



Study of the interaction of 1,4-dihydropyridine derivatives with glucocorticoid hormone receptors from the rat liver

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Abstract:

Seventeen derivatives of 1,4-dihydropyridine (DHP) series were tested *in vitro* for their ability to inhibit [1,2,4-³H]-dexamethasone binding to glucocorticoid receptor from the rat liver cytosol. Depending on structural features and inhibiting activities, the compounds can be divided into three groups. The first group (nifedipine, foridone, J-6-163, OSI-4164 and OSI-7724) had the highest activity: they inhibited specific ligand-receptor binding by 70–80% at concentrations of 10⁻⁵ M and 10⁻⁴ M, with apparent IC₅₀ values of 1.5–6.0 μ M. The second group (cerebrocrast, diethone, OSI-1211 and OSI-7265) was active at concentration of 10⁻⁴ M, and their IC₅₀ values were 23–45 μ M; compound OSI-5003 was almost inactive. Both groups are compounds with scarce water solubility, more or less lipophilic. The third group of compounds comprises ionogenic compounds (organic cations or anions with corresponding inorganic counterions): most of them are water-soluble (glutapyrone, carbatone, gammapyrone, OSI-2780, OSI-1580, OSI-2140) or liposome-forming (A-74). They lack the above-mentioned activity.

Among the first two groups, compounds possessing more bulky substituents in positions 3 and 5 are less active. The aromatic ring in the position 4 is essential for the optimal activity. It seems that there is a bell-shaped dependence of activity upon lipophilicity. In general, the compounds of the first group are strong Ca-antagonists, while the second group includes moderate Ca-antagonists, but each group comprises also compounds which lack Ca antagonistic activity. All compounds of the third group lack Ca antagonistic properties.

Key words:

1,4-dihydropyridines, glucocorticoid receptor

Abbreviations: DHP – 1,4-dihydropyridine, GC – glucocorticoid

Introduction

Steroid hormones have effects at all levels of the biological organization. The presence of receptors in the target tissue and their interaction with hormonal ligands is essential for eliciting a biological response. Receptor molecules are relatively large proteins that have specific binding sites for the hormone and are found in both the cytoplasmic and nuclear fractions of the cell [23, 31]. Binding of the steroid to its receptor molecule results in changes in structure that convert the receptor from an inactive to an active conformation. These changes result in the formation of an "activated" or "transformed" receptor-steroid complex that has a high affinity for various nuclear binding sites [24]. There is accumulating evidence that glucocorticoids (GCs) elicit their physiological and pharmacological actions through association with cytoplasmic receptors. The formation of the cytoplasmic GC-receptor complex is the first event of the regulation of specific gene transcription [6, 17]. Subsequent events after hormone-receptor complex formation include the activation of complexes that results in a decrease in the size and affinity of receptor for DNA. It has been earlier demonstrated that a number of chemical drugs influence the specific binding of GC hormones to their receptors, and thus modify their interaction with DNA. The changes in nuclear concentration of hormone receptors induced by drugs could have great clinical importance [2]. Steroid receptor antagonists have been employed as tools for elucidating the molecular basis of hormone action. The interaction of 1,4-dihydropyridine (DHP) derivatives with GC hormone receptors has not been widely investigated notwithstanding that they are widely known as biologically active compounds [7, 10, 19]. It is known that nifedipine reduces binding of [³H]-triamcinolone acetonide to GC receptors from brain cytosol fractions of rats by 58% [9].

Our previous studies [16] have revealed that derivatives of DHP may significantly alter the levels of total, transcortin-bound and free corticosterone in rat plasma. However, depending on structural features, this influence was different: cerebrocrast whose structure comprises elements of DHP calcium antagonist structure (*o*-substituted aryl group at the position 4 of the DHP ring, and ester groups at positions 3 and 5) considerably elevated the concentrations of all three forms of plasma corticosterone, whereas glutapyrone (glutamate at the position 4 of the DHP ring) acted in an opposite manner by lowering these values; carbato- (having no groups attached to position 4) was al-

most inactive. Both cerebrocrast and glutapyrone have shown strikingly long-lasting memory improving and neuroprotective effects in a variety of neurodeficit states in animals, but they differ in lipophilicity [13–15]. A suggestion was made that due to the compounds' lipophilicity, they may penetrate cell membranes and initiate steroid-like intracellular processes leading to protein synthesis. As it is well known, neurosteroids and steroids have been found to be essential molecules contributing to formation of memory processes [8, 20, 25]. However, more traditionally, the anti-inflammatory and immunosuppressive effects of glucocorticoids are described [3, 27]. Moreover, recently a strong evidence has been accumulated indicating that inflammatory processes in the brain may be attributed to neurodegeneration [1, 18]. Hence, a new anti-inflammatory treatment strategy in neurodegenerative diseases, particularly Alzheimer's disease have been started [28]. At present, non-steroidal anti-inflammatory drugs, cyclooxygenase (COX) and lipoxygenase (LOX) inhibitors are being investigated in many clinics all over the world, however, these studies stress the point that further refinement in the development of successful anti-inflammatory therapeutics is necessary [28]. In this context, a search for the new type of anti-inflammatory drugs, particularly for treatment of neuroinflammation, seems to be intriguing, taking into account by us newly discovered ability of DHP compounds to contribute to the glucocorticoid pathways. In our previous study, we showed that cerebrocrast, a derivative of DHP, which does not antagonize Ca²⁺ influx in neuronal tissues, has a preventative effect in a model of rat paw edema induced by carrageenan [12, 29].

In the present research, we studied the interaction between the rat liver glucocorticoid receptors and various DHP derivatives by examining their ability to inhibit specific ligand ([³H]-dexamethasone)-glucocorticoid receptor interaction. Six ionogenic hydrophilic compounds (derivatives of carboxylates, sulfonates, pyridinium salts) and 11 neutral, more or less lipophilic DHP derivatives were studied.

Materials and Methods

Animals

Male Wistar rats (Laboratory Animal Centre, Institute of Immunology, Vilnius, Lithuania) weighing

150–180 g each, were used for the experiments throughout the study. Experimental procedures were carried out in accordance with guidelines of the European Union, local laws and police and were approved by the Ethics Committee of Animal Experimentation.

Compounds

The following 1,4-dihydropyridine derivatives were synthesized at the Latvian Institute of Organic Synthesis (Riga, Latvia): carbatone, cerebrocrast, diethone, foridone, gammapyrone, glutapyrone, nifedipine, OSI-1211, OSI-1580, OSI-2140, OSI-2780, OSI-4164, OSI-5003, OSI-7265, OSI-7724, A-74, and J-6-163 (Tab. 1). Six compounds (carbatone, gammapyrone, glutapyrone, OSI-1580, OSI-2140, and OSI-2780) were readily soluble in water while other compounds were insoluble in aqueous medium, and the stock solutions in ethanol were prepared before diluting with the buffer to an appropriate concentration. Piracetam and corticosterone were used as reference substances.

Preparation of cytosol

Livers were perfused *in situ* through the portal vein with ice-cold saline (0.9% NaCl) by the technique described earlier [22]. Then liver slices were homogenized in the ice-cold buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM EDTA, 2mM β -mercaptoethanol, and 1 mM PMSF). The homogenate was centrifuged at $105,000 \times g$ at 0–4°C for 1 h [30]. The upper lipid layer was discarded, and the resulting supernatant was designated as cytosol.

Steroid binding

For determining the extent and specificity of steroid binding, aliquots of the cytosol were incubated with 10 nM [1,2,4- ^3H]-dexamethasone (42.0 Ci/mmol, Amersham) at 4°C for 2 h in the absence or presence of 1000-fold molar excess of non-labelled dexamethasone to determine specific steroid binding [4]. For *in vitro* competition experiments, the substances in incubation medium were tested in a concentration range from 10^{-4} M to 10^{-8} M. After incubation (at 0–4°C for 2 h) non-bound radioactivity was removed by the dextran-coated charcoal technique [11] with some modifications. The suspension of 100 μl of 5% charcoal/0.5% dextran was added to each Eppendorf tube containing 250 μl of reaction mixture. The samples were thoroughly mixed, allowed to stand in an ice-

water bath for 5 min, and then centrifuged ($12,500 \times g$, 1 min). 200 μl aliquots in 2 ml of dioxane scintillation fluid were used for radioactivity measurements. All experiments were repeated at least four times.

DEAE-cellulose chromatography

A cytosol was applied to a column (1.4×6.0 cm) of DEAE-cellulose (DE-52, Whatman) equilibrated with KPD buffer (5 mM potassium dihydrogenphosphate, 0.5 mM dithiothreitol, pH 7.6 at 0–4°C). The aliquot (0.9 ml) of the labeled cytosol (as described above) with or without an appropriate DHP (at final concentration of 10^{-4} M) was applied to the column and washed for 30 min with KPD buffer. Protein fractions containing radioactivity were eluted with a linear 5 to 400 mM KPD gradient as described previously [26]. Twenty-five fractions (2 ml each) were collected, and 0.2 ml aliquots of each fraction were added to 2 ml of dioxane scintillation fluid for the determination of radioactivity. All chromatographic procedures were performed at 4°C or 20°C.

Saturation experiments and Scatchard analysis

The mutually exclusive nature of steroid and DHP binding in such system was investigated using saturation experiments and Scatchard analysis [5]. GC receptor binding in liver cytosol was assessed using the synthetic hormone [^3H]-DEX (Amersham) in a concentration range from 2.5 nM to 25 nM, and DHPs in a concentration range from 5×10^{-4} M to 10^{-8} M for each concentration of DEX. Two controls: piracetam (negative, non-binding compound) and corticosterone (positive, binding compound) were used.

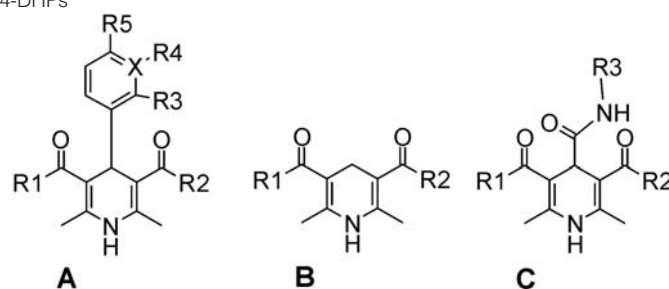
Statistical analysis

The data in Tables are expressed as the arithmetic mean \pm SE of four independent experiments. Differences between test groups (DHPs) and positive control (corticosterone) were evaluated by analysis of variance ANOVA complemented by unpaired Student's *t*-test.

Results and Discussion

In this study, we investigated the interaction between rat liver glucocorticoid receptors and various DHPs

Tab. 1. Chemical structure of 1,4-DHPs



Compound	R1	R2	R3	R4	R5	X
A group						
A-74	-OC ₃ H ₆ O(CO)C ₁₅ H ₃₁	-OC ₃ H ₆ O(CO)C ₁₅ H ₃₁	H	-CH ₃	H	N ⁺
Cerebrocrast	-OC ₂ H ₄ OC ₃ H ₇	-OC ₂ H ₄ OC ₃ H ₇	-OCHF ₂	H	H	C
Foridone	-OCH ₃	-OCH ₃	-OCHF ₂	H	H	C
J-6-163	-OC ₂ H ₄ OCH ₃	-OC ₂ H ₄ CN	H	-NO ₂	H	C
Nifedipine	-OCH ₃	-OCH ₃	-NO ₂	H	H	C
OSI-1211	-OC ₂ H ₄ OC ₂ H ₅	-OC ₂ H ₄ OC ₂ H ₅	-OCHF ₂	H	H	C
OSI-4164	-SC ₂ H ₅	-SC ₂ H ₅	H	H	-NO ₂	C
OSI-5003	cholesteryloxy	cholesteryloxy	-OCHF ₂	H	H	C
OSI-7265	-OCH ₃	-OC ₂ H ₄ OC ₂ H ₅	-OCHF ₂	H	H	C
OSI-7724	-OCH ₃	-OC ₂ H ₄ OCH(CH ₃) ₂	-OCHF ₂	H	H	C
B group						
Carbatone	-OCH ₂ COONa	-OCH ₂ COONa				
Diethone	-OC ₂ H ₅	-OC ₂ H ₅				
C group						
Gammapyrone	-OC ₂ H ₅	-OC ₂ H ₅	-C ₃ H ₆ COONa			
Glutapyrone	-OC ₂ H ₅	-OC ₂ H ₅	-CH(COONa)C ₂ H ₄ COONa			
OSI-1580	-OC ₂ H ₅	-OC ₂ H ₅	-CH(COONa)C ₂ H ₄ SCH ₃			
OSI-2140	-OC ₂ H ₅	-OC ₂ H ₅	-C ₂ H ₄ COONa			
OSI-2780	-OC ₂ H ₅	-OC ₂ H ₅	-C ₂ H ₄ SO ₃ Na			

(Tab. 1), using synthetic hormone [³H]-DEX, and examined whether DHPs can compete for GC hormone receptors. The results of concentration-response experiments for *in vitro* blockade of DEX binding by various DHPs are shown in Table 2. Potencies of a number of the most active DHPs to displace [³H]-DEX from the rat liver cytosol receptors are shown in Table 3.

Our studies of 18 compounds of DHP series have revealed great difference in their ability to compete for GC receptors. The activity to a great extent depended on structural features of the test compounds. Thus, judging from inhibiting activities, the compounds can be divided into three groups. The first

group (nifedipine, foridone, J-6-163, OSI-4164, and OSI-7724) inhibited specific ligand-receptor binding by 70–80% at concentrations 10⁻⁵ M and 10⁻⁴ M (Tab. 2), with apparent IC₅₀ values 1.5–6.0 μM (Tab. 3). Compounds of the second group (cerebrocrast, diethone, OSI-1211, OSI-7265) were active at concentration 10⁻⁴ M (Tab. 2), and their IC₅₀ values were 23–45 μM (Tab. 3); compound OSI-5003 was almost inactive. The third group (A-74, carbatone, gammapyrone, glutapyrone, OSI-1580, OSI-2140, OSI-2780) involved DHPs with charged (cationic or anionic) groups: carboxylic or sulfonic groups in amino acid moieties in position 4 of DHP; carboxylic groups in substituents in positions 3 and 5 of DHP; or pyridin-

Tab. 2. Competition of various 1,4-DHPs with [³H]-dexamethasone for glucocorticoid receptors *in vitro* (% of control)

Compound tested	[³ H]-DEX binding activity at compound tested concentration [M]				
	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
A-74	111.6 ± 9.3	116.8 ± 8.8	103.3 ± 12.1	113.3 ± 14.3	102.1 ± 12.0
Carbatone	98.6 ± 10.3	105.2 ± 11.6	118.4 ± 3.3	107.6 ± 12.5	109.0 ± 14.0
Cerebrocrast	101.8 ± 6.6	105.7 ± 11.5	91.6 ± 10.2	73.9 ± 7.5	34.8 ± 1.2**
Diethone	106.1 ± 3.4	100.0 ± 5.9	97.4 ± 6.3	94.0 ± 10.4	20.8 ± 1.6**
Foridone	105.1 ± 6.5	103.2 ± 5.9	82.2 ± 8.7	36.3 ± 8.9**	10.8 ± 0.4**
Gammapyrone	105.0 ± 6.5	103.6 ± 5.4	105.3 ± 13.1	99.6 ± 7.0	106.8 ± 10.0
Glutapyrone	99.9 ± 5.8	100.5 ± 9.7	99.9 ± 6.2	119.2 ± 8.6	108.4 ± 8.1
OSI-1211	n.a.	119.5 ± 9.9	100.8 ± 8.4	87.5 ± 6.5	25.0 ± 2.3**
OSI-1580	n.a.	104.7 ± 6.1	119.6 ± 12.3	107.6 ± 11.8	99.1 ± 14.3
OSI-2140	n.a.	103.3 ± 8.1	96.1 ± 15.3	112.6 ± 12.4	107.2 ± 11.3
OSI-2780	n.a.	118.9 ± 7.9	113.3 ± 14.3	114.4 ± 12.6	119.1 ± 10.1
OSI-4164	97.0 ± 9.1	97.0 ± 8.5	85.0 ± 8.3	14.9 ± 5.1**	12.5 ± 0.3**
OSI-5003	n.a.	80.8 ± 10.5	76.3 ± 11.4	81.4 ± 12.1	90.2 ± 8.3
OSI-7265	105.5 ± 10.0	104.2 ± 6.7	95.2 ± 3.0	74.5 ± 4.8	37.5 ± 4.7**
OSI-7724	99.8 ± 10.3	97.4 ± 8.3	83.3 ± 9.1	28.3 ± 2.3*	11.3 ± 1.2**
J-6-163	111.7 ± 9.8	98.5 ± 5.7	77.2 ± 8.4*	22.3 ± 6.0*	4.8 ± 0.8**
Nifedipine	109.7 ± 7.6	109.6 ± 10.6	97.2 ± 20.8	29.9 ± 8.7**	15.9 ± 2.3**
Negative control	113.8 ± 4.5	103.2 ± 8.0	91.0 ± 19.8	101.6 ± 13.9	77.2 ± 2.3
Positive control	64.8 ± 12.5	23.9 ± 8.6**	1.3 ± 0.9**	0.6 ± 0.4**	n.a.

Significant difference at * $p < 0.01$; ** $p < 0.001$; n.a. – not analysed. Piracetam was used as a negative control (non-binding compound). Corticosterone was used as a positive control (binding compound)

ium group in position 4 of DHP. These compounds were inactive in the studied systems. One can notice several tendencies by considering structural features of these groups:

1. Compounds possessing anionic or cationic groups are inactive; neutral DHPs possess higher or lower activity;
2. Compounds possessing more bulky substituents in positions 3 and 5 are less active, e.g. OSI-1211, cerebrocrast, OSI-7265, especially OSI-5003; the aromatic ring in the position 4 is essential for the optimal interaction with the glucocorticoid receptor (diethone);
3. Compounds possessing pronounced calcium antagonistic properties are more active (nifedipine, foridone), but there are exclusions; perhaps OSI-4164 lacks calcium antagonistic properties;
4. Water-soluble compounds (almost all compounds of the third group; A-74 forms liposomes in water) lack activity; lipophilic compounds possess activity; it seems that the most lipophilic compounds (OSI-1211,

cerebrocrast) possess moderate activity: maybe there is an optimum lipophilicity.

It seems that there is no direct relationship with memory-stimulating activity: cerebrocrast and glutapyrone showed cognition enhancer effects at low doses [13, 21], but they act quite differently in the present study; on the other hand, nifedipine and foridone which are among the most active in the present study did not show a memory-stimulating activity [21].

The recent publications demonstrate that the 90 kD heat shock protein plays a crucial role in the activation of glucocorticoid hormone receptors [24]. It is known that this transformation is closely associated with the conformational changes of receptor molecule: the increase in positive charges at the receptor surface has been detected. Both forms (non-activated and activated) of GC hormone receptor can be easily separated using DEAE-cellulose chromatography [11]. The capability of DHPs to change the activation process was studied as described in Materials and

Tab. 3. Potencies of the best [³H]-dexamethasone displacers

Compound tested	Apparent IC ₅₀ value [μM]
J-6-163	1.59 ± 0.05*
Nifedipine	3.52 ± 0.08*
Foridone	4.53 ± 0.23*
OSI-7724	5.93 ± 0.12*
OSI-4164	6.01 ± 0.36*
OSI-1211	23.71 ± 0.95*
Cerebrocrast	40.74 ± 3.26*
OSI-7265	42.16 ± 0.51*
Diethone	45.32 ± 2.27*
Corticosterone	0.06 ± 0.001**

Significant difference at * p < 0.01; ** p < 0.001

Methods using foridone and J-6-163 as the most active compounds. Results are shown in Figure 1A. The [³H]-DEX-labeled GC receptors from the rat liver cytosol in the absence of dihydropyridine have been eluted from DEAE-cellulose columns as a double peak (at 4°C): the first peak was eluted at 0.1 M KH₂PO₄ and represented the activated receptor form, the second peak (at 0.32 M KH₂PO₄) contained non-activated receptors. Similar results have been obtained when the liver cytosol was incubated with labeled dexamethasone in the presence of foridone, but DEX binding to the GC receptor was more than two times lower (Fig. 1A). When steroid-receptor complexes with or without foridone have been incubated at 20°C (during such treatment all receptors became transformed), the single radioactivity peak has been eluted at 0.1 M KH₂PO₄ both in the presence and absence of dihydropyridine (Fig. 1B). It can be concluded that foridone does not influence the activation process. J-6-163 exhibited the same properties (data not shown).

The binding of 1,4-DHPs to glucocorticoid receptor was investigated using saturation experiments and Scatchard analysis or double-reciprocal plotting as described in Materials and Methods (some data are shown in Fig. 2). According to these results, J-6-163, foridone and OSI-1211 are more similar to competitive inhibitors (apparent K_d for dexamethasone changed from 9.9 nmol to 163.9, 11.0, and 35.1 nmol, respectively, when the final concentrations of all compounds were 1 × 10⁻⁴ M). It seems that nifedipine-dependent inhibition of dexamethasone binding be-

longs to non-competitive type, but, keeping in mind that apparent K_d for DEX increased two-fold from 9.9 nmol to 21.4 nmol in the presence of nifedipine (1 × 10⁻⁴ M), there are some indications of competition. The inhibition pattern of all tested DHPs was too complicated to allow univocal conclusions to be made and true values of K_i to be calculated.

In conclusion, it has been demonstrated that a number of DHPs can influence the specific binding of [³H]-dexamethasone to glucocorticoid hormone receptor *in vitro*, and subsequently it might be supposed that such interactions can modify the formation of GC receptor complexes with DNA. The obtained results provide an evidence of involvement of fine subcellular mechanisms through which the pharmacological effects of DHP in the cell can be elicited. The obtained data indicate new useful avenue in understanding the action of DHP molecules as drugs with steroid-like action. First of all the anti-inflammatory and immunosuppressive effects may be observed. As

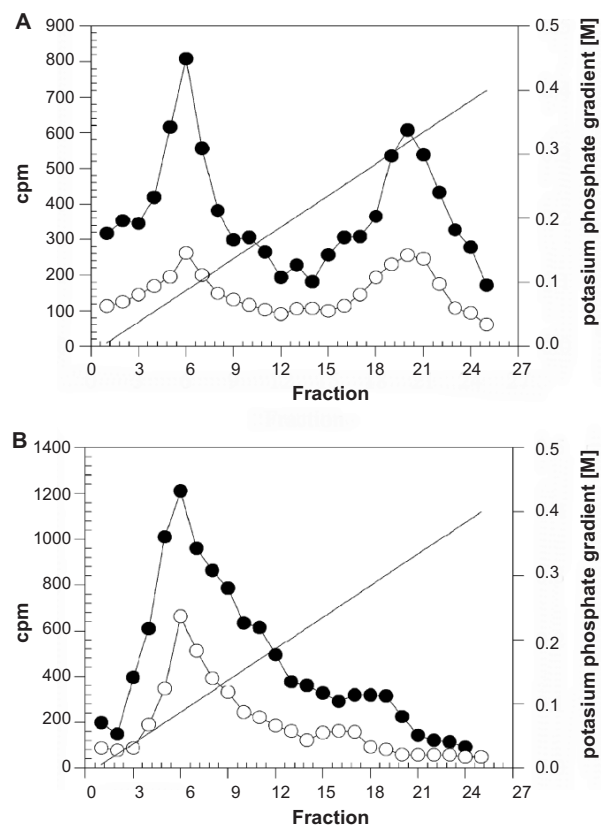


Fig. 1. Influence of foridone on the formation of the activated form of glucocorticoid receptor. Incubation of the cytosol and DEAE-cellulose chromatography was performed at 4°C (A) and 20°C (B). Black symbols – without foridone, open symbols – in the presence of 10⁻⁴ M of foridone

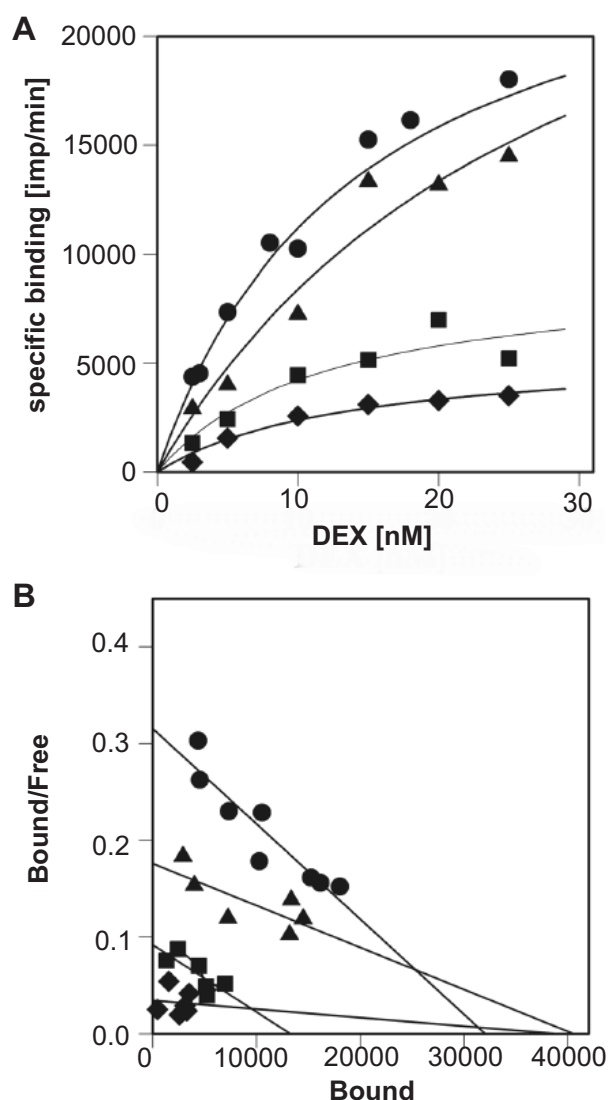


Fig. 2. Saturation curves (**A**) and Scatchard plot (**B**) of glucocorticoid hormone receptors with dexamethasone in the presence or absence of various DHPs. Circles – dexamethasone (control), squares – nifedipine, triangles – OSI-1211, diamonds – J-6-163. The final concentrations of DHPs were 10^{-4} M

it is well known, glucocorticoids are essential molecules contributing to regulation of activity of inflammatory and immune mediators [3, 27]. Particularly intriguing may be an idea of anti-inflammatory action of some of active neuroprotectants of the DHP series (e.g. cerebrocrast) in neuroinflammatory processes which now are considered as crucial in development of neurodegenerative diseases such as Alzheimer's disease [18].

Search for new type of anti-inflammatory drugs which have no typical glucocorticoid or non-steroidal anti-inflammatory drug side effects and which may

effectively regulate intracellular processes by maintaining cell life-span and by protecting it from neurodegeneration, seems to be rational and may involve further studying the DHP molecules in inflammatory processes.

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