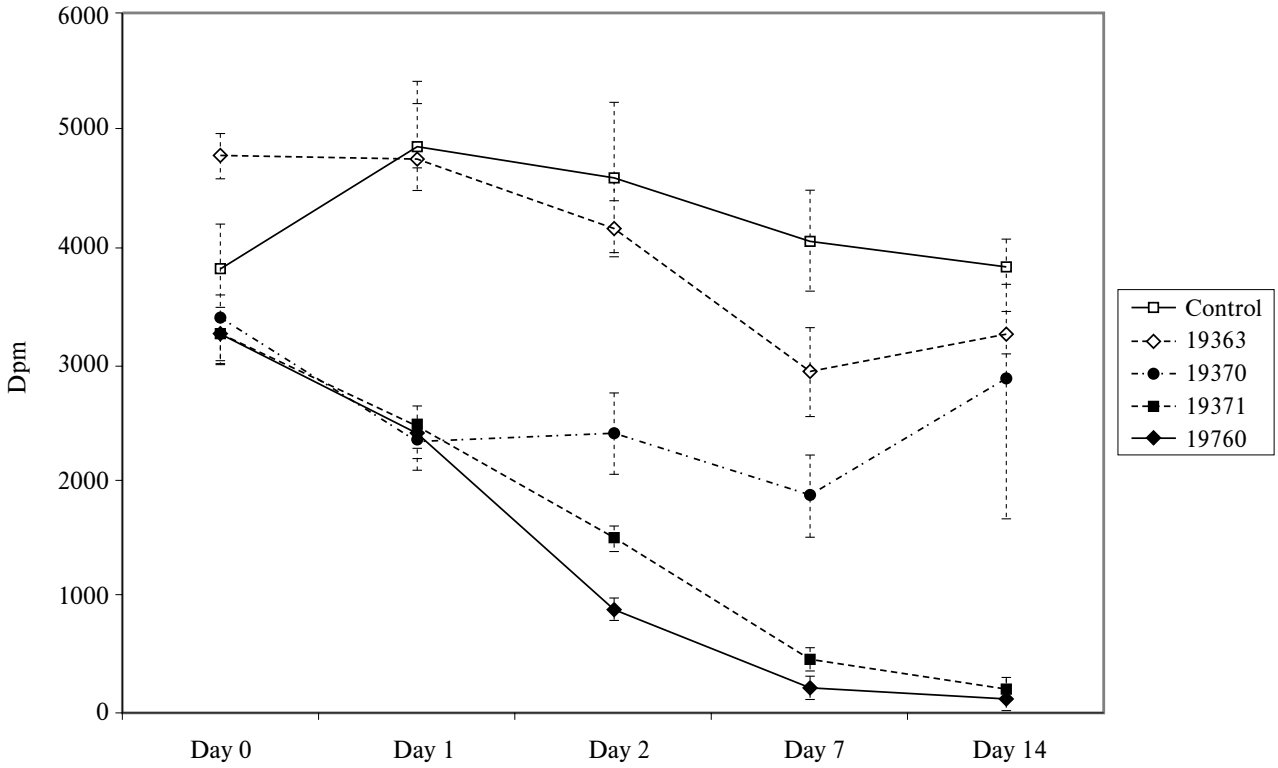


Fig. 5: Time profile of GAD activity in the presence of the VÚFB compounds tested.
GAD concentration in a sample: 5.62 mg prot/l. The enzyme activities are presented in dpm per sample. Each point represents the mean of four parallel samples.

ties, which enabled us to express and compare the results of different experiments in which the same concentrations of the enzyme were used (Fig. 2–4). Even at longer intervals, no significant effect of any compound studied was found when the highest concentration of GAD (56,25 mg/ml) was used (Fig. 2). With a decreasing concentration of GAD, differences appeared, which were not significant but were more marked during later intervals of experiments: A certain non-significant decrease in GAD activity was found with the compound VÚFB 19363 with protein concentrations 11.25 and 5.62 mg prot/l, and a small relative increase in GAD activity in comparison with controls in the case of other compounds with a protein concentration of 5.62 mg prot/l (Fig. 3 and 4). When the absolute results are analyzed, it is obvious that the effect is caused by decreasing GAD control activities, while the enzyme activities in the presence of compounds VÚFB 19370, 19371, and 19760 are more stable (see Fig. 5).

Conclusions

1. No immediate inhibition of aminotransferases and GAD by the compounds VÚFB 19363, 19369, 19370, 19371, and 19760 was found.
2. Incubation of aminotransferases or GAD with the compound VÚFB 19363 under various conditions caused no significant changes in the activities of these enzymes.
3. In the case of compounds VÚFB 19369, 19370, 19371, and 19760 there was a tendency to stabilize GAD.
4. The lack of immediate effect of the compounds VÚFB 19363, 19369, 19370, 19371, and 19760, and the stability of the enzymes during their incubation with the compounds VÚFB 19369, 19370, 19371, and 19760 are favorable and support the prospective of further testing and using these compounds as potential drugs.

Acknowledgment

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