Abstract. Since novel synthesized deprenyl-related derivatives of nitroxides, named "JSAs", have been shown to possess antioxidative properties, their cytotoxicity on neuronal-like PC-12 cells line was examined. The antiproliferative effect of two selected JSAs was examined and expressed as IC10, IC50 and IC90, and compared with those of the parent nitroxide (Nx-640), model nitroxide TEMPO and deprenyl. There were substantial differences in the dose-dependence of all the observed antiproliferative and cytotoxic effects. Compared to anticancer drugs and apoptosis inducers with topoisomerase inhibitor properties (etoposide and camptothecin), novel compounds displayed cytotoxicity at considerably higher concentrations. The dose-dependent anti-apoptotic potency of JSAs and Nx-640 was also investigated and compared to TEMPO and deprenyl effects. The observed structure-dependent correlation was very encouraging and prompted us to screen and to compare the in vivo time-dependent effects of JSAs, Nx-640 and deprenyl administration on the rat intact nigrostriatal neurones. TH-immunochemistry was applied as the test method and marker for the changes in the state of the rat catecholaminergic system, also giving evidence that low-toxic and cell-permeable JSAs can cross the blood-brain barrier, which is the mandatory prerequisite for the therapeutic application of antioxidants and drugs to the brain. Taken together, it can be concluded with great certainty that novel deprenyl-related JSAs might be especially good candidates for further anticancer investigations in vitro and in vivo and future pharmacological applications.

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Cytotoxicity, Cytoprotection and Neurotoxicity of Novel Deprenyl-related Propargylamines, Stable Nitroxide Free Radicals, In Vitro and In Vivo

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Apoptosis and the search for apoptotic "stimuli" are now important topics in anticancer research and they are commonly assumed to explain the action of chemotherapeutic drugs in cancer and resistance of tumor cells to the conventional therapeutic regimens (1-3). Very recent investigations gave evidence indicating the involvement of oxidative stress in many aspects of oncology, where ROS (reactive oxygen species) accumulated in excess mediate deleterious effects in vivo and are frequently associated with cytotoxicity, being described as damaging, harmful or toxic (4-7). Recently, it has become well known and widely accepted that ROS (superoxide, hydroxyl radical, hydrogen peroxide) may act as signaling molecules for the initiation and execution of apoptotic death in many, if not in all, current models of apoptosis (2-4, 7-13). Thus, a new strategy that could be introduced would be to employ low-toxic antioxidants to control both the degradative (oxidative) processes and apoptotic signaling pathways mediated by ROS, and the ways in which tumor cells modulate the processes to promote their survival by adaptive responses (2-4, 7-13).

We have previously found that low-toxic and cell-permeable nitroxide antioxidants acting as SOD-mimics can improve the cellular "antioxidant reserve", which may contribute to cell protection against oxidative stress and ROS cytotoxicity. Notably, growing evidence suggested that nitroxides may as well play a direct role in cancer cell death in vivo and paradoxically they can induce rather than inhibit apoptosis (14-18).

In our continued search for novel neuroprotective nitroxide compounds, we have designed a number of newly synthesized, deprenyl-related N-propargylamine derivatives of nitroxy, named "JSAs" (Figure 1) (19). The reactivity and antioxidative potency of two selected JSAs was examined in model solution systems and compared with those of deprenyl
(DEP, N-methyl-N-propargyl amphetamine), the most investigated component of adjunct therapies in experimental and clinical neuropathology and recently also in anticancer research (20-23). Notably, it is known that DEP and structurally related propargylamines, MAO B inhibitors, may increase neuronal survival, in part by decreasing apoptosis, participating in specific pro-apoptotic signal transduction pathways, by scavenging oxygen radicals or by reducing their generation (19-21, 23). Also, this potency of propargylamines and DEP could affect mitochondrial membrane permeability, which constitutes an essential element of the intrinsic pathway leading to apoptosis (2, 8, 10, 13, 20, 22).

The complex structure of novel JSAKs, with the presence of nitroxyl and propargylamine moieties (Figure 1), could warrant their ability to regulate, block or reduce the undesirable progression of the neurotoxic cascade of brain damage and clearly supports our idea that they might have potential implications for new experimental therapies, in both systematic and intracranial neoplasms and neurodegenerative events, where ROS mediate deleterious effects as well (3-9, 11-13, 19). This study was undertaken with the aim of examining and comparing the cytotoxicity of two selected JSAKs, 641 and 648, with those of the parent nitroxyl (Nx-640), TEMPO, DEP and two topoisomerase inhibitors,
camptothecin and etoposide (Figure 1), which are first-line chemotherapeutics against many kinds of malignances (24-27). Because of the rapid, specific and extensive induction of apoptosis by camptothecin and etoposide, they are convenient model compounds in studies on the anti-apoptotic activity of other substances. In this study they were used to examine the protective, anti-apoptotic efficiency of JSAKs and to compare it with those of Nx-640, TEMPO and DEP (Figure 1) in vitro. The neuronal-like pheochromocytoma cell line PC-12 used here is a well established model system to investigate the influence of oxidative stress as mediator of induced apoptosis, having biochemical characteristics that resemble those of dopaminergic neurons (28).

The cytotoxic effects of JSAK treatment on dopaminergic neurons of rats were also investigated in vivo and compared with DEP and Nx-640 actions under the same experimental conditions. Tyrosine hydroxylase (TH)-immunochemistry was applied as the test method, having in mind that TH is the rate-limiting enzyme for catecholamine synthesis and an excellent marker for changes of the catecholaminergic system in mammalian brains, because of the vulnerability of the pigmented nigrostriatal dopaminergic neurons to toxic substances (29, 30).

**Materials and Methods**

**Materials.** All chemicals of analytical grade used for synthesis of the novel nitroxide derivatives (Figure 1, A-C) were purchased from Fluka (Buchs, Switzerland), Nx-640 (4-(dimethylamino)-2,2,6,6-tetramethylpiperidine-1-oxyl,4-dimethylamino-TEMPO), JSAK-648 (4-(N-methyl-N-propargylamino-TEMPO) and JSAK-641 (4-[(N,N-dimethyl)-propargylammonio-2,2,6,6-tetramethylpiperidine-1-oxyl bromide) were synthesized as described previously (19). (R)-(-)-deprenyl hydrochloride (DEP, Figure 1 D) was purchased from Tocris Cookson Ltd (Bristol, UK). Topoisomerase inhibitors, etoposide (Figure 1 E) and camptothecin (Figure 1 F), and all other reagents of analytical grade were from Sigma-Aldrich (Poznan, Poland). The rat pheochromocytoma cell line PC-12 was obtained from the American Type Culture Collection (Manassas, USA) and cultured in RPMI 1640 medium supplemented with 10% horse serum and 5% fetal bovine serum. The cells grew in adherent manner in plastic culture vessels (Nalge Nunc International, Roskilde, Denmark) coated with collagen isolated from rat tail tendons. Cells were subcultured every week and were plated in 96-well microplates (5000 cells per well) for viability testing experiments.

**Cells viability assays in vitro**

**MTT viability assay.** The principle of the MTT cell viability assay is based on the reduction of MTT tetrazolium salt by viable cells to insoluble colored formazan crystals, which are subsequently

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**Table I. Comparative cytotoxicity* of the investigated compounds on PC-12 cells line.**

<table>
<thead>
<tr>
<th>Nr</th>
<th>Compound</th>
<th>SIC (µM)</th>
<th>IC50 (µM)</th>
<th>MIC(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>TEMPO</td>
<td>8.0</td>
<td>80.0</td>
<td>2.0</td>
</tr>
<tr>
<td>2.</td>
<td>Nx-640</td>
<td>20.0</td>
<td>2000.0</td>
<td>-</td>
</tr>
<tr>
<td>3.</td>
<td>JSAK-641</td>
<td>20.0</td>
<td>200.0</td>
<td>-</td>
</tr>
<tr>
<td>4.</td>
<td>JSAK-648</td>
<td>100.0</td>
<td>1000.0</td>
<td>5.0</td>
</tr>
<tr>
<td>5.</td>
<td>etoposide**</td>
<td>1.0</td>
<td>5.0</td>
<td>0.015</td>
</tr>
<tr>
<td>6.</td>
<td>camptothecin ***</td>
<td>0.10</td>
<td>0.35</td>
<td>0.001</td>
</tr>
<tr>
<td>7.</td>
<td>Deprenyl (DEP)</td>
<td>100.0</td>
<td>800.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

* SIC (subinhibitory concentration) - the highest concentration where still no effect of the compound on cell survival (>90% survival) was seen; IC50 - the concentration of compound causing 50% survival of cells; MIC (minimal inhibitory concentration) - the lowest concentration of compound where the maximal decrease of cell survival (< 10%) was observed; ** proapoptotic inhibitor of topoisomerase II; *** proapoptotic inhibitor of topoisomerase I.
Figure 3. Susceptibility of neuronal-like pheochromocytoma (PC-12) cells to etoposide (left) and camptothecin (right) and concentration-dependent effects of pre-treatment with nitroxide compounds.*

* - control, □ - 0.01, □ - 0.1, □ - 0.5 and □ - 2.0 mM of the investigated compounds, respectively. A, TEMPO; B, Nc-640; C, JSAK-641 and D, JSAK-648.

Experimental conditions as described in Materials and Methods. Mean ± S.E.M. of five separate experiments, t-test: p<0.05.
solubilised and assayed spectrophotometrically (31). Viability of PC-12 cells was determined after 24 hours or 3 days of exposure of cells to specific concentrations of toxic compounds. MTT solution was added to the culture medium to a final concentration of 0.2 mg/ml, the cells were incubated at 37°C for 2 hours, washed with PBS and formazan crystals were solubilised in 100 μl of dimethylsulfoxide. The formazan concentration (proportional to the number of viable cells) was determined spectrophotometrically in a StatFax 2100 (Awareness Technology, Palm City, USA) microplate reader at 580 nm.

Neutral red viability assay. The principle of the neutral red cell viability assay is based on the accumulation of the lysosomotropic dye neutral red in acidic lysosomes of viable cells. Viability of PC-12 cells was determined after 24 hours or 3 days of exposure to specific concentrations of toxic compounds. Neutral red solution was added to the culture medium to a final concentration of 0.05 mg/ml, the cells were incubated at 37°C for 3 hours, washed with PBS and fixed with 0.5% formaldehyde. Dye bound within the cells was solubilised in 1% acetic acid in 50% ethanol and its concentration was determined spectrophotometrically in a microplate reader at 545 nm.

LDH efflux viability assay. The principle of the LDH efflux viability assay is based on the release of the cytoplasmic enzyme lactate dehydrogenase from damaged cells (cells with compromised membrane integrity) - thus, it may be used to measure acute cellular toxicity rather than general viability or death by apoptotic mechanisms. The viability of PC12 cells was determined after 3 days of exposure to specific concentrations of toxic compounds. Cell growth medium was then transferred to another 96-well microplate, where it was mixed with the LDH assay solution containing (final concentrations): 50 mM sodium lactate, 0.4 mM iodonitrotetrazolium violet (INT), 1 mM NAD, 0.2 mM phenazine methosulfate in 0.2 M Tris-HCl buffer, pH 8.0. After 15 minutes of incubation at room temperature, the reaction was stopped by addition of sodium oxamate (LDH inhibitor) to a final concentration of 20 mM and the formed soluble formazan was determined spectrophotometrically in a microplate reader at 492 nm. As positive control (100% of damaged cells), cells lysed with 1% Triton X-100 were used.

Animals and experimental procedures in vivo. The experiments were carried out in compliance with the Animal Protection Bill published in Poland’s Official Current Legislation Gazette (No 11, 1997, item 724) and according to the NIH guide for the Care of Laboratory Animals. Eighty female Buffalo rats weighing 170 - 200 g, approximately 3 months old (Central Animal Farm, MU, Wroclaw, Poland), were housed in groups of 10 under standardized conditions (temperature 22°C, 12 h light: 12h dark cycle, free access to standard granular diet containing 24% crude protein and water). The experimental protocols were approved by the appropriate institutional Governmental Agency.

In this study rats were subjected to the following 5 treatments: single injections (i.p.) of DEP, Nx-640, JSAK-641, JSAK-648, respectively (2.5 mg/kg) (Figure 1) or saline (1.0 mg/kg) as control. Animals were sacrificed by decapitation at the appropriate time of the experiment, respectively. The brains were rapidly dissected, rinsed twice with cold PBS, dried on blotting paper and halved in the sagittal plane. One half was weighed and fixed in 10% buffered
Figure 5. Photomicrographs of typical examples of rat nigrostriatal cells stained with anti-TH antibody after DEP (left) or Nx-640 (right) pre-treatment.*

*A – 30 min, B – 2 h, C – 24 h and D – 48 h after DEP or Nx-640 administration, respectively.
Experimental conditions as described in Materials and Methods. Magnification = x 200.
formalin. These fixed tissues were embedded in paraffin and representative coronal sections (6 micrometers thick) were obtained. Brain sections were immunostained for tyrosine hydroxylase (TH) using the ARK method (DAKO ARK, Animal Research Kits: K 3954 and 3955). The paraffin sections were deparaffinized, rinsed twice (10 min) with water and cooked in 0.01 M sodium citrate (pH 6.0) for 8 min at 350 Watt in a microwave device. The samples were rinsed again (10 min) with water. The biotinylated ex tempore antibody (anti-tyrosine hydroxylase mouse monoclonal antibody, NCL-TH, Novocastra) was applied for 15 min. The sections were rinsed with PBS twice (10 min) and streptavidin-peroxidase was added for the next incubation (15 min). Then samples were washed again with PBS (twice) and treated with DAB + chromogen. The sections were rinsed with water twice for at least 5 min each time, counterstained with hematoxylin for 1 min and covered with coverlips for the histological examination (30).

Results and Discussion

Comparative cytotoxicity of nitroxide derivatives and topoisomerase inhibitors to PC-12 cells: relevance to the deprenyl effects. The antiproliferative potency of the model nitroxide, TEMPO, parent nitroxyl, Nx-640 or the novel propargylamine nitroxide derivatives, JSAK-641 and 648 (Figure 1) was estimated and compared with those of DEP or the model anticancer drugs - topoisomerase inhibitors, etoposide and camptothecin (Figure 1): the results were expressed as SIC (subinhibitory concentration), IC₅₀ and MIC (minimal inhibitory concentration), respectively (Table I). As can be seen, the degree of cytotoxicity of the investigated nitroxides was found to be dependent either on the substitution at the piperidine ring (Figure 1), the structural factor playing an important role in their antioxidative potency (14-19) and on the presence of the propargyl moiety in the molecule. It was evident that JSAKs and Nx-640 affect the PC-12 cells viability to a lesser extent than TEMPO or the apoptosis inducers (24-27), etoposide and camptothecin, respectively (Table I). Notably, the presented results were obtained in the concentration range between 1 µM - 10.0 mM (for all investigated nitroxyls), 0.1 - 200 µM (etoposide) and 0.1 - 100 µM (camptothecin), respectively. Compared to the latter compounds, JSAKs displayed cytotoxicity at considerably higher concentrations. The compound with cytotoxicity comparable to DEP was JSAK-648, whereas JSAK-641 was about four times more toxic (Table I). It is important to emphasize here that these results concerning the novel nitroxides, Nx-640 and JSAKs are in accordance with recently reported data of investigations of other nitroxides, indicating that they can be used in cell cultures at usual (i.e. milimolar) concentrations (14-19, 32). Taken together the investigated compounds (Figure 1) revealed the sequence of growth inhibition: camptothecin > etoposide > TEMPO > JSAK-641 > DEP > JSAK-648 > Nx-640. This pattern was specific to the viability assay method: in the neutral red
assay DEP showed higher toxicity (IC_{50} in the range of 100 mM) than TEMPO or any of the novel derivatives (IC_{50} > 0.5 mM) (not shown). Thus, this difference is probably related to the principle of MTT assay which is thought to be dependent on mitochondrial membrane potential and highly sensitive to any redox effects.

Both tested topoisomerase inhibitors were found to mediate strong inhibition of PC-12 cell growth and induction of cell death (Table I). Camptothecin was more toxic to the cells than etoposide, showing maximal inhibition of cell growth already at submicromolar concentrations. A significant difference was seen between IC_{50} values for camptothecin determined in the MTT and neutral red viability assays (not shown), whereas such a difference was detected for etoposide, which suggests a different sequence of cell death events upon induction of apoptosis by these two compounds, despite their overall similarity in mechanism of action. As expected, virtually no acute (necrotic) toxic effects of etoposide and camptothecin on PC-12 cells were seen in the LDH viability assay (not shown), which confirms their exclusively apoptotic mechanism of toxicity. Notably, while etoposide is known to induce apoptosis in neuronal cells and cell lines including PC-12 cells (33), no results of detailed studies of the cytotoxicity of these drugs to PC-12 cells are available. As for topoisomerase inhibitors acting as apoptosis inducers during the execution phase of apoptosis (33), no significant acute (necrotic) toxicity of all the investigated compounds was detected in the LDH assay of the viability in the tested concentrations range.

**Protective effect of nitroxide propargyl derivatives in PC-12 cells exposed to highly toxic topoisomerase inhibitors: comparison with DEP.** As can be seen in Figure 2 (A and B), DEP was able to exert some concentration-dependent protective effect on PC-12 cells. The extent of this effect was investigated in the concentration range of DEP between 0.01 µM - 100 µM in the presence of etoposide (1.0 - 10 µM) or camptothecin (50 - 200 nM), respectively. Notably, at the concentration of 100 µM DEP and 10 µM etoposide (Figure 2A) or 200 nM camptothecin (Figure 2B), respectively, the viability of PC-12 cells was increased by more than 90%. Thus, concerning the concentrations of DEP and topoisomerase inhibitors, the rescue effects should be independent of MAO-B inhibition (23) and the mechanism of this protection is not yet known. Under the same concentration range of etoposide, nitroxide propargyl derivatives (JSAK-641 and JSAK-648) were not able to mimic or even surpass the protective effects seen for DEP (c.f. Figure 2A and Figure 3 left). As can be seen in Figure 3B (left), Nx-640 at a concentration of 2 µM showed a protective effect (about 30%) similar to 1 µM of etoposide, which was not observed when propargyl nitroxide derivatives were applied (concentration range of 0.01 - 2 mM) (Figure 3C and D). This protective effect seems to be specific for the nitroxide moiety in the absence of the propargyl group, which might be related to interference with redox reactions involved in the initiation of the intrinsic pathway of apoptotic cells death caused by etoposide. It remains to be explored, because the low dose of etoposide and protector (Nx-640) effect (Figure 3B, left) suggests that a novel, hitherto unknown mechanism of the anti-apoptotic action of nitroxyls, related to their interference with redox reactions in the initiation of the intrinsic pathway of apoptosis caused by etoposide, could be involved. Nx-640 showed a slight protective effect to PC-12 cells at 2 mM concentration and 200 nM camptothecin (Figure 3B, right). As is shown in Figure 3C (right), JSAK-641 was able to mimic the protective action of DEP at a 50 times lower concentration, but only in the case of camptothecin (100 - 200 nM)-mediated cytotoxicity.

**Evaluation of the in vivo time-dependent effects of novel nitroxide derivatives on rat dopaminergic neurons: comparison with DEP.** The purpose of the performed study (Figures 4-6) was to determine and to compare the possible neurotoxic effects per se of JSAKs (641 or 648) and Nx-640 treatment on mammalian (rat) dopaminergic neurons. These investigations are in line with a recent concept that in vitro (cell cultures) chemosensitivity assays do not always allow for a prediction of the in vivo effects of the novel designed compounds (drugs). Moreover, it is still not known whether apoptotic cell death pathways in mammalian neurons are identical to those of the neuronal-like PC-12 cells (34). Hence, we applied the test assay involving in vivo time-course investigations of the effects of rat treatment with the investigated novel compounds (Figure 1, A-C), in conjunction with a sensitive TH-immunohistochemistry in vitro as an excellent marker for the state of the dopaminergic system of mammalian brains (30, 32). Dopaminergic neurons with the TH-antibody were easily detectable in the representative microphotographs of TH-immunostaining in the rat SN (substantia nigra) (35). As is shown in Figure 4A, in the saline-treated control group, TH-immunoreactivity revealed homogenous and weakly-stained neuronal cell bodies and fibres. At a very early stage after JSAK-648 treatment (30 min postinjection up to 2 h) and 48 h later (Figure 4B and C), the histopathological examination did not show any marked modification of the immunoreactivity of neurocytes when compared with the control (Figure 4A), clearly indicating that JSAK-648 administration was not able to profoundly impair both cytoplasm and fibres. Contrasting with these results, the pre-treatment with DEP (Figure 1D) resulted in time-dependent changes (Figure 5). Thirty min after the single dose of DEP, an increased TH-reactivity in cell
bodies and a homogenous TH-reaction in fibres were revealed (Figure 5A, left) which decreased 2 h later (Figure 4B, left). Notably, in the single fibres adjacent to the cell body (proximal part of axons) the TH-reaction had a visible granular expression after 24 h (Figure 5C, left). Forty-eight h later, the TH-reaction (Figure 5D, left) was intensive in cell bodies and the numbers of fibres was slightly elevated but still visibly lower as compared with control (Figure 4A) or earlier stages after DEP administration (Figure 5 A-C, left). Thus, during the investigated time (30 min - 48 h), DEP administration impaired the dopaminergic system, which raises a question: how can the effect per se of DEP on the TH-immunoreactivity of SN-neurocytes in vivo be explained in light of its known multiple pharmacodynamic actions (19-21)? Thus, the detailed biochemical mechanisms of DEP action on brain cells remain to be investigated and clarified.

The pre-treatment of rats with Nx-640 (Figure 1A) resulted in medium, granular TH-positivity in cell bodies with weakly TH-positive fibres, which began at 30 min postinjection and was continued up to 24 h (Figure 5 A-C, right) when no TH-positive fibres were observed. Forty-eight h later, the histopathological examination showed a strong and somewhat edematous TH-positive reaction in cell bodies and strongly TH-abundant positive fibres were seen (Figure 5D, right). The overall picture was very similar to those observed for DEP at the same time (c.f. Figure 5 D, left and right), except the fibres "edema", typical for Nx-640 action.

As can be seen in Figure 6, in the JSAK-641 pre-treated group, the TH-immunoreactivity was comparable to that observed for DEP at an early stage of its administration (30 min) (c.f. Figure 5A, left and Figure 6A). Two h later (Figure 6B), the fibres began to be slightly edematous, but their number and the intensity of the TH-reaction in both cell bodies and fibres were comparable with those seen 48 h after DEP or Nx-640 administration, respectively (Figure 6B and Figure 5D, left and right). After 24 - 48 h (Figure 6C and D) there was no edema observed in fibres and the overall picture was similar to all others, observed 48 h after the administration of the investigated compounds.

The grade of the time-dependent changes of the TH-immunoreactivity and the morphology of intact neurocytes increased in the order JSAK-648 < JSAK-641 < DEP < Nx-640 as compared with those of control (saline-treated group), suggesting a difference strongly dependent on the nitroxide structure and the presence of the propargylamine moiety in the molecule as well. We would like to emphasize here that the results of the in vivo investigations of neurotoxicity differ from these obtained for the neuronal-like cells PC-12 chemosensitivity (viability) assay in vitro. However, at this stage of our work, the ultimate mechanistic question still remains unsolved, even if we accept this difference probably results from well defined but differing conditions of the in vitro and in vivo experiments: how the structure of the novel JSAKs (Figure 1) is related to the site(s) of action within cells and their antioxidant properties (18, 19). Thus, the test systems studied cannot comprise the whole scale of possible biological "scenarios" concerning JSAKs action either in vitro or in vivo, but they nevertheless cover a representative range of standard testing and indicator reactions, which have relevant connections to in vivo situations.

In conclusion, this work provides the basis for further investigations of the novel, low-toxic and BBB (blood-brain barrier)-permeable propargylamine derivatives of nitroxide, JSAKs, since the presented results are indicative of their intracellular activation and cell permeability. These properties of JSAKs could be associated with their structure-dependent activities (Figure 1): the anticancer, antiproliferative and antioxidant activities of nitroxide free radical moiety (14-19), and the antiapoptotic potency of propargylamines (20-23). The data clearly shows that these new synthesized analogues may find application in acute oxidative stress, in brain cancer development, where ROS are generated in excess (4, 13, 19, 30), either to block or reduce the neuronal damage and death.

Further studies are in progress in our laboratories to confirm and evaluate the beneficial effects of JSAKs administration and to elucidate the cellular mechanisms of their activities both in vitro and in vivo models.

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