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Non-steroidal anti-inflammatory drugs potentiate 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death by promoting the intracellular accumulation of MPP^+ in PC12 cells

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a) NSAIDs potentiate MPP^+-induced cell toxicity.

b)

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c)

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d)

Abbreviations: MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NSAID, non-steroidal anti-inflammatory drug; PBN, phenyl-*N*-butyl-nitrone; NAC, N-acetyl-L-cysteine; NS-398, N-[2-(cyclohexyloxy)-4-nitrophenyl]methanosulfonamide; MK 571, (3-(3-(2-(7-chloro-2-quinolinyl) ethenyl) phenyl ((3-dimethyl amino-3oxo-propyl) thio) methyl) propanoic acid; GW9662, 2-chloro-5-nitro-*N*-phenylbenzamide; PPARγ, peroxisome proliferator-activated receptor γ; MRP, multidrug resistance protein; COX, cyclooxygenase; WST-1, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; 1-methoxy PMS, 1-methoxy-5-methylphenazinium methylsulfate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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Abstract

In this study, we investigated the effects of non-steroidal anti-inflammatory drugs (NSAIDs) on 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death in PC12 cells. Co-incubation of PC12 cells with indomethacin, ibuprofen, ketoprofen or diclofenac, but not aspirin or NS-398, significantly potentiated the MPP^+-induced cell death. In contrast, these NSAIDs had no effect on rotenone-induced cell death. The potentiating actions of these NSAIDs were not suppressed by treatment with phenyl-*N*-butyl-nitrone (PBN), a radical scavenger, N-acetyl-L-cysteine (NAC), an antioxidant, Ac-DEVD-CHO, a selective caspase-3 inhibitor, or GW9662, a selective antagonist of peroxisome proliferator-activated receptor γ (PPARγ). Furthermore, we observed that DNA fragmentation which is one of the hallmarks of apoptosis was not induced by co-incubation with MPP^+ and NSAIDs. We confirmed that co-incubation of PC12 cells with 30 μM of MPP^+ and 100 μM of indomethacin, ibuprofen, ketoprofen or diclofenac led to a significant increase in the accumulation of intracellular MPP^+ compared incubation with 30 μM MPP^+ alone. In addition, these NSAIDs markedly reduced the efflux of MPP^+ from PC12 cells. MK 571, which is an inhibitor of multidrug resistance proteins (MRPs), mimicked the NSAIDs-induced effects, increasing cell toxicity and promoting the accumulation of MPP^+. Moreover, some types of MRPs mRNA were detected in PC12 cells. These results suggest that some NSAIDs might cause a significant increase in the intracellular accumulation of MPP^+ via the suppression of reverse transport by the blockade of MRP, resulting in the potentiation of MPP^+-induced cell death.

Key words

1-methyl-4-phenylpyridinium, non-steroidal anti-inflammatory drugs, cell toxicity, multidrug resistance protein, PC12 cell, WST-1 assay

Introduction

The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) causes a selective degeneration of nigrostriatal dopaminergic neurons and has been investigated extensively as part of an etiological model for Parkinson's disease (Tipton and Singer, 1993; Blum et al., 2001). The neurotoxic effects in vivo of MPTP are dependent on the conversion to its active metabolite 1-methyl-4-phenylpyridinium (MPP^+) by monoamine oxidase B in glial cells and the uptake into neurons through transporters (Tipton and Singer, 1993; Kitayama et al., 1998). MPP^+ induces an inhibition of complex Ⅰ activity of the mitochondrial respiratory chain and cellular ATP depletion and a loss of mitochondrial transmembrane potential, resulting in cell death (Bates et al., 1994).

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used for their anti-inflammatory, anti-pyretic, and analgesic properties. The molecular basis for the therapeutic effects of NSAIDs is the ability to inhibit cyclooxygenase (COX) activity and thereby suppress the production of prostaglandins (Vane, 1971). In addition to alleviating inflammation and pain, NSAIDs have in some cases been suggested to have pharmacological effects which are independent of the

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inhibition of COX activity. For example, aspirin and salicylate inhibit NF-κB (Kopp and Ghosh, 1994) and the activity of IκB kinase-β (Yin et al., 1998). Avramovich et al. (2002) demonstrated that NSAIDs can activate ERK mitogen-activated protein (MAP) kinase in SH-SY5Y neuroblastoma cells and PC12 cells. Lehmann et al. (1997) indicated that several NSAIDs bind and activate peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor. Moreover, it has been demonstrated in an overexpressing cell system that some NSAIDs, including indomethacin and ibuprofen, inhibit the activity of multidrug resistance protein 4 (MRP4), which is believed to be the active efflux transporter for the release of prostaglandins from the cells that produce them (Reid et al., 2003a).

Several recent studies have reported inconsistent effects of NSAIDs on the neurotoxicity of MPTP or MPP^+ in vivo. It has been demonstrated that aspirin, a nonselective COX inhibitor, and meloxicam, a selective COX-2 inhibitor, provide neuroprotection from MPTP at the striatal and nigral levels (Teismann and Ferger, 2001). In contrast, other studies have demonstrated that indomethacin and diclofenac, both nonselective COX inhibitors, have no protective effect on MPTP toxicity, while celecoxib, which is a specific COX-2 inhibitor, aggravated

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MPP^+-induced striatal dopamine depletion in rats (Sairam et al., 2003). The effects of NSAIDs on MPTP or MPP^+-induced cell death are therefore controversial. Thus, in the present study, the possible influences of several NSAIDs on the toxicity of neurotoxins in cultured rat PC12 cells were investigated. It was found that indomethacin, ibuprofen, ketoprofen and diclofenac significantly potentiated the MPP^+-induced cell death in PC12 cells. These NSAIDs may evoke a significant increase in the intracellular accumulation of MPP^+ as a result of a blockade of the outflow of MPP^+ from PC12 cells.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceutical Co. (Tokyo, Japan). MPP^+ iodide, indomethacin, ibuprofen, ketoprofen, diclofenac sodium, phenyl-*N*-butyl-nitrone (PBN), N-acetyl-L-cysteine (NAC), and rotenone were obtained from Sigma Chemical Co. (St. Louis, MO). N-[2-(cyclohexyloxy)-4-nitrophenyl]methanosulfonamide (NS-398) and (3-(3-(2-(7-chloro-2-quinolinyl) ethenyl) phenyl ((3-dimethyl amino-3oxo-propyl) thio) methyl) propanoic acid (MK 571) were provided by Cayman Chemical Co. (Ann Arbor, MI). Ac-DEVD-CHO was purchased from BIOMOL Research Labs. (Plymouth Meeting, PA), 2-chloro-5-nitro-*N*-phenylbenzamide (GW9662) from Tocris Cookson (Bristol, UK), WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) and 1-methoxy PMS (1-methoxy-5-methylphenazinium methylsulfate) from Dojindo Laboratories (Kumamoto, Japan), and [^{3}H]-MPP^+ (2590 GBq/mmol) from PerkinElmer Life Sciences (Boston, MA). All other reagents used were of analytical grade and from Nacalai Tesque (Kyoto, Japan), Katayama Chemical

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(Osaka, Japan) or GibcoBRL (Gaithersburg, MD). Indomethacin, ibuprofen, ketoprofen, aspirin and GW9662 were each dissolved in ethanol. MPP^+, diclofenac, NAC and Ac-DEVD-CHO were dissolved in distilled H_2O. NS-398, PBN, rotenone and MK 571 were dissolved in DMSO. The final concentrations of all solvents for treatment of the cells, including control cultures, were maintained at 0.25%.

Cell culture and drugs treatment

PC12 cells were maintained in DMEM supplemented with 10% horse serum (HS), 5% fetal calf serum (FCS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in an atmosphere of 5% CO_2/95% air at 37℃. All experiments were carried out 24 hours after cells were seeded.

MPP^+ toxicity assay

Cells were treated with various concentrations of MPP^+, either alone or together with NSAIDs, in low glucose DMEM supplemented with 2% HS, 1% FCS, and penicillin/streptomycin. The quantitative cell toxicity was determined using WST-1, a water soluble formazan dye. Briefly, after incubation with various

concentrations of MPP^+ for several hours in the absence or presence of test drugs, cells were washed with Krebs Ringer HEPES-buffered (KRH) solution. Thereafter, cells were incubated with 100 μl of a solution containing 0.5 mM WST-1 and 20 μM 1-methoxy PMS for 3 hours at 37℃. Optical density was measured using a microplate reader at a wavelength of 450 nm (reference wavelength: 600 nm).

Detection of DNA fragmentation

After their incubation with the drugs, cells were washed with phosphate-buffered saline (PBS) and DNA was extracted using a DNeasy tissue kit (QIAGEN). Thereafter, the DNA was subjected to 1.5% agarose gel electrophoresis in TAE buffer. The gel was subsequently stained with 0.5 mg/ml of ethidium bromide and visualized under ultraviolet light and photographed.

MPP^+ accumulation assay

Cells were incubated in low glucose and serum DMEM containing 30 μM of [^{3}H]-MPP^+ in the absence or presence of NSAIDs for 9 hours. After the incubation, the medium was removed and cells were rapidly washed with an

ice-cold KRH solution. Then, they were solubilized with 2N NaOH for the measurement of radioactivity with a liquid scintillation counter.

MPP$^+$ efflux assay

Cells were preloaded with 30 μM MPP^+ in low glucose and serum DMEM at 37℃ for 6 hours, washed with KRH solution, then incubated in fresh low glucose and serum DMEM containing test reagents and 100 μM of cocaine to prevent the outflow or reuptake of [^{3}H]-MPP^+ via the monoamine transporters. After 3 hours, the medium was harvested, and cells were solubilized with 2N NaOH. The radioactivity in the incubation medium along with that remaining in the cells was measured separately using the liquid scintillation counter. Released [^{3}H]-MPP^+ was expressed as a percent of total cell count (sum of counts in the incubated medium and in the cells at the end of incubation).

RT-PCR analysis

Total RNA was isolated from PC12 cells or rat brain using TRIZOL® Reagent (Gibco BRL, Rockville, MD) according to the manufacturer's directions and used to synthesize cDNA with ReverTra Ace (TOYOBO) and a random hexamer

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primer. To amplify the several types of rat MRPs mRNA or rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, PCRs were performed with rat MRPs-specific primers (MRP1, forward; 5'-GCACTGGCTTCTAACTATTGG, reverse; 5'-TCTCATTGAAGTGTGAGTACAC, MRP2, forward; 5'-GGGATAAATCTCAGTGGT, reverse; 5'-ATATGCTCCACAGAGTTG, MRP3, forward; 5'-TAAGGTGGATAGCAACCAG, reverse; 5'-CCTCTGGCCAACACTGAGAT, MRP4, forward; 5'-GGACACTGAACTAGCAG, reverse; 5'-GTCGCTGTCAATGATGG, MRP5, forward; 5'-GGAACGGCAGTTGTTAT, reverse; 5'-CTTGACAGCCACCTTG) or rat GAPDH primers (forward; 5'-GAGCGAGATCCCGTCAAGATCAAA, reverse; 5'-CACAGTCTTCTGAGTGGCAGTGAT) and AmpliTag Gold™ (Perkin Elmer) at 95℃ for 10 min followed by 35 cycles of 92℃ for 30 s, 40℃ (MRP1), 47℃ (MRP2, 4 or 5), 51℃ (MRP3) or 50℃ (GAPDH) for 30 s, and 72℃ for 2 min with a final extension at 72℃ for 5 min. The resulting PCR products were analyzed on a 1.5% agarose gel and had the size expected from the known cDNA sequence.

Statistical analysis

Data are expressed as the mean ± SE of at least three independent experiments. Differences between means were determined using a one-way analysis of variance (ANOVA) with a pairwise comparison by the Bonferroni method. Differences were considered to be significant when the P value was less than 0.05.

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Results

Effects of NSAIDs on MPP^+-induced cell death. In the present study, MPP^+ caused the death of PC12 cells in a time and dose-dependent manner (Fig. 1 or 2a). Previously, we have confirmed that the norepinephrine transporter was expressed in PC12 cells (Ikeda et al., 2001). The cell toxicity of MPP^+ was almost completely suppressed on co-incubation with 1 mM cocaine or 10 μM nisoxetine (data not shown), suggesting that the cell death was induced via the uptake of MPP^+ from the norepinephrine transporter. Incubation of PC12 cells with 30 μM MPP^+ significantly induced cell death for 72 hours (Fig. 2a). Treatment with 100 μM MPP^+ for 24 hours induced cell death. Cell viability after incubation for 24, 48, and 72 hours with 100 μM MPP^+ was 68.19±1.09, 34.21 ±1.33, and 27.21±2.44 (% of control), respectively. The effects of NSAIDs on the MPP^+-induced death of PC12 cells was examined using indomethacin, ibuprofen, ketoprofen, aspirin, diclofenac and NS-398. At 100 μM, indomethacin or ibuprofen significantly potentiated the MPP^+-induced cell death when PC12 cells were co-incubated with MPP^+ for 48 or 72 hours but not 24 hours (Fig. 2a or 2b). Figure 3 shows the effects of NSAIDs on the dose-dependent toxicity of MPP^+. Incubation with 10 or 100 μM indomethacin significantly potentiated

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MPP^+-induced cell death (Fig. 3a). At 100 μM, ibuprofen or ketoprofen and diclofenac also significantly potentiated MPP^+-induced cell death (Fig. 3b-d). In contrast to these four NSAIDs, aspirin and NS-398 did not enhance the cytotoxicity of MPP^+ (Fig. 3e or 3f). None of the NSAIDs tested alone had any effect on cell viability at the doses used in this study.

Potentiation by NSAIDs of MPP^+-induced cell death does not involve the generation of ROS, caspase activity or PPARγ. Several studies have demonstrated that the toxicity of MPP^+ is related to an increase in the generation of ROS and that there is a crucial association between the production of ROS and NSAIDs-induced apoptosis in many types of cells. Therefore, to investigate the involvement of ROS in the stimulatory effects of indomethacin in PC12 cells, we examined the influence of PBN, a radical scavenger, and NAC, an antioxidant, on the MPP^+-induced cell death potentiated by indomethacin. At 1 mM, neither PBN nor NAC affected the potentiating action of indomethacin on the MPP^+-induced cell death (table 1). In addition, these drugs had no effect on the potentiation by ibuprofen or ketoprofen (data not shown). Thus, these results suggested that ROS is not involved in the potentiation induced by some NSAIDs.

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A number of observations have indicated that the activation of caspases is involved in the toxicity of MPP^+. Moreover, caspases have been demonstrated to play pivotal roles in NSAIDs-induced apoptosis in several culture systems. Therefore, to confirm the involvement of caspases in the stimulatory actions of NSAIDs, we examined the effect of Ac-DEVD-CHO, a selective caspase-3 inhibitor, on the MPP^+-induced cell death potentiated by three NSAIDs. Ac-DEVD-CHO could suppress neither the cell toxicity induced by MPP^+ alone nor the MPP^+-induced cell toxicity enhanced by indomethacin (table 1), ibuprofen and ketoprofen (data not shown), suggesting that caspase-3 at least is not involved in these events. Furthermore, the effects of indomethacin or ibuprofen on DNA fragmentation in PC12 cells are shown in Fig. 4. Treatment with 30 μM of MPP^+, either alone or together with 100 μM of these NSAIDs, for 48 hours did not induce DNA fragmentation. From these results in PC12 cells, it does not seem that a caspase-3-apoptotic cell death pathway is involved in the cytotoxic effect of MPP^+ potentiated by these NSAIDs. Recent studies have demonstrated that some NSAIDs induce gene transcription through the activation of PPARγ. We next investigated whether the activation of PPARγ is involved in the MPP^+-induced cell death potentiated by indomethacin, ibuprofen

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or ketoprofen. Co-incubation of PC12 cells with 1 or 5 μM of GW9662, a selective PPARγ inhibitor, did not suppress the stimulatory effect induced by indomethacin (table 1). GW9662 had no influence on the actions of the other two NSAIDs either (data not shown). These results suggested that the effects of NSAIDs on MPP^+-induced cell death is independent of the activation of PPARγ in PC12 cells.

NSAIDs have no influence on rotenone-induced cell death. Neurotoxins are required to be taken up into neuronal cells to initiate their neurotoxic effects. MPP^+ is thought to be taken up through several transporter systems, especially catecholamine transporters on neurons. Rotenone, another neurotoxin used to develop models of Parkinson's disease, diffuses freely into neuronal cells, and potently inhibits complex Ⅰ. Thus, we investigated the effects of some NSAIDs on rotenone-induced cell death in PC12 cells. As shown in Fig. 5, rotenone exhibited a concentration-dependent cytotoxicity in PC12 cells. However, neither indomethacin, ibuprofen nor ketoprofen affected the rotenone-induced cell death (Fig. 5).

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NSAIDs evoke an increase in the intracellular accumulation of [^{3}H]-MPP$^+$ through a decrease in the efflux of [^{3}H]-MPP$^+$ from cells. The specific enhancement of MPP$^+$ toxicity may indicate that the stimulatory effect induced by these NSAIDs is dependent on the function of transport systems in the plasma membrane. Thus, to elucidate whether the increase in the accumulation of MPP$^+$ is involved in the potentiating actions of some NSAIDs, the effects of three NSAIDs on the amount of MPP$^+$ accumulated in PC12 cells were examined. Co-incubation of PC12 cells with 30 μM of MPP$^+$, 10 or 100 μM of indomethacin, and 100 μM of ibuprofen, ketoprofen or diclofenac, but not 1 mM of aspirin, for 9 hours led to a significant increase in the accumulation of [^{3}H]-MPP$^+$ compared with that in the cells treated with 30 μM MPP$^+$ alone (Fig. 6a). Furthermore, the effects of these NSAIDs on the efflux of MPP$^+$ from PC12 cells were examined. Cells were preloaded with [^{3}H]-MPP$^+$ for 6 hours, and then the efflux of [^{3}H]-MPP$^+$ in to the medium was measured. When 100 μM of indomethacin, ibuprofen, ketoprofen or diclofenac, but not 1 mM of aspirin, was present in the extracellular medium, the release of [^{3}H]-MPP$^+$ from cells significantly decreased compared with that from vehicle-treated cells (Fig. 6b).

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Involvement of MRP in the NSAIDs-potentiated cell toxicity. It has been demonstrated that indomethacin specifically inhibits the MRP activity in human cell lines overexpressing MRP (Draper et al., 1997). Furthermore, Reid et al. (2003a) have suggested that some NSAIDs, including indomethacin, ibuprofen and ketoprofen, inhibit the activity of MRP4. Thus, to verify the involvement of MRPs in the NSAIDs-potentiated action, we first examined the expression of some types of MRPs mRNA in PC12 cells using RT-PCR analysis. Each cDNA fragment of the expected size (MRP1; 511 bp, MRP3; 766 bp, MRP4; 229 bp, MRP5; 310 bp) except for MRP2 (868 bp) was amplified after reverse transcription (Fig. 7a). Each amplicon for MRP1, 3, 4 or 5 was also found in rat brain or for MRP2 was found in rat liver (Fig. 7a). Co-incubation of PC12 cells with 30 μM of MPP^+ and 20 μM of MK 571, which is a MRP inhibitor, for 9 hours led to a significant increase in [^{3}H]-MPP^+ compared with incubation with 30 μM MPP^+ alone (Fig. 7b). The increase is similar to that observed when cells were co-incubated with NSAIDs in the present study. Furthermore, 20 or 50 μM of MK 571 mimicked the inhibitory effect on the efflux of [^{3}H]-MPP^+ by NSAIDs (Fig. 7c). In addition, the effect of MK 571 on the toxicity of MPP^+ in PC12 cells was investigated. Treatment with 20 μM MK 571 significantly potentiated the

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MPP^+-induced cell death (Fig. 7d).

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Discussion

Recently, evidence has emerged that some NSAIDs have effects not correlated with inhibitory action towards COX isozymes. For example, NSAIDs induced apoptosis in a variety of cells (Lu et al., 1995; Han et al., 2001; Yamazaki et al., 2002). In the present study, the treatment of PC12 cells with NSAIDs alone, such as indomethacin, ibuprofen, ketoprofen or diclofenac, did not affect the cell viability. However, co-incubation with these NSAIDs markedly enhanced the MPP^+-induced cell death. The other NSAIDs used in this study, including aspirin and NS-398, had no effect on the toxicity. Thus, the potentiating effect of these four NSAIDs on the MPP^+-induced cell death did not seem to be associated with their ability to inhibit COX enzymes, because all NSAIDs used in this study did not have the potentiating action, and the concentration of NSAIDs required to increase the toxicity of MPP^+ was several orders of magnitude higher than that required to suppress COXs activities. For example, it has been demonstrated that IC_{50} values of indomethacin for inhibition of human platelet COX-1 and endotoxin-stimulated human monocyte COX-2 were 0.36±0.10 μM and 0.70±

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0.20 μM, respectively (Patrignani et al., 1994). Furthermore, several studies have reported the doses of aspirin required to inhibit NF-κB or IκB kinase-β are at mM range, much higher than doses used in the present study (Kopp and Ghosh, 1994; Yin et al., 1998).

To better understand the potentiating effects of these NSAIDs on the MPP^+-induced cell death, we examined the involvement of ROS in the NSAIDs-potentiated actions. It would appear that the actions of NSAIDs are not associated with an increase in ROS, because neither PBN, a radical scavenger, nor NAC, an antioxidant, had any influence on the NSAIDs-potentiated actions. Moreover, we confirmed that these drugs did not affect the MPP^+-induced cell death in PC12 cells. It has been demonstrated that ROS or a ROS–mediated signal plays an essential role in 6-hydroxydopamine-induced but not MPP^+-induced cell death in a dopaminergic neuronal cell line (Choi et al., 1999, Kim et al., 2001). Seyfried et al. (2000) have suggested that the generation of ROS might not be the primary mechanism of MPP^+ toxicity in PC12 cells. In contrast, it has been demonstrated that ROS contributed to the apoptotic cell death induced by NSAIDs in cultured gastric cells (Kusuhara et al., 1999). Thus, one possible explanation for the discrepancy is that the mechanism of ROS

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generation, the susceptibility to ROS or the cell-death signal triggered by ROS are cell-specific. Another question arises as to whether the activation of caspases, which is one of the key factors in apoptosis, is associated with the NSAIDs-potentiated effect in PC12 cells. It has been demonstrated that several NSAIDs cause apoptosis through a caspase-dependent cascade (Kusuhara et al., 1998; Klampfer et al., 1999; Pique et al., 2000). In the present study, Ac-DEVD-CHO, a specific and potent caspase-3 inhibitor, suppressed neither the MPP^+-induced cell death nor the NSAIDs-potentiated effects. Consistent with our result, Han et al. (2003) have suggested that caspase-independent cell death pathways operate in primary dopaminergic neurons after MPP^+ treatment, while caspase-dependent pathways are involved in the cell death induced by 6-hydroxydopamine. Moreover, we observed that DNA fragmentation, which is one of the hallmarks of apoptosis, was not induced by incubation of PC12 cells with MPP^+ alone or together with NSAIDs. Taken together, NSAIDs are unlikely to potentiate the cell loss induced by MPP^+ through a general apoptotic cascade in the present system.

Several NSAIDs, including indomethacin and ibuprofen, act as a direct ligand for PPARγ (Lehmann et al., 1997; Jiang et al., 1998). Recently, evidence has

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emerged that the direct activation of PPARγ is involved in the apoptosis induced by NSAIDs in several types of cells (Yamazaki et al., 2002; Kusunoki et al., 2002). In the present study, however, the potentiating effect of NSAIDs on MPP^+-induced cell death in PC12 cells was not associated with the activation of PPARγ, because a selective PPARγ antagonist GW9662 did not affect the NSAIDs-potentiated action, and $15dPGJ_2$ or ciglitizone, which are the agonists for PPARγ, did not mimic the effects of NSAIDs in PC12 cells (unpublished observation).

Rotenone is another neurotoxin used to develop models of Parkinson's disease. The mechanisms of action of MPP^+ and rotenone overlap in many regards. Both reagents inhibit mitochondrial complex Ⅰ activity, resulting in a reduction in intracellular ATP levels and subsequent cell death. However, the mechanism of cellular entry used by these compounds is different; MPP^+ is a cationic molecule and enters cells on transporters such as the dopamine and norepinephrine transporter. In contrast, rotenone is a lipophilic molecule and enters cells freely. We found that some NSAIDs did not potentiate the rotenone-induced cell death in PC12 cells. Thus, the mechanisms for MPP^+-induced cell death with or without NSAIDs is yet remained to be elucidated, the present results indicate the

possibility that the regulation of the membrane-transport systems of MPP^+ might be associated with the potentiating actions of NSAIDs. Actually, we demonstrated that incubation of PC12 cells with some NSAIDs significantly increased the intracellular accumulation of [^{3}H]-MPP^+. In contrast, aspirin, which has no influence on the MPP^+ toxicity, did not affect the intracellular accumulation of [^{3}H]-MPP^+. Moreover, we confirmed that the efflux of [^{3}H]-MPP^+ into the incubation medium from cells preloaded with [^{3}H]-MPP^+ was significantly decreased compared with that from vehicle-treated cells, when the cells were incubated with each NSAIDs. These results suggest that NSAIDs raise intracellular MPP^+ concentrations via a blockade of the outflow of MPP^+ from cells.

It has been demonstrated that indomethacin specifically inhibits the MRPs activity in human cell lines overexpressing MRP (Draper et al., 1997). The MRPs belong to a superfamily of ATP-binding cassette transporters, and have been implicated in the resistance of tumor cells to chemotherapeutic drugs (Cole et al., 1992; Borst et al., 2000). In the present study, MRP1, 3, 4 and 5 mRNA were detected in PC12 cells. In addition, MK571, a MRP inhibitor (Reid et al., 2003b; Rius et al., 2003), mimicked the potentiation of cell toxicity, the enhancement of

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[^{3}H]-MPP$^+$ accumulation and the reduction of [^{3}H]-MPP$^+$ efflux in response to NSAIDs. Therefore, these results suggest that [^{3}H]-MPP$^+$ efflux occurs through MRPs expressed in PC12 cells and NSAIDs reduced MPP$^+$ efflux by the blockade of MRPs. We could not identify the distinct subtype(s) which contribute to the NSAIDs-induced effects because of lack of MRP subtype-selective inhibitor. Although we could not rule out the possibility that one or some MRPs might contribute to the NSAIDs-induced effects, we have speculated MRP4 is one of the most important factor in the action of NSAIDs as several following reasons. It has been recently demonstrated that MK-571 might selectively inhibit both MRP1 and MRP4 transport (Chen et al., 2001; Dallas et al., 2003). Indomethacin affects the some MRPs including MRP4 (Draper et al., 1997). On the other hand, ibuprofen, ketoprofen or diclofenac have inhibitory effect on MRP4 rather than other MRPs (Reid et al., 2003a). Moreover, the concentrations of NSAIDs required to enhance the MPP$^+$ toxicity is nearly equal to the IC_{50} for these NSAIDs against MRP4 transport (5-50 μM; Reid et al., 2003a). Taken together, it could be expected that NSAIDs first block the activity of MRPs including MRP4 and inhibit the efflux of MPP$^+$ from PC12 cells, resulting in the retention of a high concentration of MPP$^+$ in cells and subsequent potentiation of

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cell toxicity. The cytoplasmic concentration of MPP^+ in monoaminergic neurons is dependent on the influx of MPP^+ through plasma membrane monoamine transporters and the sequestration of MPP^+ into synaptic vesicles through vesicular monoamine transporter. In addition to these well known transporter systems for MPP^+, other transporter systems are expected. The present results suggest that MRPs also regulated the concentration of MPP^+ in a causal relationship with its toxicity in PC12 cells.

In conclusion, our results demonstrated that several NSAIDs potentiate MPP^+-induced cell death through a blockade of MRPs rather than the inhibition of COXs activities in PC12 cells. Our report provides new insights as follows; 1) some types of MRPs are expressed in PC12 cells and these are one of the transport systems involved in discharging intracellular MPP^+ outside of the cell. 2) Some NSAIDs including indomethacin, ibuprofen, ketoprofen and diclofenac inhibited the activities of MRPs including MRP4 in PC12 cells. 3) The blockade of MRPs by these NSAIDs led to an increase in the intracellular concentration of MPP^+ and aggravation of cell toxicity. It has been demonstrated that NSAIDs had inconsistent effects on MPP^+-induced cell death; a protective, an aggravative or no effect depending on the drug and cells used or experimental

conditions. The present results suggest that differences in the transport systems in cell types and in the action of NSAIDs on these transport systems are critical causes of the discrepancies. NSAIDs are split into various subclasses, including salicylic acid derivatives, indoleacetic acid derivatives, propionic acid derivatives and so on. Therefore, it might also be important to evaluate the effects of each subclass or each compound itself on MRPs.

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Legends

Fig. 1 Dose-dependent effects of MPP^+ on cell viability. The viability of PC12 cells treated with various concentrations of MPP^+ for 48 hours is shown. Data represent the mean±SEM (bars) for three to seven independent experiments. *P<0.05, **P<0.01 compared with the value for untreated cells.

Fig. 2 Time-dependent effects of indomethacin or ibuprofen on MPP^+-induced cell death. PC12 cells were incubated with 30 μM MPP^+ for the periods indicated in the absence or presence of 100 μM of indomethacin or ibuprofen. Data represent the mean±SEM (bars) for three to seven independent experiments. **P<0.01 compared with the value for the cells treated with MPP^+ alone for the corresponding incubation period.

Fig. 3 Effects of NSAIDs on MPP^+-induced cell death. PC12 cells were incubated with various concentrations of MPP^+ for 48 hours in the absence or presence of indomethacin (a; 10 or 100 μM), ibuprofen (b; 10 or 100 μM), ketoprofen (c; 10 or 100 μM), diclofenac (d; 10 or 100 μM), aspirin (e; 300 μM or 1 mM) or NS-398 (f; 10 or 30 μM). Data represent the mean ± SEM (bars) for three to nine

independent experiments. *P<0.05, **P<0.01 compared with the value for the cells treated with the corresponding dose of MPP^+ alone.

Fig. 4 Effect of NSAIDs on internucleosomal DNA cleavage in PC12 cells. PC12 cells were incubated without (lane 1) or with 30 μM MPP^+ alone (lane 2) or MPP^+ plus 100 μM of indomethacin (lane 3) or ibuprofen (lane 4) for 48 hours. Lane M indicates DNA size markers.

Fig. 5 Effects of NSAIDs on rotenone-induced cell death. PC12 cells were incubated with various concentrations of rotenone for 48 hours in the absence (●) or presence of indomethacin (100 μM; ▲), ibuprofen (100 μM; ▼) or ketoprofen (100 μM; ■). Data represent the mean±SEM (bars) for three to five independent experiments.

Fig. 6 Effects of NSAIDs on the intracellular accumulation or efflux of [^{3}H]-MPP^+ in PC12 cells. a) Effects of NSAIDs on the intracellular accumulation of [^{3}H]-MPP^+ in PC12 cells. PC12 cells were incubated with 30 μM [^{3}H]-MPP^+ for 9 hours in the absence (control) or presence of 10 or 100 μM of indomethacin

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(indo) and 100 μM of ibuprofen (ibup), ketoprofen (keto), or diclofenac (diclo) or 1 mM of aspirin (asp). Data represent the mean±SEM (bars) for three to five independent experiments. *P<0.05, **P<0.01 compared with the value for cells treated with 30 μM of MPP^+ alone. b) Effects of NSAIDs on the efflux of [^{3}H]-MPP^+ into the culture medium from PC12 cells. Cells were preloaded with 30 μM [^{3}H]-MPP^+ for 6 hours. They were then incubated for 3 hours in fresh medium alone (control) or containing 100 μM of indomethacin (indo), ibuprofen (ibup), ketoprofen (keto), or diclofenac (diclo) or 1 mM of aspirin (asp). Data represent the mean±SEM (bars) for three to five independent experiments. *P<0.05, **P<0.01 compared with the value in the medium treated with vehicle alone.

Fig. 7 MK 571, a MRP inhibitor, mimics the NSAIDs-induced effects in PC12 cells. a) RT-PCR analysis of MRPs mRNA expression in PC12 cells. Each lane represents the cDNA fragments of MRP1~5 (MRP1; 511 bp, MRP2; 868 bp, MRP3; 766 bp, MRP4; 229 bp, MRP5; 310 bp) amplified from the RNA of PC12 cells (lane 1, 4, 7, 10 or 13), rat brain (lane 3, 9, 12 or 15) or rat liver (lane 6). Lane 2, 5, 8, 11 or 14 indicate the product of PCR with the RT reaction omitted

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as a negative control. The integrity of the isolated RNAs was examined using GAPDH-specific primers (lane 1; PC12 cells, lane 2; negative control mentioned above, lane 3; rat brain, lane 4; rat liver). The cDNA fragment of GAPDH was 330 bp. Lane M indicates size markers (100 bp DNA Ladder). b) Effects of MK 571 on the intracellular accumulation of [^{3}H]-MPP^+ in PC12 cells. PC12 cells were incubated with 30 μM [^{3}H]-MPP^+ for 9 hours in the absence or presence of 2 or 20 μM of MK 571. Data represent the mean ± SEM (bars) for five independent experiments. **P<0.01 compared with the value for the cells treated with 30 μM of MPP^+ alone. c) Effects of MK 571 on the efflux of [^{3}H]-MPP^+ from PC12 cells. After being preloaded as mentioned in Fig. 6b, cells were incubated for 3 hours in fresh medium alone (control) or containing 2, 20 or 50 μM of MK 571. Data represent the mean ± SEM (bars) for three to five independent experiments. *P<0.05, **P<0.01 compared with the value in the medium treated with vehicle alone. d) Effects of MK 571 on MPP^+-induced cell death. PC12 cells were incubated with various concentrations of MPP^+ for 48 hours in the absence or presence of MK 571 (2 or 20 μM). Data represent the mean±SEM (bars) for five independent experiments. *P<0.05, **P<0.01 compared with the value for the cells treated with the corresponding dose of MPP^+ alone.

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Table 1

Effects of several kinds of inhibitors on MPP^+-induced cell death in PC12 cells

All values indicate the cell viability when PC12 cells were incubated with 30 μM of MPP^+ alone or together with several kinds of inhibitors for 48 hours. The data represent the mean±SEM for four independent experiments.

	Cell viability (% of control)
MPP^+ 30 μM	82.73±3.61
+ indomethacin 100 μM	43.19±4.93
+ PBN 1 mM	74.48±5.11
+ NAC 1 mM	85.48±7.45
+ Ac-DEVD-CHO 100 μM	83.57±1.95
+ indomethacin 100 μM + PBN 1 mM	33.4±1.83
+ indomethacin 100 μM + NAC 1 mM	39.27±0.17
+ indomethacin 100 μM + Ac-DEVD-CHO 100 μM	49.95±0.71
+ indomethacin 100 μM + GW9662 1 μM	42.59±2.97
+ indomethacin 100 μM + GW9662 5 μM	47.12±5.52

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Figure 1

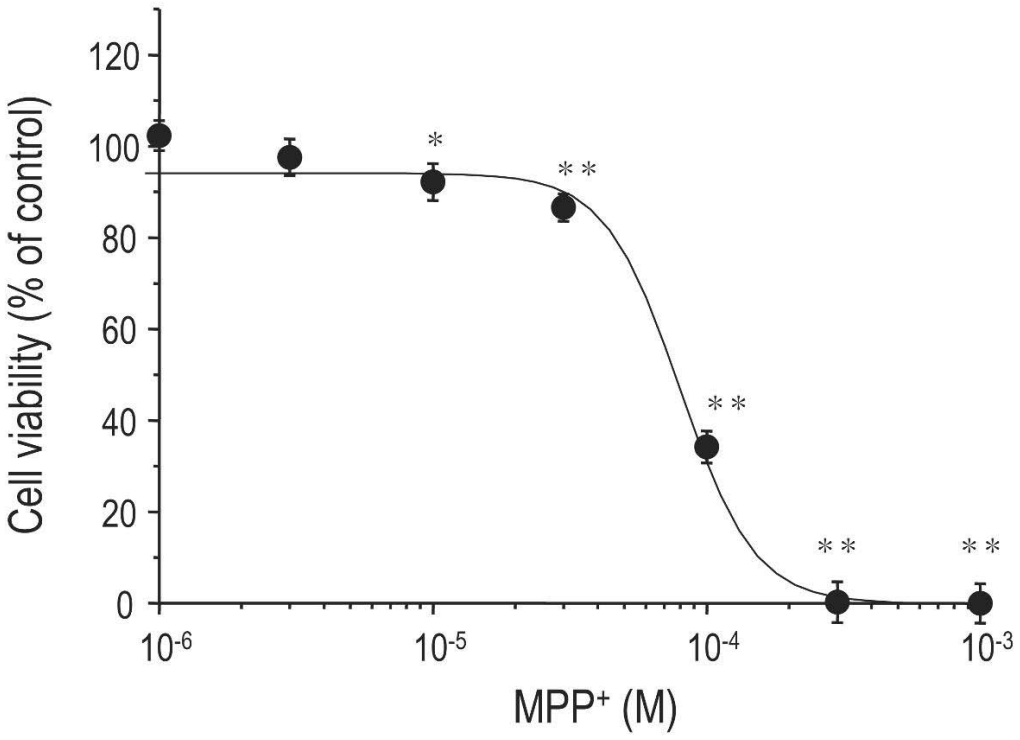

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Figure 2

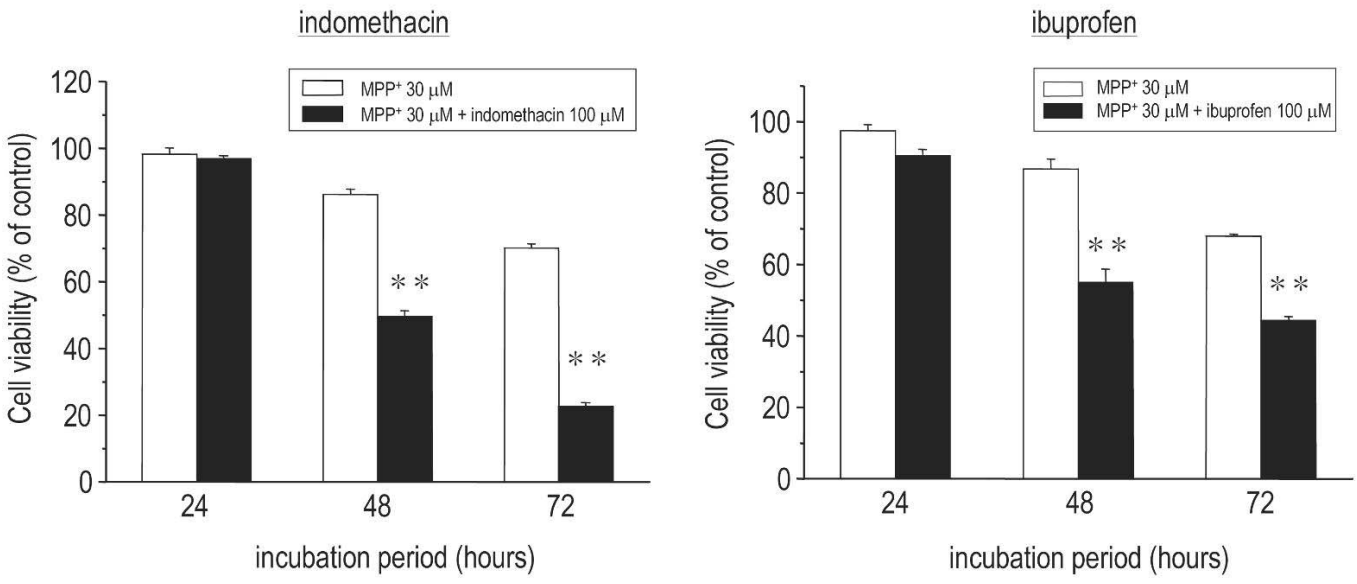

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Figure 3

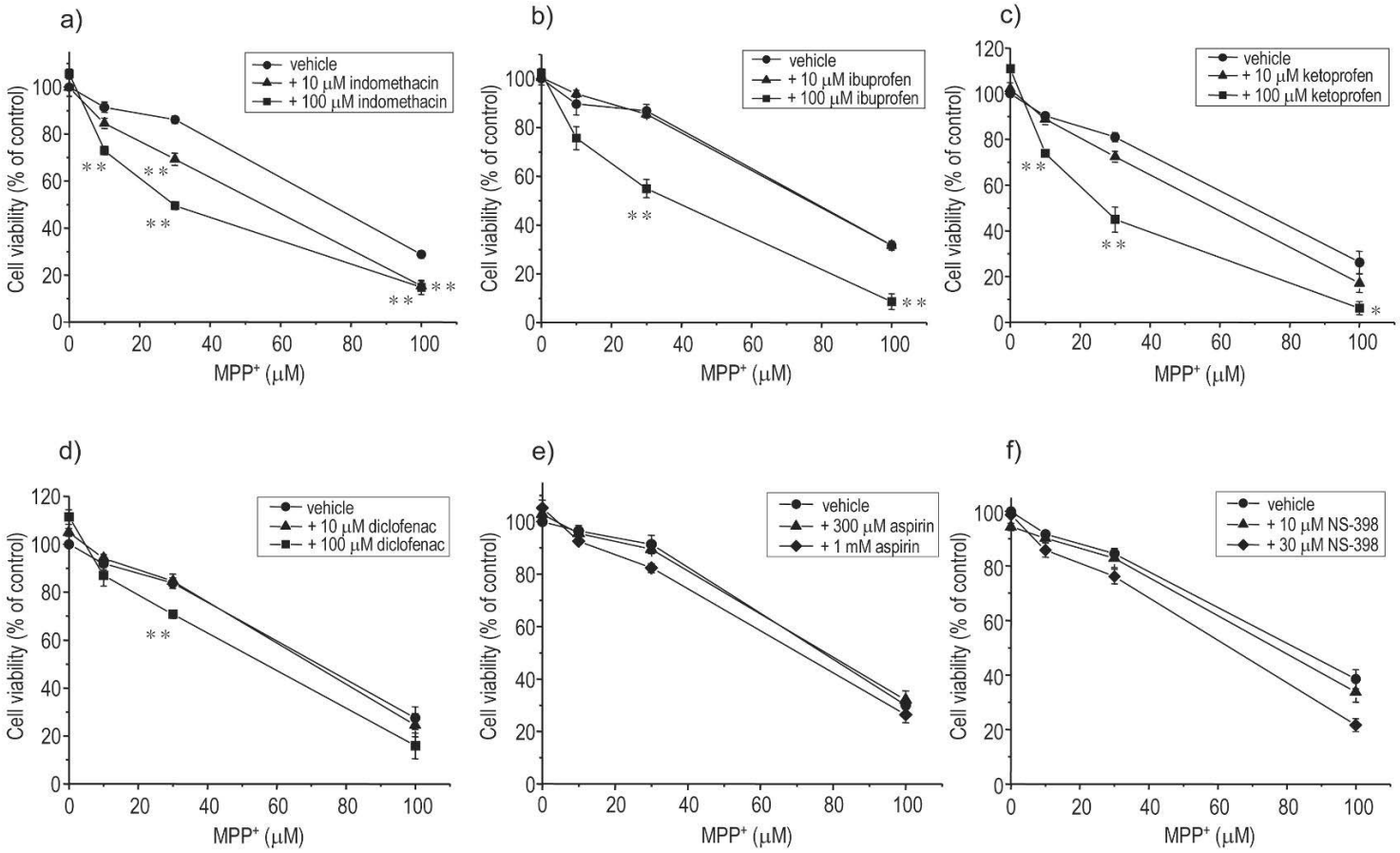

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Figure 4

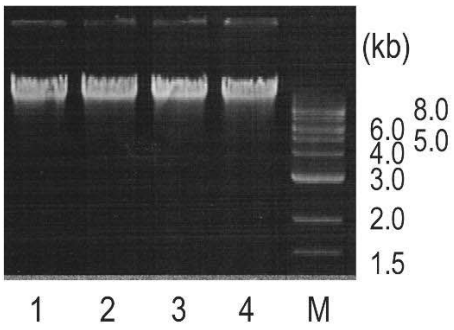

Figure 5

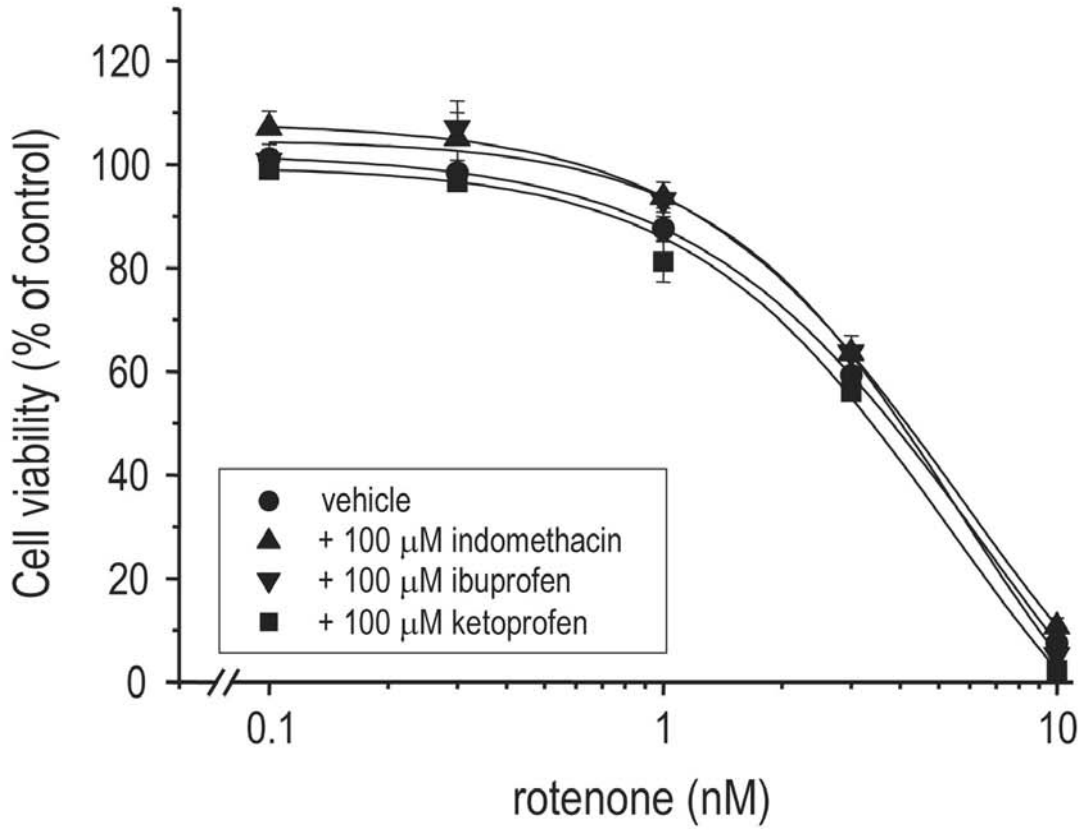

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Figure 6

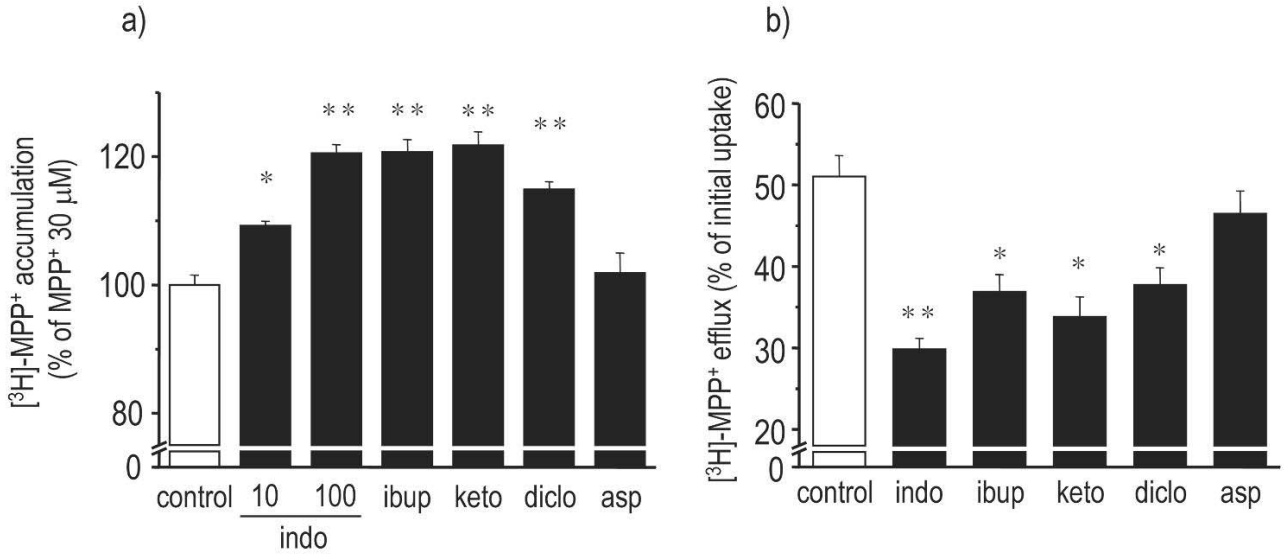

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Figure 7