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The Sarin-like Organophosphorus Agent bis (isopropyl methyl)phosphonate Induces Apoptotic Cell Death and COX-2 Expression in SK-N-SH Cells

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ABSTRACT

Organophosphorus compounds, such as sarin, are highly toxic nerve agents that inhibit acetylcholinesterase (AChE), but not cholinesterase, via multiple mechanisms. Recent studies have shown that organophosphorus compounds increase cyclooxygenase-2 (COX-2) expression and induce neurotoxicity. In this study, we examined the toxicity of the sarin-like organophosphorus agent bis(isopropyl methyl)phosphonate (BIMP) and the effects of BIMP on COX-2 expression in SK-N-SH human neuroblastoma cells. Exposure to BIMP changed cell morphology and induced caspase-dependent apoptotic cell death accompanied by cleavage of caspase 3, caspase 9, and poly (ADP-ribose) polymerase (PARP). It also increased COX-2 expression, while pretreatment with a COX inhibitor, ibuprofen, decreased BIMP-dependent cell death and COX-2 expression in SK-N-SH cells. Thus, our findings suggest that BIMP induces apoptotic cell death and upregulates COX-2 expression.

Key words: *Sarin, Organophosphates, Apoptosis, COX-2*

Organophosphorus compounds have been used as pesticides and nerve agents worldwide; their toxicity is due to irreversible inhibition of acetylcholinesterase (AChE)[6,7,11,15,26,43)]. The resulting accumulation of acetylcholine in cholinergic synapses results in overstimulation of cholinergic neurons, an event known as an acute cholinergic crisis. Moreover, a recent study showed that organophosphorus compounds cause multiple toxicities[27)], for example, DNA damage[3,39)], mitochondria dysfunction[21)], and an inflammatory response[4)]. These toxicities cannot be explained only by toxicity with canonical acetylcholinesterase targeting toxicity. Elucidation of various toxic mechanisms of organophosphorus compounds is very important for optimal treatment.

Organophosphorus compounds can also elicit toxicity independently of their acetyl cholinesterase inhibitory functions; this toxicity is due to mitochondrial dysfunction[32,44)]. Mitochondria have an important role in the production of adenosine triphosphate and reactive oxygen species (ROS), and are also key players in some types of caspase-dependent cell death. Given their central role in several pathways, dysfunctional mitochondria elicit multisystem disorders. For example, oxidative damage alters mitochondrial membrane potential, thereby inducing cell death. Moreover, mitochondria-dependent cell death pathways, such as apoptosis and necrosis, can lead to changes in mitochondrial function and morpholo-

Abbreviations: AChE, acetylcholinesterase; COX, cyclooxygenase; BIMP, bis(isopropyl methyl)phosphonate; ROS, reactive oxygen species; PGE_2, prostaglandin E_2; NSAID, nonsteroidal anti-inflammatory drug; PARP, poly (ADP-ribose) polymerase; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; LDH, lactate dehydrogenase; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; HRP, horseradish peroxidase; SEM, standard error of the mean; BPMP, bis(pinacolyl methyl)phosphonate

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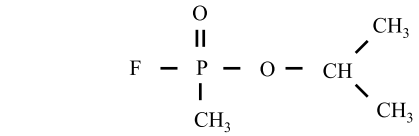
gy[22,33]. Organophosphorus compounds can induce ROS and mitochondrial dysfunction-dependent cell death[8,25], and we previously reported that a soman-like organophosphate agent alters the mitochondrial membrane[19]. However, the precise mechanisms through which organophosphate compounds induce mitochondria-dependent cell death are unclear.

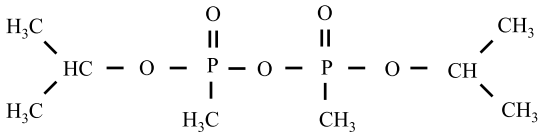
Inflammation is an important response of the immune system to injury or infection[16]; however, excessive inflammatory responses are associated with the pathology of many degenerative diseases[5,42]. Cyclooxygenase (COX) is an important inflammatory molecule that catalyzes the first step in the formation of prostaglandins from arachidonic acid. There are two isoforms of COX, COX-1 and COX-2; COX-1 is responsible for basal and constitutive prostaglandin synthesis, while COX-2 is an inducible enzyme whose expression is increased in response to various stimuli[35]. COX-2 expression and production of inflammatory factors, such as prostaglandin E_2 (PGE_2), can induce dopaminergic neuronal cell disorder[17]. Interestingly, organophosphorus compounds can modulate inflammatory responses[10,20,41], and soman, a compound that is similar to sarin, increases inducible COX-2 expression and inflammation in rats[2,41]. Thus, organophosphorus compounds may modulate inflammatory responses, such as COX-2 overexpression, thereby contributing to the onset of neurodegenerative disorders. Moreover, ibuprofen, a nonsteroidal anti-inflammatory drug (NSAID) that inhibits both COX-1 and COX-2[13,31], has been widely used to relieve pain and inflammation and may also have neuroprotective effects[5,18,36].

Sarin is a highly toxic organophosphorus agent, and its synthesis and usage are strictly regulated in Japan. Therefore, in a previous study, we synthesized a sarin-like organophosphate agent, bis(isopropyl methyl)phosphonate (BIMP), that has a phosphonate group (isopropyl methylphosphonate) that is similar to that of sarin. This imparts sarin-like activity on BIMP, as it acts as an AChE antagonist (Fig. 1). After intravenous injection, the LD_{50} value of BIMP in mice was 0.8 mg/kg, and AChE activity in murine blood and brain cells reduced in a dose-dependent manner[28]. Consistent with this mechanism of action, BIMP exhibits a toxicity profile similar to that of sarin[28]. Importantly, BIMP has almost no volatility at room temperature and is easily hydrolyzed in water, making BIMP suitable for experiments in ordinary laboratories[30,40]. Therefore, BIMP may be a suitable alternative compound for mechanistic studies of sarin toxicity.

In this study, we used BIMP to elucidate the mechanisms through which organophosphorus compounds mediate their cytotoxic effects. To this end, we examined the effect of BIMP on both COX-2 expression and cytotoxicity in the human neuroblastoma cell line, SK-N-SH.



Sarin



BIMP

Fig. 1. Chemical structures of sarin and BIMP. BIMP has similar phosphonate group with sarin.

MATERIALS AND METHODS

Reagents

BIMP was prepared as previously described[28,30]. Anti-caspase 3, anti-cleaved caspase 9, anti-nuclear poly (ADP-ribose) polymerase (PARP), and anti-COX-2 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and ibuprofen were purchased from Wako (Japan).

Cell culture and BIMP treatment

Human neuroblastoma SK-N-SH cells were obtained from the European Collection of Cell Cultures (ECACC, UK) and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin-neomycin (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2. Culture media were replaced every 3 days. For treatment, BIMP was dissolved in DMSO, and cells were exposed to various concentrations of BIMP for 0-24 hr in medium containing 0.1% FBS.

Measurement of cell viability

Cell viability was measured using the MTT assay (Cell Proliferation Kit I [MTT]; Roche Applied Science, Germany). The cells were seeded in 96-well plates (2×10^4 cells/well) and exposed to BIMP for 24 hr. MTT was then added to the cells, and the plates were incubated at 37°C for 4 hr in a humidified atmosphere with 5% CO_2. Finally, the cells were lysed in lysis buffer, and the quantity of formazan product was measured by determining the absorbance at 490/650 nm, which is directly proportional to the number of living cells in culture. Results were expressed as a percentage of the control cells.

Detection of lactate dehydrogenase (LDH) activity

Cytotoxicity was measured by determining the total LDH content (Cytotoxicity Detection Kit Plus

[LDH]; Roche Applied Science); LDH release was considered an indicator of cytotoxicity. Cells were seeded in 96-well plates (2×10^4 cells/well) and exposed to BIMP for 24 hr. The cellular extract was mixed with LDH reaction mixture (1:1), and the mixture was then incubated in the dark at room temperature for 20-30 min. The reaction was stopped with 0.2N HCl. The absorbance was measured at 490/630 nm in a microplate reader, and the results were expressed as a percentage of the control cells.

Western blot analysis

Cells were seeded in 6-well plates (4×10^5 cells/well) and exposed to BIMP. Cells were then lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% sodium deoxycholate, 1% NP-40, and 0.1% sodium dodecyl sulfate [SDS]) containing 1 mM Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, USA) on ice. Collected cells were broken by sonication on ice and centrifuged at 20,000 × *g* for 20 min at 4°C. The protein concentration was measured using a BCA protein assay kit (Thermo Scientific). Next, 20 μg of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were incubated in the presence of different primary antibodies at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-linked secondary antibodies for 1 hr. Immunoreactivity was visualized using VersaDoc (Bio-Rad, Hercules, CA, USA), and protein bands were analyzed by Quantity one (Bio-Rad).

Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA followed by the Tukey-Kramer method. Differences with p values less than 0.05 were considered significant. Error bars represent the mean ± standard error of the mean (SEM).

RESULTS

BIMP-dependent cytotoxicity reduces SK-N-SH cell viability

To evaluate whether BIMP could induce SK-N-SH cell death independently of its acetyl cholinesterase inhibitory activity, we measured the effects of BIMP on cell viability and cytotoxicity. Cells were exposed to BIMP (100-750 μM) and incubated for 24 hr. MTT assays revealed that BIMP significantly reduced cell viability in a concentration-dependent manner (Fig. 2A). Additionally, LDH assays showed that BIMP significantly induced cytotoxicity in a concentration-dependent manner (Fig. 2B). Ob-

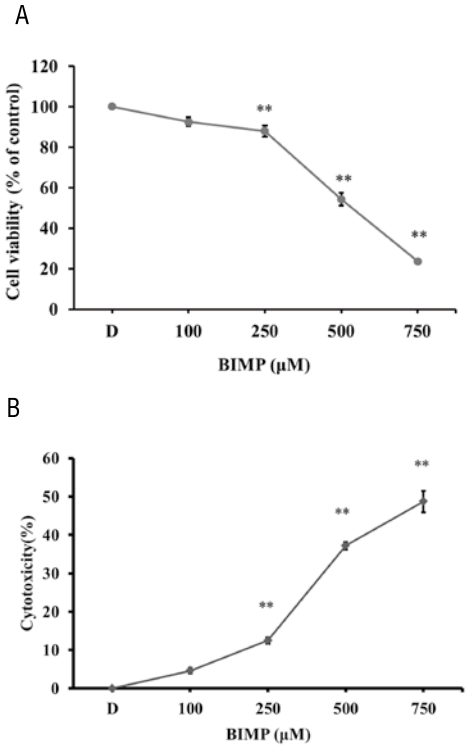


Fig. 2. BIMP reduced cell viability and induced cytotoxicity in SK-N-SH cells.
Cells were exposed to various concentrations of BIMP (0-750 μM) for 24 hr in medium containing 0.1% FBS. (A) Cell viability was measured using an MTT assay after 24 hr (mean ± SEM, n = 6). (B) Cytotoxicity was measured using an LDH release detection assay after 24 hr (mean ± SEM, n = 6). **p < 0.01 vs. control, one-way ANOVA followed by the Tukey-Kramer method.

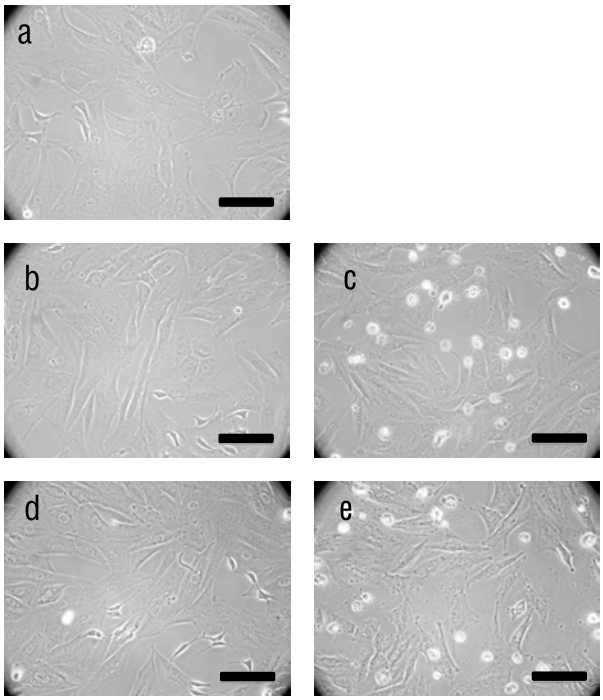


Fig. 3. Light microscopy of SK-N-SH cell morphology.
(a) Untreated SK-N-SH cells. (b, d) Cells were exposed to 0.1% DMSO for 12 (b) or 24 hr (d). (c, e) Cells were exposed to 500 μM BIMP for 12 (c) or 24 hr (e). All incubations were performed in medium containing 0.1% FBS. The bar represents 100 μm.

servation of cell morphology by light microscopy revealed that treatment with 500 μM BIMP for 12 or 24 hr caused morphological changes and increased the numbers of floating cells, consistent with the onset of cell death (Fig. 3c and 3e). Taken together, these data suggested that BIMP induced SK-N-SH cell death and morphological changes.

BIMP-dependent viability reduction is associated with markers of apoptosis in SK-N-SH cells

To determine the type of BIMP-induced cell death in SK-N-SH cells, we treated them with various concentrations of BIMP (100-750 μM) for 24 hr and then performed western blot analysis for detection of cleaved caspase 3, cleaved caspase 9, and cleaved PARP in whole-cell lysates (Fig. 4). Cleaved caspase 3 expression was significantly increased in a concentration-dependent manner. The same was also true for caspase 9, with a significant increase in cleavage following exposure to 750 μM BIMP. Levels of cleaved PARP were also significantly increased following exposure to 500 μM BIMP. Interestingly, cleaved PARP expression was lower in cells treated with 750 μM BIMP when compared with the 500 μM dose. This is likely due to the substantial amount of cell death observed at a high BIMP concentration, which leads to general cellular degradation. Owing to its dramatic and reproducible biological effects, we subsequently used 500 μM BIMP in all other experiments. Further analysis revealed that levels of cleaved caspase 3 increased in a time-dependent manner following treatment with BIMP over a 24-hr period (Fig. 4E). Taken together, these results suggested that BIMP induced caspase-dependent apoptotic cell death.

BIMP induces COX-2 expression in SK-N-SH cells

Next, we examined the effects of BIMP on COX protein expression in SK-N-SH cells exposed to 500 μM BIMP over a 24-hr period. Western blot analysis (Fig. 5) revealed that COX-2 expression

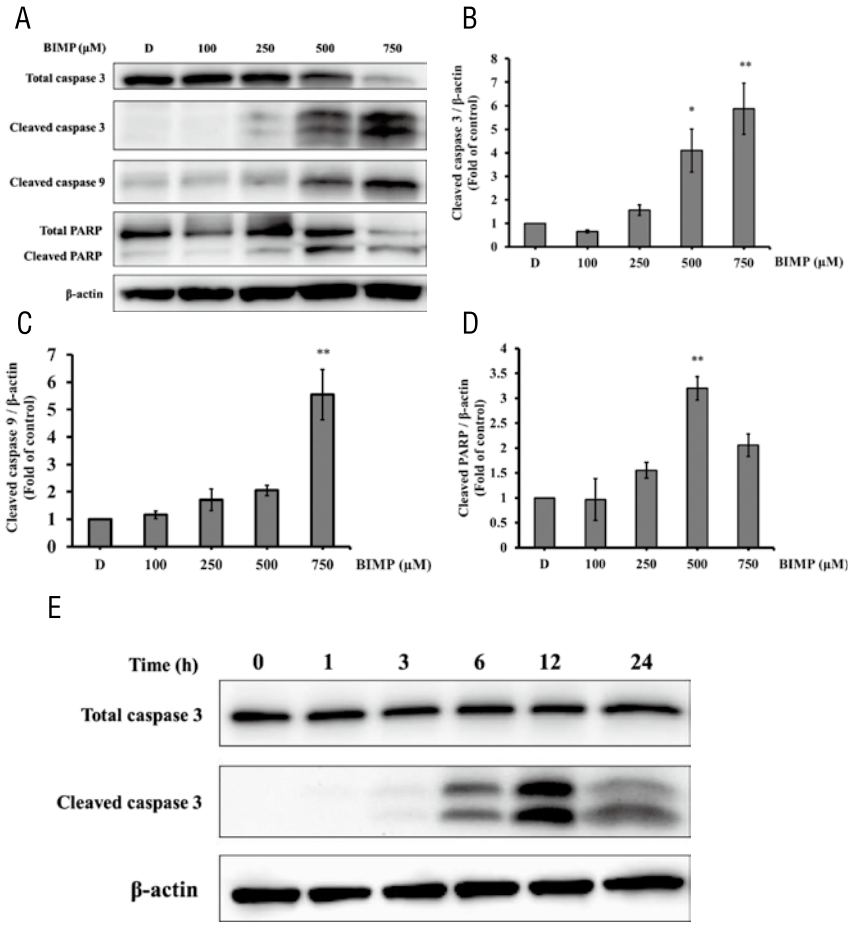


Fig. 4. Expression of apoptosis-related proteins following treatment with BIMP in SK-N-SH cells.
(A) Western blot analysis of cleaved caspase 3, cleaved caspase 9, cleaved PARP, and β-actin. Cells were exposed to various concentrations of BIMP (0-750 μM) for 24 hr in medium containing 0.1% FBS. (B) Densitometry analysis of western blots for cleaved caspase 3, (C) cleaved caspase 9, and (D) cleaved PARP. The results were normalized individually to the level of the loading control (β-actin). Data are shown as the mean ± SEM (n = 3). *$p < 0.05$, **$p < 0.01$ vs. control, one-way ANOVA followed by the Tukey-Kramer method. (E) Western blot analysis of cleaved caspase 3. Cells were exposed to 500 μM BIMP for 0, 1, 3, 6, 12, or 24 hr in medium containing 0.1% FBS.

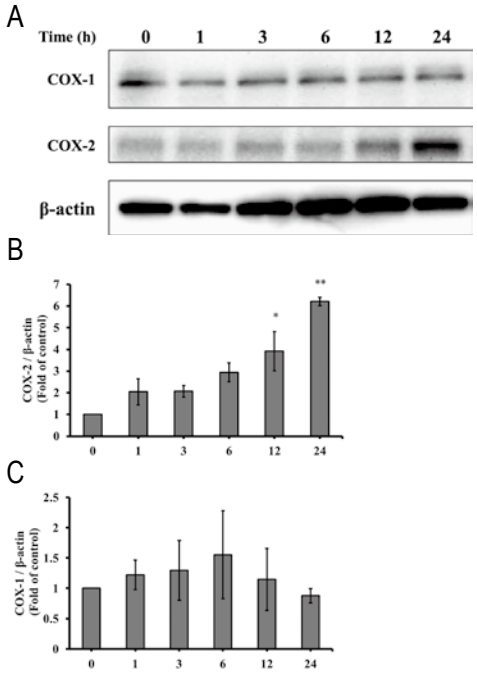


Fig. 5. Expression of COX-1 and COX-2 following treatment with BIMP in SK-N-SH cells.
(A) Western blot analysis of COX-1, COX-2, and β-actin. The cells were treated with 500 μM BIMP for 0, 1, 3, 6, 12, or 24 hr in medium containing 0.1% FBS. (B, C) Densitometric analysis of western blots for COX-1 (B) and COX-2 (C). The results were normalized individually to the loading control (β-actin). Data are shown as the mean ± SEM (n = 3). *p < 0.05, **p < 0.01 vs. the control, one-way ANOVA followed by the Tukey-Kramer method.

was significantly increased in a time-dependent manner (Fig. 5B). However, COX-1 expression was not significantly altered. These data showed that stimulation with BIMP induced the expression of COX-2, but did not affect the expression of COX-1.

Ibuprofen protects against BIMP-induced cell death

To investigate the effects of ibuprofen on BIMP-induced cell death, we exposed SK-N-SH cells to

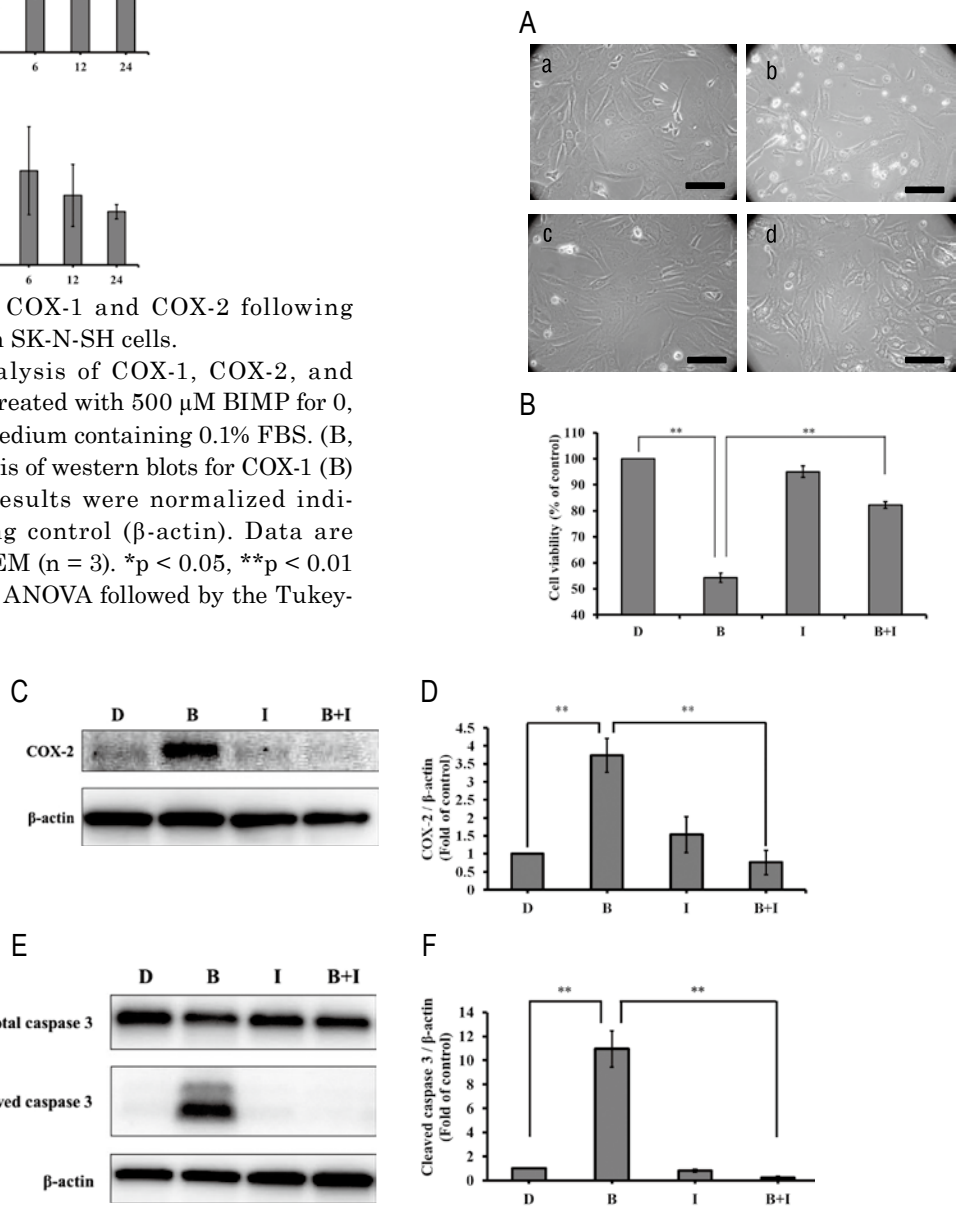


Fig. 6. Effects of ibuprofen on BIMP-treated SK-N-SH cells.
(A) Light microscopy of cell morphology. (a) Untreated SK-N-SH cells. (b) Cells were exposed to 500 μM. (c) Cells were treated with 1 mM ibuprofen. (d) Cells were pretreated with 1 mM ibuprofen for 30 min prior to treatment with 500 μM BIMP. All incubations were performed in medium containing 0.1% FBS for 24 hr. The bar represents 100 μm. (B) Cells were exposed to 500 μM BIMP with or without 1 mM ibuprofen, and after 24 hr, cell viability was measured using an MTT assay (mean ± SEM, n = 6). (C-F) Western blot analysis of COX-2 (C and D) and cleaved caspase 3 (E and F). Cells were pretreated with 1 mM ibuprofen for 30 min and then exposed to 500 μM BIMP for 24 hr in medium containing 0.1% FBS. The results were normalized individually to the loading control (β-actin). Data are the mean ± SEM (n = 3). *p < 0.05, **p < 0.01, one-way ANOVA followed by the Tukey-Kramer method. D = DMSO (0.1%), B = BIMP (500 μM), I = ibuprofen (1 mM).

500 μM BIMP for 24 hr after pretreatment for 30 min with ibuprofen, and then observed cell morphology by microscopy. This revealed a clear protective effect of the NSAID. However, cell morphology was still altered compared with that of cells treated with 0.1% DMSO (Fig. 6A). Additionally, as shown using MTT assays, ibuprofen itself elicited no toxicity, while the drug significantly decreased BIMP-induced death (Fig. 6B). These data suggest that ibuprofen had the potential to attenuate BIMP-induced toxicity in SK-N-SH cells.

Ibuprofen inhibited BIMP-induced COX-2 expression and apoptotic cell death

Due to its mechanism of action, we next determined whether the protective effects of ibuprofen were associated with changes in the expression of inflammatory markers. Cells were exposed to 500 μM BIMP for 24 hr after pretreatment for 30 min with 1 mM ibuprofen, and western blot analysis was performed for detection of the pro-inflammatory enzyme, COX-2, and cleaved caspase 3 expression. Interestingly, BIMP-dependent induction of COX-2 and caspase 3 cleavage was significantly decreased by ibuprofen (Fig. 6C-F). These results showed that ibuprofen effectively blocked BIMP-induced COX-2 expression and apoptotic cell death.

DISCUSSION

In this study, we examined the mechanisms through which the sarin-like organophosphate agent BIMP affects viability in neuroblastoma cells. We found that BIMP induced toxicity in SK-N-SH cells, resulting in altered cell morphology and reduced cell viability. In our previous study, we found that the soman-like organophosphate bis (pinacolyl methyl)phosphonate (BPMP) induced mitochondrial vacuolation and stellation in rat astrocytes[19]. Here, we observed that BIMP induced similar phenotypes, as it caused loss of neuronal cell projections, and subsequent cell rounding. Thus, our findings suggest that BIMP could induce neurotoxicity.

Previous studies have shown that organophosphorus compounds induce apoptotic cell death[8,25]. Similarly, our results showed that BIMP induced apoptotic cell death in SK-N-SH cells, as indicated by the induction of cleaved caspase 3, cleaved caspase 9, and cleaved PARP. Caspase 3 is a critical executioner of apoptosis, as it is either partially or completely responsible for the proteolytic cleavage of many key proteins, including PARP[29]. Cleavage of both these proteins is a hallmark of apoptosis. Additionally, caspase 9 is an important member of the caspase family that further processes other caspases, including caspase 3, upon cytochrome c release from mitochondria[34]. Therefore, we conclude that BIMP induced mitochondrial dysfunction and

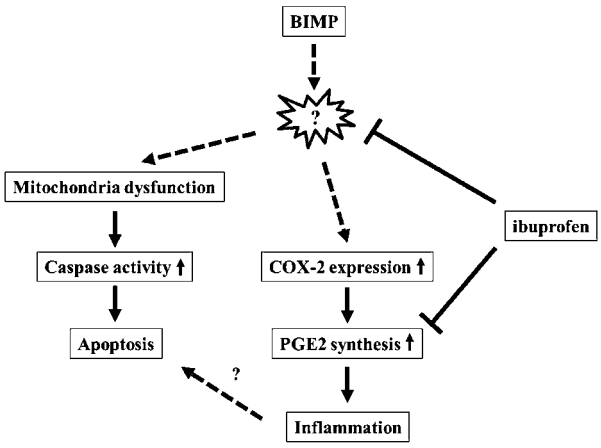


Fig.7. Schematic model for BIMP induced neurotoxicity in SK-N-SH cells

mediated the activation of caspase-9, leading to mitochondria-dependent apoptosis.

Interestingly, while exposure to BIMP did not affect COX-1 expression, there was a significant time-dependent increase in the level of COX-2. COX-2 induces production of the inflammation product PGE_2, which promotes apoptotic cell death in rat cortical cells[37]. The mechanism through which COX-2 induces neurotoxicity is still unknown. However, high expression of COX-2 has been shown to affect the nervous system adversely. Moreover, consistent with our results, organophosphorus compounds increase COX-2 expression both *in vivo* and *in vitro*[9,23], and sarin vapor has been shown to increase prostaglandin levels in guinea pigs[24] and rats[14]. Our data also indicate that BIMP induced COX-2 expression and caused inflammation, similar to the effects of other organophosphorus compounds. Thus, BIMP may directly induce inflammation in neurons involving COX-2 expression. Further studies are required to elucidate the details of these mechanisms.

In this study, we found that ibuprofen could attenuate BIMP-induced toxicity. Treatment of the cells with ibuprofen inhibited COX-2 expression and reduced levels of cleaved caspase 3, concurrent with suppression of BIMP-induced cell death. In previous studies, treatment with NSAIDs increased survival rates and attenuated brain edema in mice following exposure to soman[1]. Moreover, the organophosphorus compound VX induces COX-2 expression and PGE_2 release in rat neurons, while treatment with NSAIDs suppressed these effects and reduced VX-dependent cell death[38]. Moreover, the NSAIDs, indomethacin and NS-398 reduced COX-2 expression in the human neuroblastoma cell line, MSN[12]. Thus, NSAIDs have an important role in attenuating inflammation induced by organophosphorus compounds, which is consistent with our current results. In addition, our findings indicate that anti-inflammatory drugs, such as ibuprofen, could exert neuroprotec-

tive effects by suppressing the cytotoxic effects of BIMP.

In conclusion, we found that the sarin-like organophosphate BIMP induces apoptotic cell death via mitochondrial dysfunction and activation of inflammation, resulting in increased expression of COX-2. Additionally, COX-2 expression played an important role in BIMP-induced neurotoxicity, and this effect was attenuated by ibuprofen (Fig. 7). These results are consistent with those of other recent studies that show the inflammatory response is intimately involved in the toxicity of organophosphorus compounds, and that treatment of inflammation might prevent some of the toxicity of the organophosphorus compounds. However, further studies are required to determine the precise mechanisms of BIMP-induced neurotoxicity and inflammation.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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