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Neuroprotective effects of (24*R*)-1,24-dihydroxycholecalciferol in human neuroblastoma SH-SY5Y cell line☆

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Abstract

The active form of Vitamin D_3 has been reported to prevent neuronal damage caused by a variety of insults, however, it may also induce undesirable hypercalcemic effects. In the present study, we evaluated effects of (24*R*)-1,24-dihydroxycholecalciferol (PRI-2191) on hydrogen peroxide- and excitatory amino acid-induced neuronal damage in human neuroblastoma (SH-SY5Y) cell line. Exposure of SH-SY5Y cells to *N*-methyl-D-aspartate (NMDA; 5 mM), kainate (0.2 mM) and hydrogen peroxide (0.1–1 mM) significantly enhanced lactate dehydrogenase release. Furthermore, the neurotoxic effects of hydrogen peroxide was dependent on c-Jun N-terminal kinase (JNK)- and p38- mitogen-activated protein kinase (MAPK) activity. Both secosteroids at nanomolar concentrations inhibited neuronal damage, but their efficacy varied depending on the toxic agent. PRI-2191 was equipotent as 1α,25-dihydroxyVitamin D_3 in protecting SH-SY5Ycells against NMDA toxicity, and had stronger effect against hydrogen peroxide-induced damage, but was less efficient against kainate-induced injury. The obtained results suggest potential usefulness of PRI 2191 in the treatment of neurodegenerative diseases.

Keywords: (24*R*)-1,24-dihydroxyVitamin D_3; PRI-2191; Toxicity; Hydrogen peroxide; Kainate; NMDA; SH-SY5Y cells; MAP kinases

1. Introduction

It has been postulated that the active form of Vitamin D_3-1α,25-dihydroxyVitamin D_3, is not only involved in regulation of the calcium homeostasis in peripheral tissue, but also has a potent influence on function of the central nervous system [1]. 1α,25-DihydroxyVitamin D_3 acts via specific nuclear receptors (Vitamin D receptor; VDR), which have been identified in multiple brain regions, such as hippocampus, cortex, amygdala and thalamus [2]. VDRs, which heterodimerize with retinoic acid receptors, act as ligand-activated transcription factors and regulate the expression of target genes, which play a key role in cellular growth, neuronal development and neurotransmitter synthesis. 1α,25-DihydroxyVitamin D_3 has also been shown to increase synthesis of trophic factors, such as nerve growth factor (NGF), neurotrophins 3 and 4 and glia-derived neurotrophic factor (GDNF) [3,4]. These effects may be linked to recently postulated neuroprotective properties of this secosteroid. In fact, 1α,25-dihydroxyVitamin D_3 protects neurons against NMDA, glutamate, 6-hydroxydopamine and reactive oxygen species in various neuronal cell cultures [5,6]. Moreover, 1α,25-dihydroxyVitamin D_3 slows neuronal aging and reduces ischemia-related brain damage in animal models [3,7]. Besides stimulation of neurotrophin gene expression, these effects seem to be associated with down-regulations of L-type calcium channel expression, stimulation of acetylcholine synthesis and increase in intracellular glutathione content [1]. Furthermore, 1α,25-dihydroxyVitamin D_3 can inhibit proinflamatory cytokine and nitric oxide synthesis [8]. These facts strongly suggest that 1α,25-dihydroxyVitamin D_3 may be useful in therapy of some neurodegenerative disorders including Parkinson's and Alzheimer diseases. The main drawback of 1α,25-dihydroxyVitamin D_3 as a potential neuroprotective agent is its ability to induce hypercalcemia and hyperphosphatemia, which in turn may lead to renal insufficiency. Importantly, the neuroprotective effects of 1α,25-dihydroxyVitamin D_3 do not seem to depend on peripheral calcium homeostasis, which indicates that some 1α,25-dihydroxyVitamin D_3 analogues with low or devoid of calcemic activity may be considered as potential drugs for treatment of neurodegenerative disorders.

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The aim of our study was to evaluate effects of (24*R*)-1,24-dihydroxyVitamin D_3 (PRI-2191), a low calcemic analogue of 1α,25-dihydroxyVitamin D_3, on hydrogen peroxide-, *N*-methyl-D-aspartate (NMDA)-, and kainate-induced damage in human neuroblastoma (SH-SY5Y) cells. The analysis of structure-biological activity relationships suggests that PRI-2191 should retain neuroprotective properties of the Vitamin D_3 active form.

2. Materials and methods

2.1. Cell culture

The SH-SY5Y (human neuroblastoma) cell line was obtained from the American Type Culture Corporation (ATCC). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% foetal bovine serum (Gibco-BRL), 50 units/ml of penicillin and 50 μg/ml of streptomycin (Sigma Co), and kept in humidified atmosphere of 5% CO_2/95% O_2 at 37 °C.

Three to four days before experiment the cells were seeded at 15000 cells per well in 96-well plates in medium containing reduced amount of serum (5% FBS). In the first part of this study, effect of different concentrations of hydrogen peroxide (0.1–1 mM) on lactate dehydrogenase (LDH) release was estimated after the oxidant had been present in the medium for 24 h. Next, the effect of c-Jun N-terminal kinase (JNK) inhibitor SP 600125 (1 μM) and p38- kinase inhibitor SB 203580 (1 μM) on hydrogene peroxide (0.5 mM)–induced LDH release was measured. The inhibitors were dissolved in DMSO (final concentration 0.5%) and added to culture medium for 24 h, 20 min before hydrogen peroxide.

In the second part of this study, 1α,25-dihydroksyVitamin D3 (calcitriol) and (24*R*)-1,24-dihydroxyVitamin D_3 (tacalcitol, PRI-2191; both of Pharmaceutical Research Institute, Warsaw, Poland) dissolved in a mixture of ethanol/water were added at indicated concentrations to culture medium for 24 h. After 20 min, hydrogen peroxide (0.5 mM), NMDA (5 mM; RBI) or kainate (0.2 mM; Tocris) were added for 24 hours. Control cells were treated with the same volume of appropriate solvent.

2.2. Determination of LDH activity

Neurotoxicity was detected by measuring the efflux of LDH into the culture media 24 h after treatment with toxic agent (hydrogen peroxide, NMDA or kainate). LDH activity was determined in medium using colorimetric method (Cytotoxicity Detection Kit, Roche Diagnostic GmbH), according to which the amount of colored hydrazone, formed in a reaction of pyruvic acid with 2,4-dinitrophenylhydrazine, was inversely proportional to the LDH activity in the sample and could be quantified by measuring the absorbance at 400–550 nm.

2.3. Statistics

Statistical analysis of the results was performed using ANOVA followed by Duncan's test. Value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of SP 600125, SB 203580, 1α,25 dihydroxyVitamin D_3 and PRI-2191 on toxicity induced by hydrogen peroxide in SH-SY5Y cells

Incubation of SH-SY5Y cells for 24 h with hydrogen peroxide (0.1–1 mM) enhanced up to sixfold in a concentration-dependent manner the LDH level in the medium (Fig. 1.). Hydrogen peroxide concentration of 0.5 mM has been chosen for further study. In order to verify involvement of mitogen-activated protein (MAP) kinases in neurotoxic mechanism of hydrogen peroxide in SH-SY5Y cel1s, as postulated for other cell cultures [9], the effects of SP 600125 (a selective inhibitor of JNK kinase) and SB 203580 (inhibitor of p38 kinase) were determined. Both SP 600125 and SB 203580 completely inhibited hydrogen peroxide-induced LDH release (Fig. 1.). 1α,25-dihydroxyVitamin D_3 at 50 and 500 nM, but not at 5 nM, significantly decreased the hydrogen peroxide-induced toxicity by 25 and 27%, respectively (Fig. 2). Even stronger neuroprotective effect was observed after incubation of the cells with the analogue of 1α,25-dihydroxyVitamin D_3, PRI-2191, which at 5, 50 and 500 nM inhibited the hydrogen peroxide-evoked toxicity by 36, 41 and 43%, respectively.

3.2. Effect of 1α,25 dihydroxyVitamin D_3 and PRI-2191 on toxicity induced by NMDA in SH-SY5Y cells

Incubation of SH-SY5Y cells for 24 h with 5 mM NMDA enhanced 3-fold the LDH level in the medium as compared to control. Lower concentrations of NMDA (0.2–1 mM) had no consistent effect on LDH release (data not shown). 1α,25-dihydroxyVitamin D_3 at 5 and 50 nM attenuated the NMDA-evoked cell injury by 70 and 62%, respectively (Fig. 3). PRI-2191 at the same concentrations showed also significant, albeit weaker (59 and 42%, respectively) neuroprotective effect. Both compounds under study, when used at the lowest (0.5 nM) and the highest (500 nM) concentrations, had no significant effect on NMDA-induced LDH release.

3.3. Effect of 1α,25 dihydroxyVitamin D_3 and PRI-2191 on toxicity induced by kainate in SH-SY5Y cells

Exposure of the cell culture to 0.2 mM kainate for 24 h elevated LDH release by ca. 30% as compared to control. The kainate-induced toxicity was attenuated by

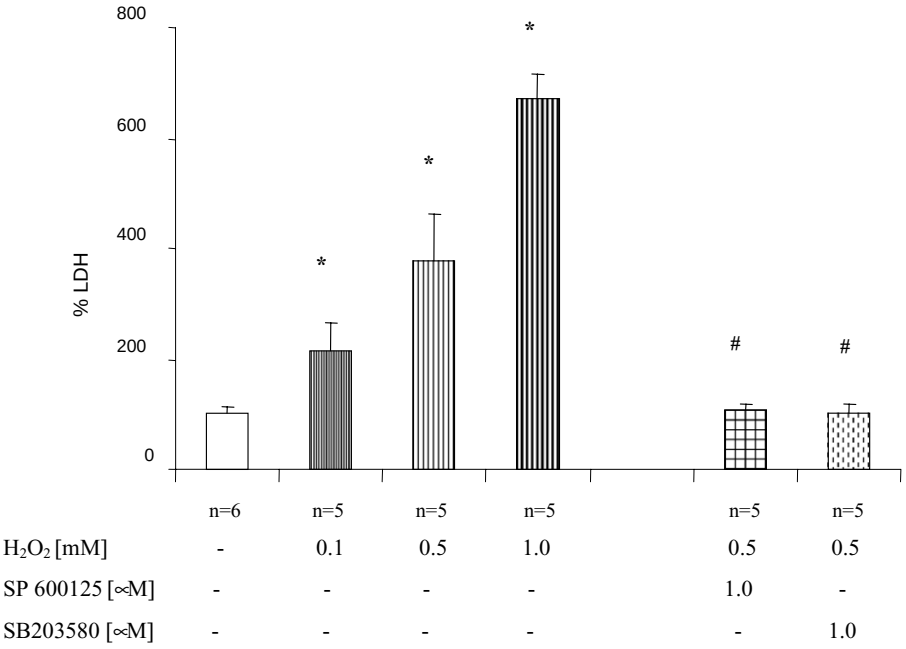

Fig. 1. Concentration-dependent effect of hydrogen peroxide (H_2O_2) on lactate dehydrogenase (LDH) efflux from SH-SY5Y cells. The effect of SP 600125 and SB 203580 on hydrogen peroxide (0.5 mM)—induced LDH efflux. The data were normalized to the amount of LDH released from control cells receiving vehicle only (100%). Results are shown as a mean ± S.D., and the significance of differences between the means was evaluated by the Duncan's test following a one-way or two-way analysis of variance, respectively (*$P < 0.05$ vs. control culture; $^{\#}P < 0.05$ vs. 0.5 mM H_2O_2); *n*: number of cultures.

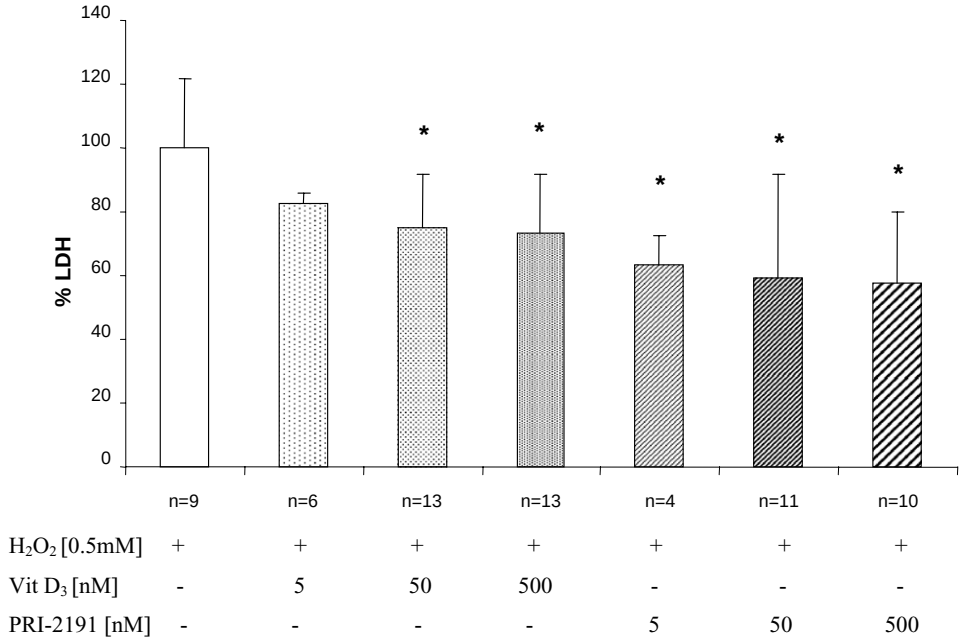

Fig. 2. The effect of 1α,25-dihydroxyVitamin D_3 (vit. D_3) and its analogue PRI –2191 on 0.5 mM hydrogen peroxide (H_2O_2)-induced lactate dehydrogenase (LDH) efflux from SH-SY5Y cells. The data were normalized to the amount of LDH released from vehicle-treated cells exposed to H_2O_2 (100%). Results are shown as a mean ± S.D., and the significance of differences between the means was evaluated by the Duncan's test following a two-way analysis of variance (*$P < 0.05$ vs. control culture); *n*: number of cultures.

1α,25-dihydroxyVitamin D_3 (50 and 500 nM) by 76 and 51%, respectively, whereas PRI-2191 had significant effect (66%) only when used at 500 nM. Lower concentrations of the secosteroids were without effect (Fig. 4).

4. Discussion

Despite an enormous need and many years of extensive research, no neuroprotective drug acting specifically on

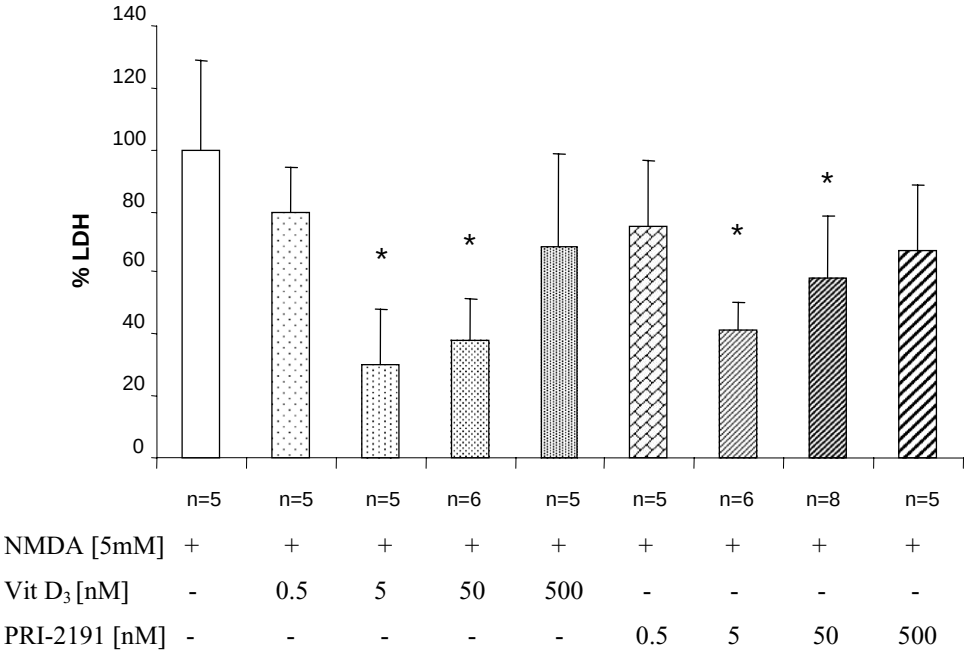

Fig. 3. The effect of 1α,25-dihydroxyVitamin D_3 (vit. D_3) and its analogue PRI –2191 on NMDA-induced lactate dehydrogenase (LDH) efflux from SH-SY5Y cells. The data were normalized to the amount of LDH released from vehicle-treated cells exposed to H_2O_2 (100%). Results are shown as a mean ± S.D., and the significance of differences between the means was evaluated by the Duncan's test following a two-way analysis of variance (*$P < 0.05$ vs. control culture); *n*: number of cultures.

neuronal cells has been introduced to the clinical practice. Numerous clinical trials with substances inhibiting particular steps of excitotoxic and/or apoptotic cascades, such as excitatory amino acid antagonists, voltage-dependent sodium and calcium channel blockers, antioxidants, inhibitors of nitric oxide synthases, etc. due to low efficacy or undesired effects proved disappointing. Some expert opinions suggest that a neuroprotective drug should be searched for among agents which can act simultaneously on several mechanisms critical to neuronal survival. The fact that

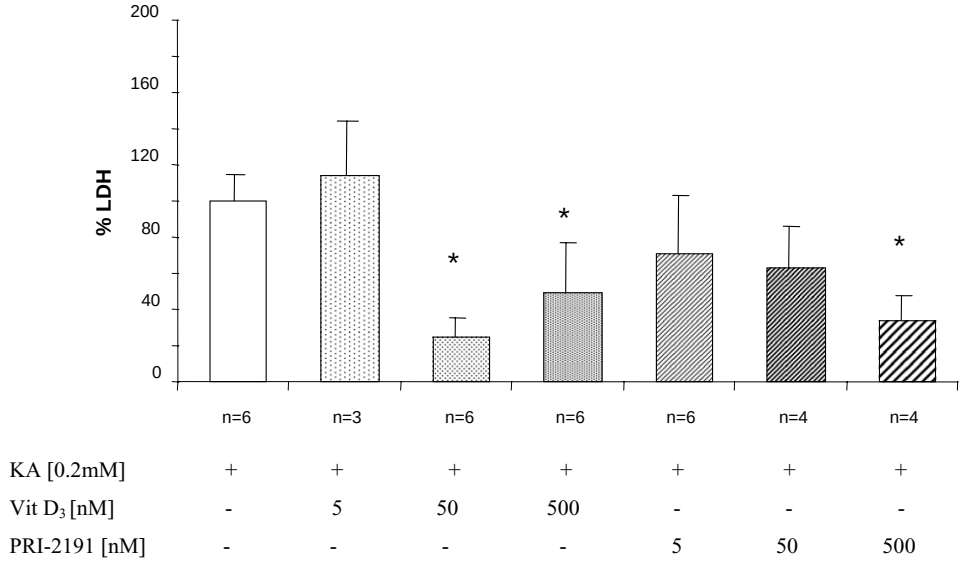

Fig. 4. The effect of 1α,25-dihydroxyVitamin D_3 (vit D_3) and its analogue PRI–2191 on kainate-induced lactate dehydrogenase (LDH) efflux from SH-SY5Y cells. The data were normalized to the amount of LDH released from vehicle-treated cells exposed to H_2O_2 (100%). Results are shown as a mean ± S.D., and the significance of differences between the means was evaluated by the Duncan's test following a two-way analysis of variance (*$P < 0.05$ vs. control culture); *n*: number of cultures.

some low-calcemic analogues of 1α,25-dihydroxyVitamin D_3, independently of their affinity for nuclear VDRs, stimulate NGF synthesis [10] suggests a possibility to obtain a substance with stronger neuroprotective action than 1α,25-dihydroxyVitamin D_3 itself, but devoid of hipercalcemic activity. The present data showed that both 1α,25 dihydroxyVitamin D_3 and its low calcemic analogue protect human neuroblastoma SH-SY5Y cells against NMDA-, kainite- and hydrogen peroxide-induced damage.

Considering neuroprotective effects of secosteroids on excitatory amino acid-induced damage, it should be mentioned that SH-SY5Y cells present many features of mature noradrenergic neurons and express glutamate receptors (GluRs) and sodium-dependent glutamate-aspartate transporter [11,12]. Activation of both ionotropic, especially the NMDA ones, and metabotropic GluRs in this cell line increases intracellular Ca^{2+} concentration [11], whose overload is a crucial factor triggering excitotoxic and apoptotic cascades. Indeed, in the present study activation of NMDA receptor functionally coupled with calcium channels had stronger neurotoxic effect on SH-SY5Y cells than the agonist of kainate/AMPA receptors. Low nanomolar concentrations of PRI-2191 and 1α,25 dihydroxyVitamin D_3 showed similar potency in inhibiting of NMDA-induced toxicity, whereas high concentrations of both compounds were ineffective. These data are in general agreement with other reports, which showed that low concentrations (0.1–100 nM) of 1α,25 dihydroxyVitamin D_3 prevented the NMDA- and glutamate-induced toxicity of mesencephalic [6] and hippocampal cells in vitro [5]. In contrast to equivocal results concerning efficacy of neuroprotective effects of the secosteroids used at low concentrations, a uncertainty exists whether they are also effective at high concentrations. Indeed, both secosteroids at 500 nM were ineffective against NMDA-induced injury, which is in line with previous reports [5,6]. Both secosteroids had similar potency against NMDA-induced damage, whereas PRI 2191 prevented kainate-induced toxicity only when used at high concentration, and it was more potent than 1α,25 dihydroxyVitamin D_3 against hydrogen peroxide-induced injury. This suggests that neuroprotective effects of secosteroids depend on type of cell cultures and the neurotoxic agent, and points to their complex neurochemical mechanisms of action. Besides acting as transcription factors via nuclear VDR they regulate formation of second messengers and a variety of protein kinases by nongenomic membrane mechanisms [13]. Induction of differentiation and antiproliferative activity of 1α,25-dihydroxyVitamin D_3 and PRI 2191 are connected with activation of extracellular-signal regulated protein kinases (ERK 1, 2) and phophatidylinositols 3-kinase (PI3-K) [14]. PRI-2191 binds to the nuclear VDR with lower affinity than 1α,25-dihydroxyVitamin D_3, but has stronger effect on cell differentiation. Since activation of PI3-K and ERK and inhibition of JNK and p38 MAP kinase promote cell survival, a stronger effect of PRI against H_2O_2-induced toxicity may be connected with its more potent effect on protein kinase activity. Cytotoxic effect of H_2O_2 is connected with activation of JNK-, and p38-MAPK in various cell lines [9]. In line with these reports, we found that in SH-SY5Y cells, the H_2O_2-induced toxicity was also dependent on JNK- and p38-MAPK activity. Since 1α,25-dihydroxyVitamin D_3 inhibits the activation of JNK- and p38-MAPK and protects epidermal cells against hydrogen peroxide and other cytotoxic agents [15], it is not unlikely that also in SH-SY5Y cell line the neuroprotective effects of secosteroids involve inhibition of stress-activated protein kinases. However, this assumption has to be verified in future experiments by measuring effects of secosteroids on activity of these kinases.

Summing up, the present study showed that PRI-2191 was equipotent as 1α,25-dihydroxyVitamin D_3 in protecting SH-SY5Ycells against NMDA toxicity, had stronger effect against hydrogen peroxide-induced damage, but was less efficient against kainate-induced injury. The obtained results indicate that neuroprotective efficacy of PRI-2191 depends on a neurotoxic agent and suggest its potential usefulness in the treatment of neurodegenerative diseases.

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