calcein was observed in MDA-MB-231 cells treated with AAP than in control cells, suggesting that drug efflux activity is suppressed by treatment with AAP. As shown in Fig. 5E, *mrp2-5* mRNA expression was suppressed by treatment of MDA-MB-231 cells with AAP, suggesting that this down-regulation of expression is involved in AAP-induced inhibition of drug efflux activity and increase in susceptibility to anti-cancer drugs of MDA-MB-231 cells.

### 3.4. Effect of AAP on growth of tumor xenografts in nude mice

We tested whether AAP-dependent induction of differentiation of MDA-MB-231 cells affects their tumorigenic activity *in vivo*. Nude mice were inoculated subcutaneously with AAP-treated or non-treated MDA-MB-231 cells and the growth of the tumor xenografts was monitored. As shown in Fig. 6A, although tumor xenografts grew well in mice inoculated with control cells, such growth was not observed in mice inoculated with AAP-treated cells, showing that treatment of cells with AAP *in vitro* suppresses the tumorigenic activity of MDA-MB-231cells.

Next, we examined the effect of daily subcutaneous administration of AAP on the growth of tumor xenografts in nude mice with and without simultaneous weekly intravenous administration of doxorubicine. As shown in Fig. 6B, administration of either AAP or doxorubicine significantly suppressed growth of the tumor xenografts. At day 35, tumor xenografts were removed and the expression of CD44, CD24 and β-catenin was examined by immunohistochemical analysis. As shown in Fig. 6C and D, administration of AAP but not of doxorubicine affected the expression of these proteins: higher or lower expression of CD24 or CD44 and β-catenin, respectively, was observed in tumor xenografts from AAP-administered mice relative to xenografts from control or doxorubicine-administered mice. This suggests that AAP induces differentiation of MDA-MB-231 cells *in vivo*. Interestingly, administering both AAP and doxorubicine resulted in a more distinct suppression of tumor xenograft growth (Fig. 6B).

We then compared the effects of *o*-acetamidophenol on the growth of tumor xenografts in nude mice. Due to its hydrophobicity, *o*-acetamidophenol was orally administered. As shown in Fig. 7A, administration of *o*-acetamidophenol reduced the growth

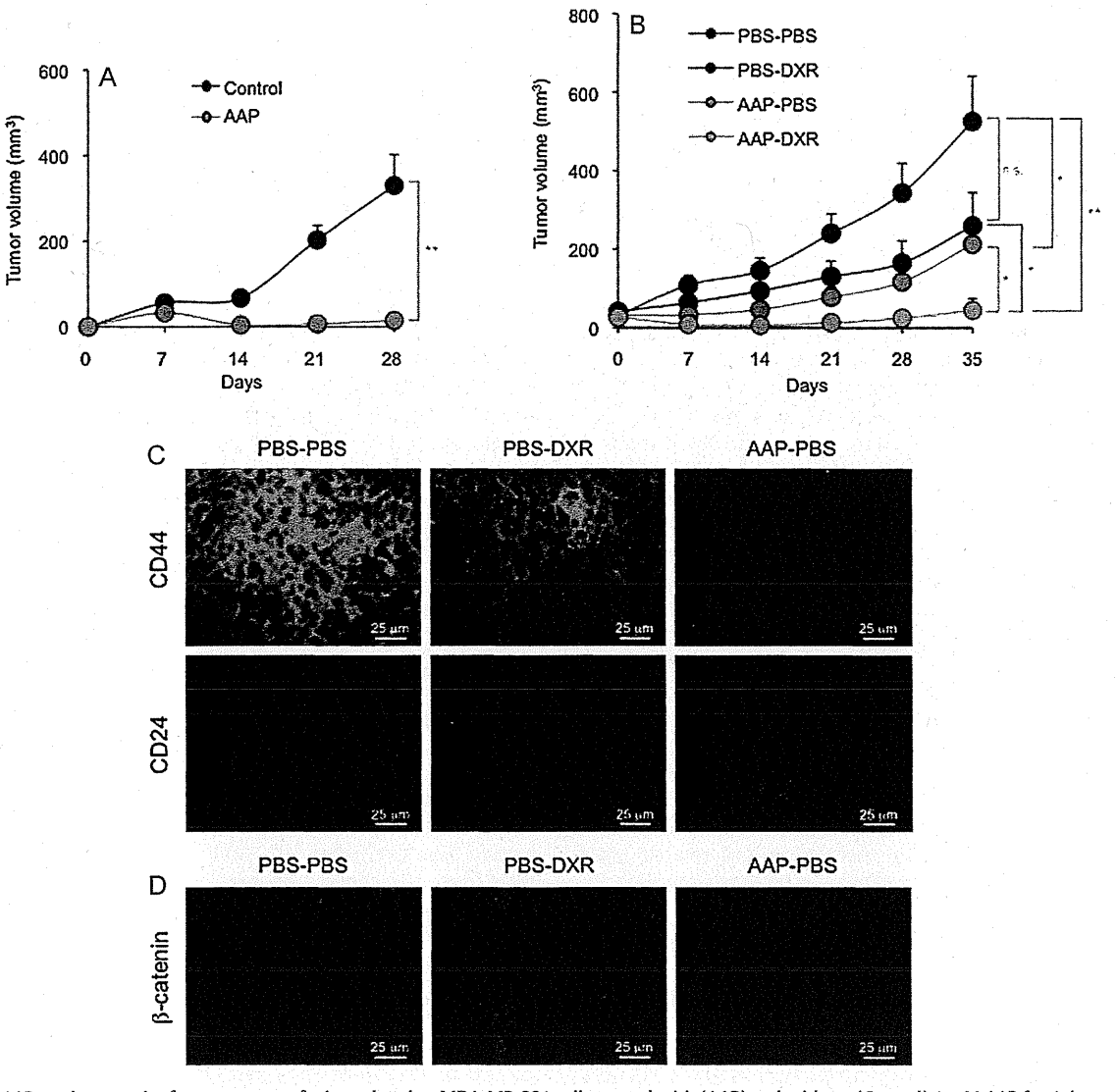
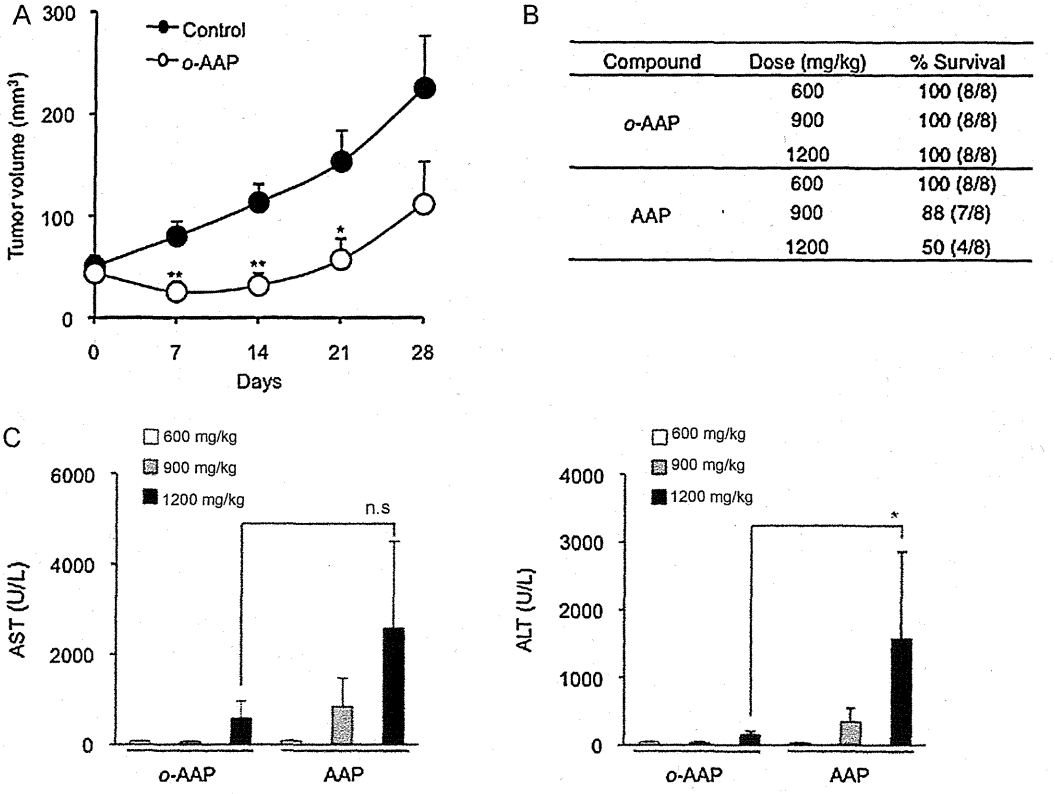



**Fig. 6.** Effect of AAP on the growth of tumor xenografts in nude mice. MDA-MB-231 cells treated with (AAP) and without (Control) 1 mM AAP for 4 days were inoculated subcutaneously into the right hind footpad of each nude mouse ($1 \times 10^7$ cells/mouse) at day 0 (A). MDA-MB-231 cells were inoculated subcutaneously into the right hind footpad of each nude mouse ($1 \times 10^7$ cells/mouse). After 2 weeks (day 0), daily subcutaneous administration of AAP (600 mg/kg) or PBS into the left hind footpad and/or weekly intravenous administration of doxorubicin (4 mg/kg, DXR) or PBS into the tail vein were initiated (B–D). Tumor sizes were measured weekly and their volumes were calculated (A and B). At day 35, tumor xenografts were removed and subjected to immunohistochemical analysis with antibodies against CD44, CD24 and β-catenin (C and D). Values are mean ± S.E.M. ($n = 6$–8). *$P < 0.05$; **$P < 0.01$; n.s., not significant.




| Compound | Dose (mg/kg) | % Survival |
|---|---|---|
| *o*-AAP | 600 | 100 (8/8) |
| | 900 | 100 (8/8) |
| | 1200 | 100 (8/8) |
| AAP | 600 | 100 (8/8) |
| | 900 | 88 (7/8) |
| | 1200 | 50 (4/8) |

**Fig. 7.** Hepatotoxicity and anti-cancer effect of *o*-acetamidophenol. MDA-MB-231 cells were inoculated subcutaneously into the right hind footpad of each nude mouse ($1 \times 10^7$ cells/mouse). After 2 weeks (day 0), daily oral administration of o-acetamidophenol (*o*-AAP) or methylcellulose (Control) were initiated. Tumor sizes were measured weekly and their volumes were calculated (A). ICR wild-type mice were orally administered the indicated doses of *o*-AAP or AAP. After 8 h, the survival rate of mice (B) and the activities of AST and ALT in plasma (C) were determined as described in Section 2. Values are mean ± S.E.M. ($n = 4$–8). $^*P < 0.05$; $^{**}P < 0.01$.

of tumor xenografts. The most serious problem with the clinical use of AAP is its hepatotoxicity, which may become an obstacle for its use as an anti-cancer drug. We compared the toxicity of AAP and *o*-acetamidophenol in wild-type ICR mice. As shown in Fig. 7B, unlike AAP, *o*-acetamidophenol did not cause mouse death at doses lower than 1200 mg/kg. Furthermore, the plasma levels of AST and ALT, indicators of hepatic injury, were higher in AAP-administered mice than in *o*-acetamidophenol-administered mice (Fig. 7C), suggesting that *o*-acetamidophenol is more safe for the liver than AAP. However, surprisingly, as shown in supplemental Fig. S2, long-term survival rate was lower with *o*-acetamidophenol than with AAP. The plasma levels of AST and ALT were lower with *o*-acetamidophenol than with AAP, suggesting that long-term treatment of mice with *o*-acetamidophenol is more toxic than AAP due to unknown and hepatic injury-independent mechanism.

## 4. Discussion

Due to the accumulating evidence suggesting that CSCs play important roles in tumor growth, metastasis and relapse after chemo- or radiotherapy, a number of studies have tried to identify drugs that specifically kill CSCs [21,22]. As an alternative strategy for cancer therapy focusing on CSCs, in this study, we searched for drugs that induce differentiation of CSCs. For this purpose, we used the breast cancer cell line MDA-MB-231, which was reported to mainly contain stem cell-like cells ($CD44^+/CD24^{-/low}$), and a chemical library consisting of drugs already in clinical use. We found that AAP (1 mM) induces differentiation of MDA-MB-231 cells *in vitro*, which was judged by cell morphological change; alteration of the expression profile of cell surface markers (from $CD44^+/CD24^{-/low}$ to $CD44^{-/low}/CD24^+$); up-regulation or down-regulation of expression of markers for differentiated cells or stem cell-like cells, respectively; inhibition of cell proliferation and invasion; and localization of ZO-1 and β-catenin at cell–cell contacts. This is the first report of a clinically used drug inducing differentiation of cancer stem cell-like cells. The reverse process of epithelial–mesenchymal transition, mesenchymal–tpithelial-transition (MET) has been paid much attention because this transition seems to suppress cancer progression [28,48]. The alterations of phenotypes associated with MET in MDA-MB-231 cells [48] were much the same as those associated with treatment of cells with AAP observed in this study. Thus, results in this study also imply that AAP induces MET in MDA-MB-231 cells.

Although as the suppression of cell proliferation is one of the alterations to phenotype that occurs with differentiation of CSCs, it is possible that alterations to other differentiation-related phenotypes are caused by suppression of cell proliferation. However, we conclude that the alterations to the phenotypes are not the result of inhibition of cell proliferation because treatment of cells with 4% ethanol caused cell growth inhibition to a similar extent as 1 mM AAP but did not induce differentiation of MDA-MB-231 cells; AAP did not induce cell death, judged by a trypan blue exclusion test, with differentiation of MDA-MB-231 cells (data not shown). Moreover, cell growth inhibition did not correlate with the induction of differentiation in experiments using various derivatives of AAP. Analysis with AAP derivatives also revealed that the anti-inflammatory activity of AAP, judged by its inhibitory effect on $PGE_2$ synthesis, correlates with its differentiation-inducing activity. However, we found that treatment of MDA-MB-231 cells with 0.1 mM indomethacin, which caused inhibition of $PGE_2$ synthesis to a similar extent as 1 mM AAP, did not induce differentiation of MDA-MB-231 cells (data not shown). Therefore, it seems that the anti-inflammatory activity of AAP is involved in, but not sufficient for, induction of the differentiation of MDA-MB-231 cells. It was recently reported that $PGE_2$ contributes to the maintenance of the undifferentiated

properties of haematopoietic stem cells [49] and a similar mechanism may be involved in the AAP-induced differentiation of MDA-MB-231 cells.

Both the TGF-β and Wnt/β-catenin canonical signaling pathways play important roles in the maintenance of the undifferentiated properties of breast CSCs and mammary gland stem cells [24–27]. However, the TGF-β signaling pathway does not seem to contribute to the maintenance of the undifferentiated properties of MDA-MB-231 cells because an inhibitor of this pathway did not induce differentiation of MDA-MB-231 cells. On the other hand, we conclude that the Wnt/β-catenin canonical signaling pathway is involved in AAP-induced differentiation of MDA-MB-231 cells as treatment of cells with AAP decreased the cellular level of β-catenin, this decrease was suppressed by an inhibitor of GSK3β, and the inhibitor suppressed the AAP-induced differentiation of MDA-MB-231 cells. At present, the mechanism whereby AAP inhibits the Wnt/β-catenin canonical signaling pathway is unknown. It was reported that the Wnt/β-catenin canonical signaling pathway plays an important role in the maintenance of self-renewal and pluripotency activities in colon CSCs and leukemia stem cells [50,51]. It has also been recently reported that the Wnt/β-catenin canonical signaling pathway plays an important role in the maintenance of self-renewal and pluripotency activities in not only mammary gland stem cells but also in brain and colon stem cells [25–27,52]. Since CSCs share with normal stem cells a mechanism for maintenance of stem cell-like properties [23], the results of this study suggest that AAP induces the differentiation of leukemia stem cells and brain and intestinal CSCs and could be effective for chemotherapy for these cancers and leukemia.

Resistance to anti-cancer drugs is one of the phenotypes of CSCs, which causes insufficient chemotherapy and relapse of cancers after chemotherapy. In this study, we have shown that pre-treatment of MDA-MB-231 cells with AAP makes cells more susceptible to anti-tumor drugs (doxorubicine and 5-FU). Since a similar increase in sensitivity was observed in MDA-MB-231 cells differentiated by overexpression of JAM-A but not in AAP-treated MCF-7 cells (a breast cancer cell line with differentiated properties), this AAP-induced increase in sensitivity of MDA-MB-231 cells to anti-cancer drugs is most likely mediated by their differentiation. We also suggest that AAP decreases the drug efflux activity of MDA-MB-231 cells and suppresses the expression of MRPs. It has been reported that doxorubicine or 5-FU is a substrate of MRP2 or MRP5, respectively [53,54]. Thus, the results of this study suggest that AAP increases the sensitivity of MDA-MB-231 cells to anti-cancer drugs through differentiation-mediated suppression of expression of MRPs and the resulting inhibition of the drug efflux activity.

We also evaluated the activity of AAP as an anti-tumor drug *in vivo*, by monitoring the growth of tumor xenografts in nude mice. We showed that pre-treatment of MDA-MB-231 cells with AAP *in vitro* decreases their tumorigenic activity. Since a previous paper suggests that the $CD44^+/CD24^{-/low}$ subpopulation of MDA-MB-231 cells has a higher tumorigenic activity than the $CD44^{-/low}/CD24^+$ subpopulation [10], the AAP-induced suppression of tumorigenic activity seems to be mediated by the induction of differentiation. Also, we have shown that subcutaneous administration of AAP to mice inhibited the growth of tumor xenografts of MDA-MB-231 cells. Administration of AAP to mice increased or decreased the expression of CD24 or CD44 and β-catenin, respectively, in tumor xenografts, suggesting that administered AAP induces differentiation of MDA-MB-231 cells *in vivo*, as seen *in vitro*. Supporting this notion, we found that the peak plasma concentration of AAP after subcutaneous administration (600 mg/kg) is about 2 mM (1 h after administration, data not shown), which is higher than that required for induction of differentiation of MDA-MB-231 cells *in vitro*.

We also showed that administration of AAP enhanced the doxorubicine-dependent suppression of tumor xenograft growth. As for the mechanism for this enhancement, an interesting idea is that AAP makes MDA-MB-231 cells more susceptible to doxorubicine by induction of differentiation, as seen *in vitro*. However, as described above, administration of AAP alone also suppressed tumor xenograft growth, it is thus, also possible that this is an additive effect of AAP and doxorubicine on tumor xenograft growth. It was recently reported that the $CD44^+/CD24^{-/low}$ subpopulation of MDA-MB-231 cells has a higher level of metastatic activity than the $CD44^{-/low}/CD24^+$ subpopulation [10]. Metastasis is a multi-step process that involves tumor cell escape from the primary site, migration, adhesion and extravasation at the secondary site, and initiation of growth and angiogenesis, and CSCs play important roles in metastasis [3]. Thus, the results of this study suggest that treatment of MDA-MB-231 cells with AAP *in vitro* or administration of AAP *in vivo* suppresses the metastatic activity of MDA-MB-231 cells.

The number of drugs reaching the marketplace has decreased year by year. This is because unexpected side effects and poor pharmacokinetics of possible drugs are being revealed in the clinical trial stage. Thus, we consider a new strategy for drug development, in which new pharmacology effects of drugs already in clinical use are identified and are used for the development of these drugs for other diseases. Therefore, in this study, we searched for drugs that are already in clinical use for chemicals that induce differentiation of MDA-MB-231 cells. We believe that development of AAP as an anti-tumor drug, or as a drug potentiating efficacies of other anti-tumor drugs, has a high probability of success because its safety and pharmacokinetics in humans have already been confirmed. However, the major obstacle for this idea is the required dose of AAP. The clinical dose of AAP for anti-inflammatory, antipyretic and analgesic effects is 1500 mg/human/day (25 mg/kg/day) and the dose required for anti-inflammatory, antipyretic and analgesic effects in animals is 150 mg/kg, which is much lower than the dose used in this study (600 mg/kg). The use of a high dose of AAP for clinical purposes is not appropriate because it would cause hepatic side effects. Therefore, a method that would decrease the dose of AAP required for achieving anti-tumor effects, such as its specific delivery to tumors, is important. Alternatively, simultaneous administration of drugs, such as N-acetylcysteine, which decrease the hepatotoxicity of AAP could be considered [55].

In conclusion, we propose that AAP becomes a new class of anti-tumor drugs, which induce the differentiation of CSCs. This type of drug would be beneficial for cancer therapy in combination with other chemotherapeutic agents, because it may overcome the obstacles of current cancer therapy: resistance to chemotherapy, metastasis and relapse.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.02.012.

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*JB Review*

# Drug discovery and development focusing on existing medicines: drug re-profiling strategy

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**As a new strategy for drug discovery and development, I focus on drug re-profiling as a way to identify new treatments for diseases. In this strategy, the actions of existing medicines, whose safety and pharmacokinetic effects in humans have already been confirmed clinically and approved for use, are examined comprehensively at the molecular level and the results used for the development of new medicines. This strategy is based on the fact that we still do not understand the underlying mechanisms of action of many existing medicines, and as such the cellular responses that give rise to their main effects and side effects are yet to be elucidated. To this extent, identification of the mechanisms underlying the side effects of medicines offers a means for us to develop safer drugs. The results can also be used for developing existing drugs for use as medicines for the treatment of other diseases. Promoting this research strategy could provide breakthroughs in drug discovery and development.**

*Keywords*: drug re-profiling/drug discovery and development/existing medicines/comprehensive analysis.

*Abbreviations*: Aβ, amyloid-β peptide; AD, Alzheimer's disease; CHOP, C/EBP homologous protein; COX, cyclooxygenase; DDS, drug delivery system; ER, endoplasmic reticulum; GGA, geranylgeranylacetone; HSF1, heat shock factor 1; HSP, heat shock protein; IBD, inflammatory bowel disease; NSAIDs, non-steroidal anti-inflammatory drugs; $PGE_2$, prostaglandin $E_2$; TJ, tight junction.

Key words to describe major industries that are likely to sustain developed countries, including Japan, in the 21st century are 'high added value' and 'knowledge-intensive'. Considering the high level of personnel costs in these countries, goods of high added value (marketable though expensive) and knowledge-intensive goods (unable to be produced in developing countries) are required. Medicines are ideal as such goods, but the pharmaceutical industry responsible for producing them must reinvent itself and continually develop in order to meet economic growth objectives.

To achieve this outcome, huge amounts of money have been invested to promote drug discovery and development. Moreover, in order to raise the efficiency of drug discovery and development, major pharmaceutical companies have repeatedly merged with each other, and novel techniques for drug discovery, such as genomic drug discovery, high-throughput screening, and combinatorial chemistry have been established. While it was thus thought that the beginning of the 21st century would be heralded by an avalanche of new medicines coming onto the market, the number of drugs reaching the marketplace has decreased year by year (Fig. 1). This is because unexpected side effects and poor pharmacokinetics of potential drugs are being revealed at various stages of clinical trials, thus rendering the drugs not fit for use on humans. I consider that this is due to the fact that a large proportion of developable drugs (high safety and good pharmacokinetics) have actually already been discovered. Thus, I would like to focus attention on a new strategy for drug discovery and development, which focuses on the use of existing medicines; in other words, to employ a drug re-profiling strategy.

## Background to the drug re-profiling strategy

In the drug re-profiling strategy, the actions of drugs already in clinical use, whose safety and pharmacokinetics in humans have already been confirmed, are examined comprehensively at the molecular level, using current and/or ground-breaking technologies, and the results used for the development of new medicines (Fig. 2). This refers not only to medicines currently in the market place, but also to medicines that have been withdrawn from the market or medicines whose clinical trials failed due to ineffectiveness (not because of safety issues).

In addition to an apparent deadlock in current drug discovery and development strategies, another aspect of the drug re-profiling strategy is the fact that among existing medicines, there are many of them for whom it is unclear how their underlying mechanisms of action give rise to their main effects and side effects. Many drugs that have been on the market for a long time (in most cases, good drugs) can be included in this group. This is because a significant proportion of them are derived from natural products that are traditionally thought to be effective for the treatment of particular

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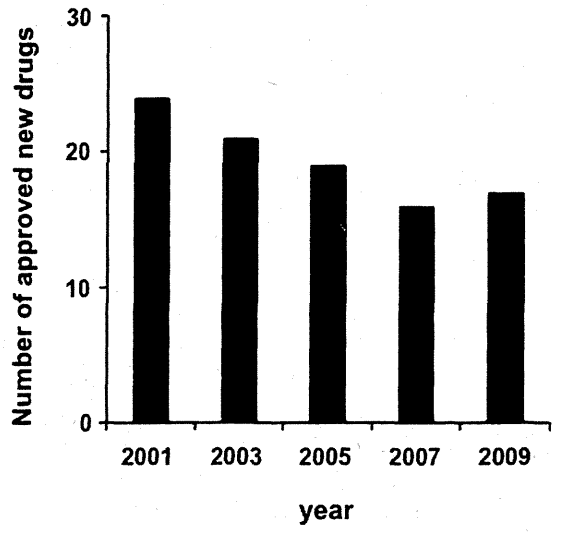


Fig. 1 Decrease in the number of new drugs approved by FDA.

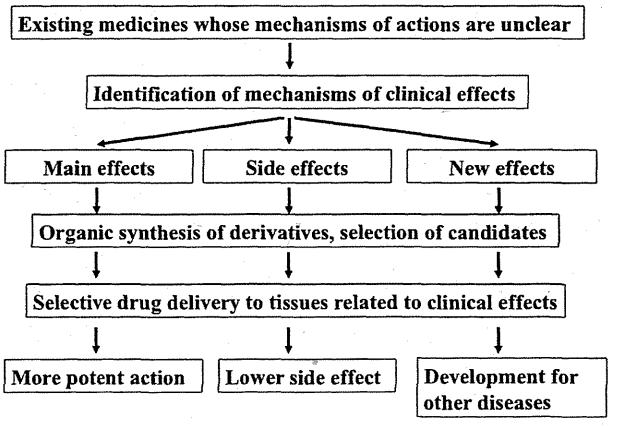


Fig. 2 Drug re-profiling strategy.

conditions. Nevertheless, the mechanisms underlying how these drugs achieve their clinical effect have not been examined. Furthermore, when such traditional medicines were developed, it was difficult, if not impossible to investigate the molecular mechanisms of action that give rise to their main effects and side effects, due to a lack of analytical technology.

On the other hand, epidemiological studies have revealed a number of novel clinical effects of existing medicines (for example, the anti-tumour and anti-Alzheimer's disease (AD) effects of non-steroidal anti-inflammatory drugs (NSAIDs), as described below); however, the mechanisms governing these novel clinical effects are unclear at present.

With this background in mind, in drug re-profiling the actions of clinically employed drugs are subjected to a comprehensive examination at the molecular level to identify the mechanisms underlying their main actions and side effects, with the aim to develop more potent and safer medicines, or to identify novel clinical effects and their underlying molecular mechanisms. This may enable the development of existing drugs as treatments for other diseases (Fig. 2). The advantage of this strategy is that there is a decreased risk for unexpected side effects and poor pharmacokinetics in humans because their safety and pharmacokinetics have already been well characterized. By employing this strategy, we could also improve the efficiency of drug development by reducing the enormous amount of time, money and energy that goes into getting a product to market. For example, pre-clinical tests (such as evaluation for safety, metabolism, absorption and excretion in animals) and phase I clinical trials in humans can be omitted. Drug re-profiling has consequently been linked to the concept of 'eco-medicine'.

This research strategy can also be considered as a new type of basic chemical biology. When the mechanisms of action of existing medicines are poorly understood, this means that they act in an unknown biological manner; in other words, the identification of such mechanisms may lead us to new biological outcomes. For example, as described below, analysis of the anti-tumour activity of NSAIDs led us to identify that tight junction (TJ)-associated proteins regulate the metastasis of tumours (*1*, *2*).

Recently, a number of successful results of indication expansion have been reported. For example, sildenafil and minoxidil were originally developed as medicines for the treatment of cardiovascular diseases. However, in the clinical setting, other pharmacological activities were identified and these drugs were re-developed for the treatment of erectile dysfunction and alopecia, respectively (*3*, *4*). In Japan, ramosetron was originally developed as an antiemetic drug and thereafter, taking into account its principal side effect, constipation, this drug was re-developed as a treatment for diarrhea-predominant irritable bowel syndrome (*5*).

In these indication expansions, the strategy was found by chance, giving rise to the possibility of there being many un-identified pharmacological activities of existing medicines. Thus, in drug re-profiling, the pharmacological activities of existing medicines are identified scientifically and comprehensively using innovative technologies and the results are used for drug development, including indication expansion.

## Methods underlying the drug re-profiling strategy

Steps in the drug re-profiling strategy can be described as follows:

(i) Targeting and selection of existing drugs to be subjected to analysis.
(ii) Comprehensive analysis of the actions of these drugs and to identify the manner in which they exert their clinical action (main effect, side effects and novel effects) with the aim to select compounds that might warrant further analysis.
(iii) Organic synthesis of derivatives of the selected drug to obtain more-effective homologues. A principle advantage of the drug re-profiling strategy is that existing medicines can be subjected to drug development in order to reduce costs associated with development and risk of

failure. However, when a drug with desirable characteristics cannot be found in existing medicines, the slight modification of existing medicines should be considered (see our study for NSAIDs with reduced gastric side effects).

(iv) Drug delivery system (DDS) studies to deliver drugs to tissues related to the drug's main effects or novel effects, or to avoid delivery in cases where side effects occur.

We applied this strategy to existing medicines to identify new possibilities for drug development. Furthermore, since some beneficial effects of existing medicines may not be identified by this strategy only, we recently prepared a library of existing medicines and applied various screening methods to these compounds in order to comprehensively search for existing medicines with clinically beneficial effects.

The following discussion provides more detailed methods for each step, which are currently being performed in our work.

### *Targeting and selection of existing medicines*

Candidate existing medicines to be subjected to drug re-profiling are selected on the basis of data from epidemiological studies and previous clinical trials (including examples of failure), as well as from analyses of existing drugs whose mechanism of action is unclear.

### *Comprehensive analysis of the mechanisms of action of targeted medicines*

*Analysis of genes whose expression is induced by the target drug.* Using DNAchip and proteinchip techniques, genes and proteins induced by the target drug are identified in various cells types (such as cells from different tissues, and cells expressing proteins related to specific diseases). As for genes possibly related to some diseases, the drug and the gene are analysed in an *in vitro* system (for example, using siRNA) and also in animal models. Through these studies, we select existing medicines that are possibly linked to new drug development strategies. On the other hand, the mechanism of action of targeted drugs is analysed to identify new biological outcomes.

*Analysis of proteins bound to the target drug.* To identify proteins bound to the target drug, total human proteins are separated by 2D gel electrophoresis and detected with the labeled drug. Analysis of the identified proteins is performed as described earlier.

*Other analyses.* Other comprehensive analyses using innovative techniques are also performed. For example, alterations in the concentrations of various signal transduction-related molecules (such as cAMP) after the treatment of cells with the drug are monitored, or systematic screening of receptors that bind the drug is performed.

*Analysis of a library of existing medicines.* A library of existing medicines is subjected to the various screening systems. When novel, clinically beneficial actions are identified, further drug development and analysis of underlying molecular mechanisms are carried out as described above.

### *Organic synthesis of derivatives and analysis of their actions*

Derivatives of existing medicines selected are synthesized. In such cases, clear strategies for the synthesis are needed. For example, in the case of the synthesis of NSAIDs with lower membrane permeabilizing activity, we computer-simulated the interaction between the target NSAID and the membrane and used the results to synthesize derivatives of the target NSAID (*6*). The activities of the newly synthesized drugs are estimated *in vitro* and *in vivo* for the subsequent selection of promising compounds as candidates for new medicines.

### *DDS-mediated modification of drugs*

DDS is a technique that permits selective drug delivery to specific tissues, and as such is essential in the quest for drug re-profiling. For example, when the underlying mechanisms related to the side effects of existing medicines are revealed, DDS can be used to avoid delivery of the drug to tissues related to the side effect. Conversely, when the novel clinical effects of drugs are revealed, DDS can be used to selectively deliver the drugs to the relevant tissues related to this effect.

Embedding of the medicine into nanoparticles and modification of the surface of nanoparticles (for example, loading antibodies that recognize tissue-specific proteins) is a useful DDS technique. Using more traditional techniques, it was impossible to embed hydrophilic drugs; however, we recently found a way around this by introducing a phosphate side chain into the drug and its insolubilization with zinc ion. By this method, we were able to embed $PGE_1$ (a stimulator of vascularization) into nanoparticles and deliver this drug to the site of vascular disorders (*7–10*). Further progression of this technique may lead us to be able to deliver the target drug to the preferable position.

## Examples of drug re-profiling

### *NSAIDs*

NSAIDs are one of the most frequently used classes of medicines in the world and account for ~5% of all prescribed medications (*11*). NSAIDs are inhibitors of cyclooxygenase (COX), a protein essential for the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. However, NSAID administration is associated with gastro-intestinal complications, such as gastric ulcers and bleeding. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications (*12*). Inhibition of COX by NSAIDs was previously thought to be fully responsible for their gastrointestinal side effects; however, recent reports suggest that some additional, unknown mechanisms might contribute to this side effect. On the other hand, a range of epidemiological studies have revealed that prolonged NSAID use reduces the risk of cancer and AD (*13–17*). However, the molecular mechanisms

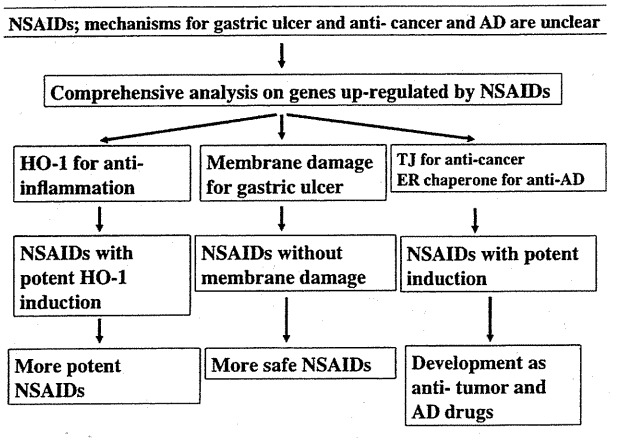


**Fig. 3 Drug re-profiling study for NSAIDs.**

governing these newly identified effects of NSAIDs are unclear at present.

In order to identify the molecular mechanism underlying the gastric side effect of NSAIDS, as well as their anti-inflammatory, anti-tumour and anti-AD effects, we comprehensively examined the actions of NSAIDs at the molecular level using a number of state-of-the-art techniques (for example, by using DNAchip analysis to search for genes whose expression is up-regulated by NSAIDs). We made several observations that can be outlined as follows: the induction of HO-1 (an anti-inflammatory protein) is involved in the anti-inflammatory action of NSAIDs (*18*); NSAID-dependent membrane permeabilization and the resulting induction of the endoplasmic reticulum (ER) stress response [induction of C/EBP homologous protein (CHOP)] and apoptosis are involved in the gastric side effects of NSAIDs (*19–23*); the COX-inhibition and induction of expression of TJ-associated proteins is involved in NSAIDs' anti-tumour effect (*1*, *2*, *24*); not only the COX-inhibition and resulting inhibition of EP2 and EP4 receptors but also the ER stress response (induction of ER chaperones) are involved in NSAIDs' anti-AD effect (*25–28*) (Fig. 3).

As for the gastric side effects of NSAIDs, we found that loxoprofen has the lowest membrane permeabilizing activity among existing NSAIDs (*29*). Loxoprofen has been used clinically for a long time as a standard NSAID in Japan, and clinical studies have suggested that it is safer than other NSAIDs, such as indomethacin. We therefore synthesized a series of loxoprofen derivatives and found that fluoro-loxoprofen does not have membrane permeabilizing activity yet still exerts an anti-inflammatory effect and causes fewer gastric ulcers in mice than loxoprofen (*6*). These results suggest that the drug re-profiling strategy used here is a useful means to identify the molecular mechanisms governing the side effects of existing medicines and to develop new drugs with reduced side effects.

Further to the above, we selected NSAIDs with potent activity for inducing the expression of TJ-associated proteins and ER chaperones, and we are developing these NSAIDs with a view to using them as anti-tumour and anti-AD drugs, respectively. From these achievements, we realized that the drug re-profiling strategy could help us to develop existing drugs for use in the treatment of other diseases.

As a consequence of our findings, we suggested that claudins transmembrane proteins consisting of TJs positively or negatively affect the migration and invasion activity of cancer cells, depending on the claudin species, and that this action plays an important role in conferring the chemopreventive effect of NSAIDs through the inhibition of metastasis (*1*, *2*). Furthermore, based on the finding that EP2 and EP4 receptors are involved in the anti-AD effect of NSAIDs, in other words, promoting the progression of AD, we examined the mechanism underlying this involvement. By using $EP_2$- or $EP_4$-receptor-null mice, we found that activation of the $EP_2$ receptor stimulates the production of amyloid-β peptide (Aβ) through the activation of adenylate cyclase, as well as causing an increase in the cellular level of cAMP and activation of protein kinase A (*28*). On the other hand, activation of the $EP_4$ receptor causes its co-internalization with PS-1 (γ-secretase) into endosomes, which in turn activates γ-secretase, resulting in the upregulation of Aβ production (*28*). These results led us to develop antagonists for these receptors as anti-AD drugs. In fact, we recently found that oral administration of an antagonist specific for the $EP_4$ receptor improves cognitive functions in AD model mice (Hoshino *et al.*, unpublished data). Thus, the drug re-profiling strategy has also enabled us to identify new biological outcomes and new targets of existing medicines.

### *Geranylgeranylacetone*

Geranylgeranylacetone (GGA) was developed 27 years ago and has been used clinically since 1983 as a standard anti-ulcer drug in Japan. However, the molecular mechanism underlying this anti-ulcer action of GGA was, until recently, unclear. Rokutan and his co-workers comprehensively examined the action of GGA at the molecular level and found that it is a non-toxic heat shock protein (HSP)-inducer (*30*). Since HSPs protect cells from various stressors (*31*, *32*), we hypothesized that GGA achieves its anti-ulcer effect by making gastric mucosal cells resistant to various gastric irritants by induction of HSPs. We successfully proved this hypothesis of the contribution of the HSP-inducing activity of GGA to its anti-ulcer activity by showing that GGA does not exhibit anti-ulcer activity in heat shock factor 1 (HSF1)-null mice, where the induction of HSPs is suppressed (*33*, *34*). These results suggest that the drug re-profiling strategy may contribute to identification of the molecular mechanisms underlying the clinical effects of existing medicines and that transgenic mice are useful tools to understand such molecular mechanisms (Fig. 4).

It was recently revealed that HSP70 has an anti-inflammatory activity by means of its inhibition of nuclear factor kappa B and a resulting suppression of pro-inflammatory cytokine and chemokine expression (*35–38*). Therefore, we consider that inducers of

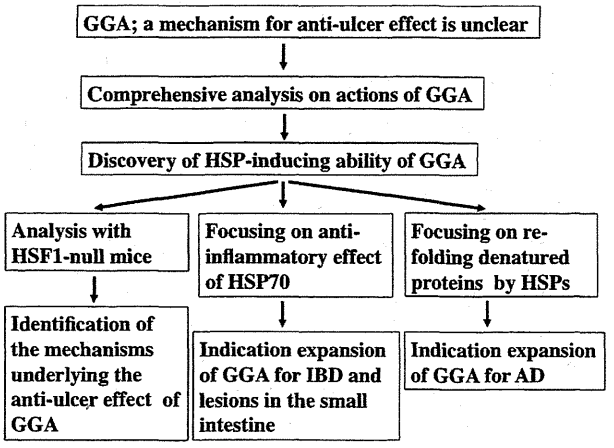


**Fig. 4** Drug re-profiling study for GGA.

HSP70, such as GGA, could be effective for treating diseases that involve stressor-induced cell death and inflammation. To commence this work, we focused on inflammatory bowel disease (IBD), which has become a significant health problem with an actual prevalence of 200–500 per 100,000 people in Western countries, with a doubling rate of just over 10 years (*39*). Recent studies suggest that IBD involves chronic inflammatory disorders in the intestine due to 'a vicious cycle'. Infiltration of leukocytes into intestinal tissues causes intestinal mucosal damage induced by reactive oxygen species that are released from the activated leukocytes, with this damage further stimulating the infiltration of leukocytes (*40*). Based on the cytoprotective and anti-inflammatory effects of HSP70, we speculated that expression of HSP70 would be effective for treating IBD. Using animal models for colitis, we found that transgenic mice expressing HSP70 are more resistant to colitis than the wild-type mice. Furthermore, we revealed that expression of HSP70 achieves this protective effect against colitis through its cytoprotective and anti-inflammatory activity (*33*). Ohkawara *et al.* (*42*) addressed this issue by employing GGA in their studies, and reported that oral administration of GGA suppressed IBD-related colitis. Furthermore, they showed that GGA up-regulated the expression of HSP70 and HSP40 but not other HSPs in the colon (*41*, *42*). These results support the idea that HSP70 is protective against IBD-related colitis and suggest that non-toxic inducers of HSP70 are therapeutically beneficial for IBD (Fig. 4).

More attention has generally been paid to NSAID-induced gastric lesions rather than lesions of the small intestine, because the latter are usually asymptomatic and their diagnosis is difficult to make. However, recent improvements in diagnostic techniques such as capsule endoscopy and double-balloon endoscopy have revealed that NSAID-induced lesions of the small intestine occur very frequently and that the small intestine is even more susceptible than gastric tissue to the detrimental effects of NSAIDs (*43*, *44*). Nevertheless, clinical protocols for the treatment of NSAID-induced lesions of the small intestine have not been established. For example, acid control drugs are not as effective for treating NSAID-induced lesions of the small intestine compared with their effect on gastric lesions (*45*, *46*). Recent studies suggest that NSAID-induced lesions of the small intestine involve the direct cytotoxicity (topical effect) of the NSAID, and inflammatory responses. Thus, it is reasonable to speculate that HSP70 protects against NSAID-induced lesions of the small intestine. Using transgenic mice expressing HSP70 and wild-type mice, we compared the development of lesions in the small intestine after administration of indomethacin. Indomethacin-induced such lesions in a dose-dependent manner in wild-type mice and this production was significantly reduced in transgenic mice expressing HSP70. We also found that expression of HSP70 achieves this protective effect through its cytoprotective and anti-inflammatory activity. Furthermore, pre-administration of GGA suppressed the indomethacin-induced lesions in a dose-dependent manner, and the GGA-induced expression of HSP70 suppressed the extent of indomethacin-induced lesions by inhibiting indomethacin-induced mucosal cell apoptosis and reducing the inflammatory response (*47*). These results strongly suggest that oral administration of GGA could be therapeutically beneficial against NSAID-induced lesions of the small intestine in humans owing to its HSP-inducing activity (Fig. 4).

Based on the activity of HSPs for re-folding denatured proteins, a group from Nagoya University speculated that GGA would be effective for treating spinal and bulbar muscular atrophy (whose major cause is protein denaturation) and succeeded in verifying this (*48*). On the other hand, we recently reported that expression of HSP70 is an effective treatment against AD in a mouse model. AD model mice showed less of an apparent cognitive deficit when they were crossed with transgenic mice expressing HSP70. Transgenic mice expressing HSP70 also displayed lower levels of Aβ, Aβ plaque deposition and neuronal and synaptic loss than control mice. These results suggest that expression of HSP70 in mice suppresses not only the pathological but also the functional phenotypes of AD (*25*). These studies on GGA suggest that the drug re-profiling strategy is useful for the indication expansion of existing medicines (Fig. 4).

## Conclusions and perspectives

In order to successfully carry out a drug re-profiling strategy, specialists in various fields of drug development, epidemiology, clinical medicine, molecular biology, genomic analysis, organic chemistry, DDS and material chemistry must pool their resources and know-how. The bringing together of such experts is currently underway in Japan, and success in the re-profiling of different drugs will surely affect the drug development strategy of pharmaceutical companies in the future. In other words, our final goals are to contribute to the development of the pharmaceutical industry and to promote efficient drug development through drug re-profiling. Many pharmaceutical companies have had disappointing economic growth because they expected a rush of new drugs to come

onto the market with the development of novel techniques such as genomic drug discovery. I want to contribute to the revival of the pharmaceutical industry by promoting a paradigm shift in their drug development strategy that is based on drug re-profiling.

If drug re-profiling is to be performed in an efficient manner then a network of researchers from universities and industry is required. In universities, numerous researchers have developed original screening systems for medicines, giving rise to the real possibility to establish a research network in which existing medicines are made available by pharmaceutical companies and subjected to such screening procedures to obtain clues for new uses of currently available drugs. Since such screening systems are closely related to the basic research carried out by the researchers in question, the identification of drugs by these screening systems also greatly contributes to the progress of their basic research. Given the potential that a university–industry network would have, it is highly recommendable that such a system be established.

**Conflict of interest**
None declared.

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# Therapeutic Effect of Lecithinized Superoxide Dismutase on Pulmonary Emphysema[S]

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## ABSTRACT

No medication exists that clearly improves the mortality of chronic obstructive pulmonary disease (COPD). Oxidative molecules, in particular superoxide anions, play important roles in the COPD-associated abnormal inflammatory response and pulmonary emphysema, which arises because of an imbalance in proteases and antiproteases and increased apoptosis. Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anions. Lecithinized human Cu/Zn- SOD (PC-SOD) has overcome a number of the clinical limitations of SOD, including low tissue affinity and low stability in plasma. In this study, we examine the effect of PC-SOD on elastase-induced pulmonary emphysema, an animal model of COPD. The severity of the pulmonary inflammatory response and emphysema in mice was assessed by various criteria, such as the number of leukocytes in the bronchoalveolar lavage fluid and the enlargement of airspace. Not only intravenous administration but also inhalation of PC-SOD suppressed elastase-induced pulmonary inflammation, emphysema, and dysfunction. Inhalation of PC-SOD suppressed the elastase-induced increase in the pulmonary level of superoxide anions and apoptosis. Inhalation of PC-SOD also suppressed elastase-induced activation of proteases and decreased in the level of antiproteases and expression of proinflammatory cytokines and chemokines. We also found that inhalation of PC-SOD suppressed cigarette smoke-induced pulmonary inflammation. The results suggest that PC-SOD protects against pulmonary emphysema by decreasing the pulmonary level of superoxide anions, resulting in the inhibition of inflammation and apoptosis and amelioration of the protease/antiprotease imbalance. We propose that inhalation of PC-SOD would be therapeutically beneficial for COPD.

## Introduction

Chronic obstructive pulmonary disease (COPD) is currently the fourth leading cause of death in the world, and its prevalence and mortality rates have been increasing (Rabe et al., 2007). COPD is a disease state defined by irreversible and progressive airflow limitation associated with an abnormal inflammatory response. The most important etiologic factor for COPD is cigarette smoking (CS) (Peto et al., 1999; Rabe et al., 2007). Pathologic characteristics of COPD include infiltration of leukocytes, enhanced mucus secretion, dysfunctional airway matrix remodeling, and destruction of parenchyma (enlargement of airspace) (Barnes and Stockley, 2005; Owen, 2005; Rabe et al., 2007). Protease/antiprotease imbalance and apoptosis play important roles in this emphysematous lung destruction. Unfortunately, there is no effective drug therapy that is able to significantly and clearly modulate disease progression and mortality (Calverley et al., 2007; Miravitlles and Anzueto, 2009).

It has been suggested that oxidative molecules play an important role in the pathogenesis of COPD (Pinamonti et al., 1998; Nadeem et al., 2005; Mak, 2008). In addition to

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**ABBREVIATIONS:** COPD, chronic obstructive pulmonary disease; BALF, bronchoalveolar lavage fluid; CS, cigarette smoking; DAPI, 4,6-diamino-2-phenylindole; DPhPMPO, 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide; ELISA, enzyme-linked immunosorbent assay; ESR, electron spin resonance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H and E, hematoxylin and eosin; IL, interleukin; IPF, idiopathic pulmonary fibrosis; KC, keratinocyte-derived chemokine; kU, kilounit; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PC, phosphatidylcholine; RT-PCR, reverse transcription-polymerase chain reaction; SOD, superoxide dismutase; PC-SOD, lecithinized human Cu/Zn-SOD; PPE, porcine pancreatic elastase; QOL, quality of life; ROS, reactive oxygen species; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

stimulation of the inflammatory response by induction of production of proinflammatory cytokines and chemokines, oxidative molecules induce pulmonary cell apoptosis, activate proteases, and inactivate antiproteases (Valentin et al., 2005; Rahman and Adcock, 2006; Greenlee et al., 2007).

Superoxide dismutase (SOD) catalyzes the dismutation of superoxide anion to hydrogen peroxide, which is subsequently detoxified to oxygen and water (Kinnula and Crapo, 2003). Of human SODs, Cu/Zn-SOD accounts for 80% of all SOD activities within the lung (Kinnula and Crapo, 2003). Altered levels of expression and activity of SOD were observed in both patients with COPD and animals treated with elastase or CS (animal models for COPD) (Kondo et al., 1994; Daga et al., 2003; Valenca et al., 2008), and transgenic mice expressing Cu/Zn-SOD were resistant to elastase- or CS-induced pulmonary emphysema (Foronjy et al., 2006). Furthermore, transgenic mice expressing another type of SOD, extracellular SOD, or knockout mice for this protein were resistant or sensitive, respectively, to elastase- or CS-induced pulmonary emphysema through attenuating oxidative fragmentation of extracellular matrix (Yao et al., 2010). These results suggest that administration of SOD could be of therapeutic benefit in the treatment of COPD. However, because of its low affinity for tissues and low stability in plasma, there is no report showing that administration of SOD is effective for the treatment of patients with COPD or elastase- or CS-induced pulmonary emphysema in animals.

Igarashi et al. (1992) developed PC-SOD, a lecithinized human Cu/Zn-SOD in which four phosphatidylcholine (PC) derivative molecules are covalently bound to each SOD dimer. This modification drastically improves the plasma stability and cellular affinity of SOD (Igarashi et al., 1992,1994; Ishihara et al., 2009). As described under *Discussion*, clinical studies showed that intravenously administered PC-SOD is effective for ulcerative colitis and idiopathic pulmonary fibrosis (IPF) (Broeyer et al., 2008; Suzuki et al., 2008a,b) Furthermore, we recently reported that inhalation of PC-SOD is effective against bleomycin-induced pulmonary fibrosis in mice (an animal model for IPF) (Tanaka et al., 2010). We believe that inhalation may be a viable option for administration of PC-SOD, which would improve the quality of life (QOL) of patients treated with this drug. In this study, we found that inhalation of PC-SOD suppresses elastase-induced pulmonary inflammation, emphysema, and dysfunction, through suppression of cell death, activation of proteases, induction of expression of proinflammatory cytokines and chemokines, and decrease in the level of α1-antitrypsin (an antiprotease). We propose that inhalation of PC-SOD would be therapeutically beneficial for COPD.

## Materials and Methods

**Chemicals and Animals.** Paraformaldehyde and porcine pancreatic elastase (PPE) were obtained from Sigma (St. Louis, MO). Novo-Heparin (5000 units) for injection was from Mochida Pharmaceutical Co. (Tokyo, Japan). Chloral hydrate was from Nacalai Tesque (Kyoto, Japan). Diff-Quik was from the Sysmex Corporation (Kobe, Japan). Terminal deoxynucleotidyl transferase was obtained from TOYOBO (Osaka, Japan). Biotin 14-ATP, Alexa Fluor 488 goat anti-mouse immunoglobulin G, and Alexa Fluor 488 conjugated with streptavidin were purchased from Invitrogen (Carlsbad, CA). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). The RNeasy kit was obtained from QIAGEN (Valencia, CA), the PrimeScript 1st Strand cDNA Synthesis Kit was from TAKARA Bio (Ohtsu, Japan), and the iQ SYBR Green Supermix was from Bio-Rad Laboratories (Hercules, CA). Cytospin 4 was purchased from Thermo Fisher Scientific (Waltham, MA), and Mayer's hematoxylin, 1% eosin alcohol solution, and mounting medium for histological examination (malinol) were from MUTO Pure Chemicals (Tokyo, Japan). Unmodified SOD (5190 U/mg) and PC-SOD (3000 U/mg) were from our laboratory stocks (Igarashi et al., 1992). The α1-antitrypsin ELISA kit was from Immunology Consultants Laboratory (Newberg, OR). ELISA kits for interleukin (IL)-1β and IL-6 were from Thermo Fisher Scientific. ELISA kits for tumor necrosis factor (TNF)-α, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1, and keratinocyte-derived chemokine (KC) were from R&D Systems (Minneapolis, MN). 4,6-Diamino-2-phenylindole (DAPI), diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid, and 2-diphenylphosphinoyl-2-methyl-3,4-dihydro-2*H*-pyrrole *N*-oxide (DPhPMPO) were from Dojindo (Kumamoto, Japan). An antibody against 8-OHdG was from Nikken SEIL (Shizuoka, Japan). Wild-type mice (6–8 weeks old, ICR, male) were used. The experiments and procedures described here were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996) and were approved by the Animal Care Committee of Kumamoto University.

**Treatment of Mice with PPE, CS, and PC-SOD.** Mice maintained under anesthesia with chloral hydrate (500 mg/kg) were given one intratracheal injection of PPE (50 or 100 μg/mouse) in phosphate-buffered saline (30 μl/mouse) by use of a micropipette (p200) to induce pulmonary emphysema. Commercial (nonfiltered) cigarettes (Peace; Japan Tobacco Inc., Tokyo, Japan) that yielded 28 mg of tar and 2.3 mg of nicotine on a standard smoking regimen were used. For exposure of mice to CS, 15 to 20 mice were placed in a chamber (volume, 45 L). Mice were exposed to the smoke of two cigarettes for 25 min, three times a day for 3 days. In the chronic model, mice were exposed to the smoke of one cigarette for 35 min, three times a day, 5 days a week, for 4 weeks. Each cigarette was puffed 15 times for 5 min.

For intravenous administration of PC-SOD, PC-SOD was dissolved in 5% xylitol and administered via the tail vein. For control mice, 5% xylitol solution was administered. The first administration of PC-SOD was performed just before PPE administration.

For the administration of PC-SOD by inhalation, five to seven mice were placed in a chamber (volume, 45 L). PC-SOD was dissolved in 10 ml of 5% xylitol, and an ultrasonic nebulizer (NE-U17 from Omron, Tokyo, Japan) that was connected to the chamber was used to nebulize the entire volume of the PC-SOD solution in 30 min. For control mice, 5% xylitol solution was subjected to nebulization. Mice were kept in the chamber for another 10 min after the 30 min of nebulization. The first inhalation of PC-SOD was performed just before PPE administration.

The amount of α1-antitrypsin in the plasma and proinflammatory mediators in BALF was measured by ELISA according to the manufacturer's protocol.

**Preparation of BALF and Cell Count.** BALF was collected by cannulating the trachea and lavaging the lung with 1 ml of sterile phosphate-buffered saline containing 50 units/ml heparin (two times). Approximately 1.8 ml of BALF was routinely recovered from each animal. The total cell number was counted using a hemocytometer. Cells were stained with Diff-Quik reagents after centrifugation with Cytospin 4, and the ratios of alveolar macrophages, lymphocytes, and neutrophils to total cells were determined.

**Measurement of Production of Superoxide Anions.** The production of superoxide anions was assayed by electron spin resonance (ESR) spin trapping with DPhPMPO as described previously (Karakawa et al., 2008). Cells collected from BALF were incubated with 0.9% NaCl containing 500 μM diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid and 10 mM DPhPMPO for 10 min at 37°C. ESR

spectra were recorded at room temperature on a JES-TE200 ESR spectrometer (JEOL, Tokyo, Japan) under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.43 GHz; microwave power, 40 mW; scanning field, 335.2 ± 5 mT; sweep time, 2 min; field modulation width, 0.25 mT; receiver gain, 400; and time count, 0.3 s. Every buffer and solution used in the reaction mixture used for ESR measurement was treated with Chelex 100 resin (Bio-Rad Laboratories) before use to remove metals.

**Histological and Immunohistochemical Analyses and Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Assay.** Lung tissue samples were fixed for 24 h at a pressure of 25 cm $H_2O$, and then embedded in paraffin before being cut into 4 μm-thick sections.

For histological examination, sections were stained first with Mayer's hematoxylin and then with 1% eosin alcohol solution [hematoxylin and eosin (H and E) staining]. Samples were mounted with malinol and inspected with the aid of an Olympus (Tokyo, Japan) BX51 microscope. Twenty lines (500 μm) were drawn randomly on the image of sections stained with H and E, and the intersection points with the alveolar walls were counted to determine the mean linear intercept. The morphometric analysis at the light microscopic level was conducted by a blinded investigator.

For immunohistochemical analysis, sections were treated with 20 μg/ml protease K for antigen activation. Sections were blocked with 2.5% goat serum for 10 min, incubated for 12 h with an antibody against 8-OHdG (1:100 dilution) in the presence of 2.5% bovine serum albumin, and then incubated for 1 h with Alexa Fluor 488 goat anti-mouse IgG in the presence of DAPI (5 μg/ml). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51).

For the TUNEL assay, sections were incubated first with proteinase K (20 μg/ml) for 15 min at 37°C, then with TdTase and biotin 14-ATP for 1 h at 37°C, and finally with Alexa Fluor 488 conjugated with streptavidin and DAPI (5 μg/ml) for 2 h. Samples were mounted with VECTASHIELD and inspected with the aid of a fluorescence microscope (Olympus BX51).

**Gelatin Zymography.** The proteolytic activities of MMP-2 and MMP-9 were assessed by SDS-polyacrylamide gel electrophoresis using zymogram gels containing 0.1% gelatin as described previously (Namba et al., 2009). The protein concentration was determined by the Bradford method (Bradford, 1976). After electrophoresis at 4°C (10 μg of protein/lane), the gels were washed with 2.5% Triton X-100 for 30 min at room temperature and incubated with zymogram development buffer for 2 days at 37°C. Bands were visualized by staining with Coomassie brilliant blue.

**Real-Time RT-PCR Analysis.** Real-time RT-PCR was performed as described previously (Namba et al., 2009) with some modifications. Total RNA was extracted from pulmonary tissues using an RNeasy kit according to the manufacturer's protocol (QIAGEN). Samples (2.5 μg of RNA) were reverse-transcribed using a PrimeScript first-strand cDNA Synthesis Kit. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument; Bio-Rad Laboratories) experiments using iQ SYBR GREEN Supermix and analyzed with Opticon Monitor Software (Bio-Rad Laboratories). Specificity was confirmed by electrophoretic analysis of the reaction products and inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, GAPDH cDNA was used as an internal standard.

Primers were designed using the Primer3 website (http://frodo.wi.mit.edu/primer3/). The primers used were (forward primer, reverse primer): TNF-α, 5′-cgtcagccgatttgctatct-3′, 5′-cggactccgcaaagtctaag-3′; IL-1β, 5′-gatcccaagcaatacccaaa-3′, 5′-ggggaactctgcagactcaa-3′; IL-6, 5′-ctggagtcacagaaggagtgg-3′, 5′-ggtttgccgagtagatctcaa-3′; MIP-2α, 5′-accctgccaagggttgacttc-3′, 5′-ggcacatcaggtacgatccag-3′; MCP-1, 5′-ctcacctgctgctactcattc-3′, 5′-gcttgaggtggttgtggaaaa-3′; KC, 5′-tgcacccaaaccgaagtcat-3′, 5′-ttgtcagaagccagcgttcac-3′; and GAPDH, 5′-aactttggcattgtggaagg-3′, 5′-acacattgggggtaggaaca-3′.

**Analysis of Lung Function.** Analysis of lung function was performed with a computer-controlled small-animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada), as described previously (Kuraki et al., 2002). Mice were anesthetized with chloral hydrate (500 mg/kg), tracheotomized with an 8-mm section of metallic tubing, and mechanically ventilated at a rate of 150 breaths/min, using a tidal volume of 8.7 ml/kg and a positive end-expiratory pressure of 2 to 3 cm $H_2O$. The single-compartment model (snap shot) and the constant-phase model (forced oscillation technique) were applied to analyze lung function. Total respiratory system elastance and tissue elastance were measured by the snap shot and forced oscillation techniques, respectively. All data were analyzed using FlexiVent software (version 5.3) (SCIREQ).

**Statistical Analysis.** All values are expressed as the mean ± S.E.M. Two-way analysis of variance followed by the Tukey test or the Student's *t* test for unpaired results was used to evaluate differences between three or more groups or between two groups, respectively. Differences were considered to be significant for values of $P < 0.05$. We repeated the experiments at least two times as independent experiments (see figure legends) and selected one set of representative data to show in the figures. The stated number of test sample is not summation of independent plural experiments but is for only one independent experiment.

## Results

**Effect of PC-SOD on Elastase-Induced Pulmonary Emphysema.** Pulmonary emphysema was induced in mice given a single (at day 0) intratracheal administration of PPE. The PPE-induced pulmonary inflammatory response can be monitored by determining the number of leukocytes (alveolar macrophages, lymphocytes, and neutrophils) in the BALF 3 days after the administration of PPE (50 μg/mouse). As shown in Fig. 1A, the total number of leukocytes and individual numbers of alveolar macrophages, lymphocytes, and neutrophils all were increased by the PPE treatment. This effect was suppressed by the simultaneous once-daily intravenous administration of PC-SOD, suggesting that PC-SOD ameliorates the PPE-induced inflammatory response. However, a higher dose of PC-SOD (30 kU/kg) did not suppress the PPE-induced inflammatory response (Fig. 1A), so in this study PC-SOD exhibited a bell-shaped dose-response profile, similar to that observed previously for intravenous administration of PC-SOD in animal models of other diseases (Ishihara et al., 2009; Tanaka et al., 2010). Intravenous administration of the higher dose (30 kU/kg) of PC-SOD alone (without PPE administration) did not affect the number of leukocytes in the BALF (data not shown).

PPE-induced pulmonary emphysema can be monitored by histopathological analysis and measurement of the mean linear intercept (an indicator of airspace enlargement caused by breakdown of the alveolar walls) 3 days after the administration of PPE. Histopathological analysis of pulmonary tissue using H and E staining revealed that PPE administration induced severe pulmonary damage (infiltration of leukocytes and breakdown of the alveolar walls) and these phenomena were suppressed by the intravenous administration of low doses (1.5 and 3 kU/kg), but not of a high dose (30 kU/kg), of PC-SOD (Fig. 1B). The mean linear intercept was increased by the administration of PPE; this increase was suppressed by intravenous administration of low doses (1.5 and 3 kU/kg) of PC-SOD but was not significantly suppressed at the higher dose (30 kU/kg) (Fig. 1C). Pulmonary tissue damage and the increase in the mean linear intercept 14

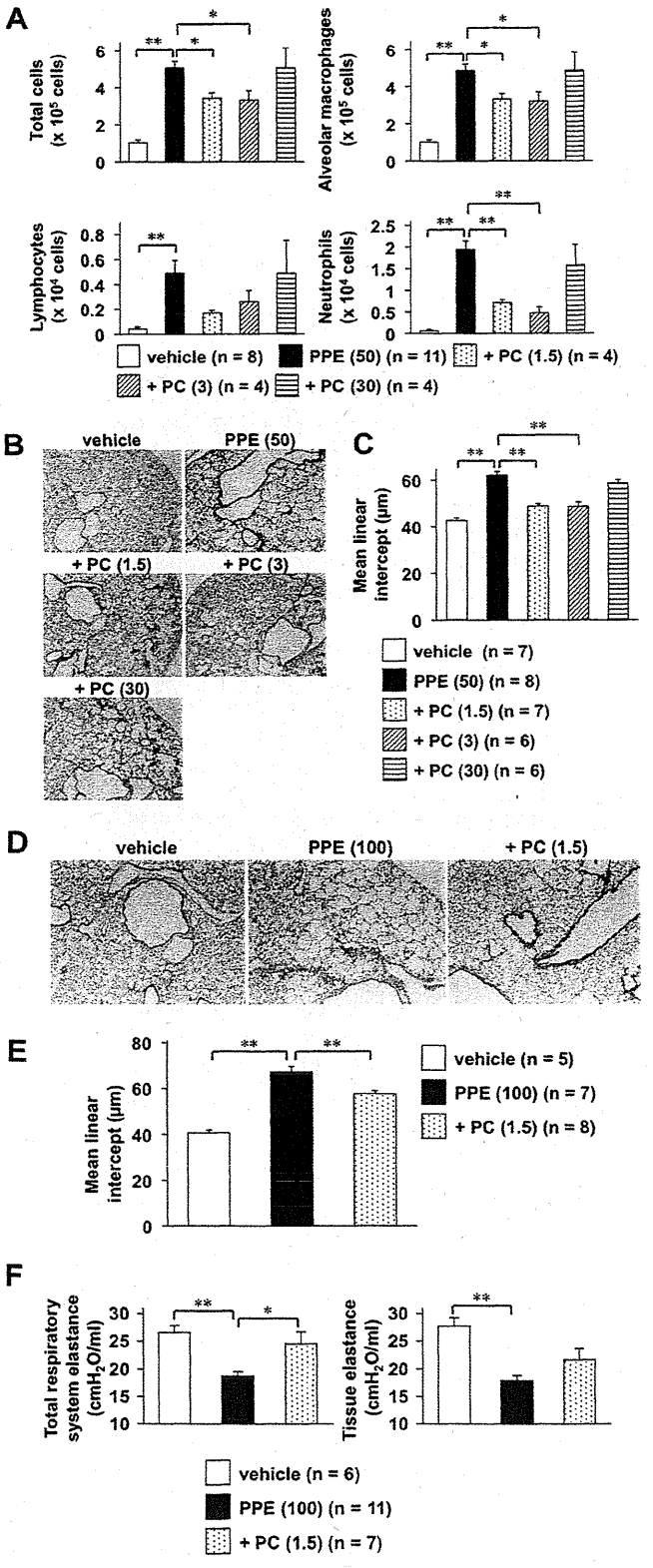


**Fig. 1.** Effect of intravenous administration of PC-SOD on PPE-induced pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 or 100 μg/mouse) once at day 0 were intravenously administered the indicated doses of PC-SOD (1.5, 3, or 30 kU/kg) once daily for 3 days (days 0–2) (A–C) or 14 days (days 0–13) (D–F). A, the total cell number and numbers of alveolar macrophages, lymphocytes, and neutrophils were determined at day 3 as described under *Materials and Methods*. B and D, sections of pulmonary tissue were prepared at days 3 or 14 and subjected to histopathological examination (H and E staining). C and E, airspace size was estimated by determining the mean linear intercept as described under *Materials and Methods*. F, at day 14, total respiratory system elastance and tissue elastance were determined as described under *Materials and Methods*. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments.

days after PPE administration were also suppressed by the intravenous administration of PC-SOD (Fig. 1, D and E). We used higher dose of PPE (100 μg/mice) to monitor pulmonary emphysema 14 days after the administration of PPE.

The alteration in lung mechanics associated with pulmonary emphysema is characterized by a decrease in elastance (Kuraki et al., 2002). We thus examined the effect of intravenous administration of PC-SOD on PPE-induced alterations to lung mechanics, using a computer-controlled small-animal ventilator. Total respiratory system elastance (elastance of total lung including bronchi, bronchiole, and alveoli) and tissue elastance (elastance of alveoli) were reduced by PPE treatment, and intravenous administration of PC-SOD increased these indexes (Fig. 1F). These results suggest that not only PPE-induced pulmonary emphysema but also PPE-induced pulmonary dysfunction is ameliorated by intravenous administration of PC-SOD.

**Effect of Inhalation of PC-SOD on Elastase-Induced Pulmonary Emphysema.** We recently reported that inhalation of PC-SOD ameliorates bleomycin-induced pulmonary fibrosis (Tanaka et al., 2010). This route of administration does not show a bell-shaped dose-response profile (Tanaka et al., 2010) and may result in higher QOL for patients treated with PC-SOD. Thus, here we examined the effect of inhalation of PC-SOD on PPE-induced pulmonary emphysema. Mice were placed in a chamber connected to an ultrasonic nebulizer, thus exposing them to PC-SOD-containing vapor. We confirmed, by high-performance liquid chromatography analysis and measurement of SOD activity, that this treatment did not affect the structure and activity of the PC-SOD (data not shown). Inhalation of PC-SOD-containing vapor was repeated once daily for 3 or 14 days, and the mice were examined for PPE-induced pulmonary disorders. As shown in Fig. 2A, inhaled PC-SOD ameliorated the PPE-induced inflammatory response. This ameliorative effect was observed with not only low doses (30 and 60 kU/chamber) but also a high dose (600 kU/chamber) of PC-SOD, suggesting that the dose-response profile for this administration route is not bell-shaped. PPE-induced emphysematous lung damage and the increase in the mean linear intercept were also suppressed by inhalation of PC-SOD (Fig. 2, B–E), suggesting that inhalation of PC-SOD ameliorates PPE-induced pulmonary emphysema. Again, a bell-shaped dose-response profile was not observed for the ameliorative effect of inhalation of PC-SOD against PPE-induced pulmonary emphysema (Fig. 2, B and C). As shown in Table 1, inhalation of unmodified SOD (600 kU/chamber) did not affect the PPE-induced pulmonary inflammatory response and emphysema. This suggests that lecithinization of SOD potentiates its ameliorative effect against PPE-induced lung disorders, as is the case for dextran sulfate sodium-induced colitis and bleomycin-induced pulmonary fibrosis (Ishihara et al., 2009; Tanaka et al., 2010). We also found that inhalation of PC-SOD sup-

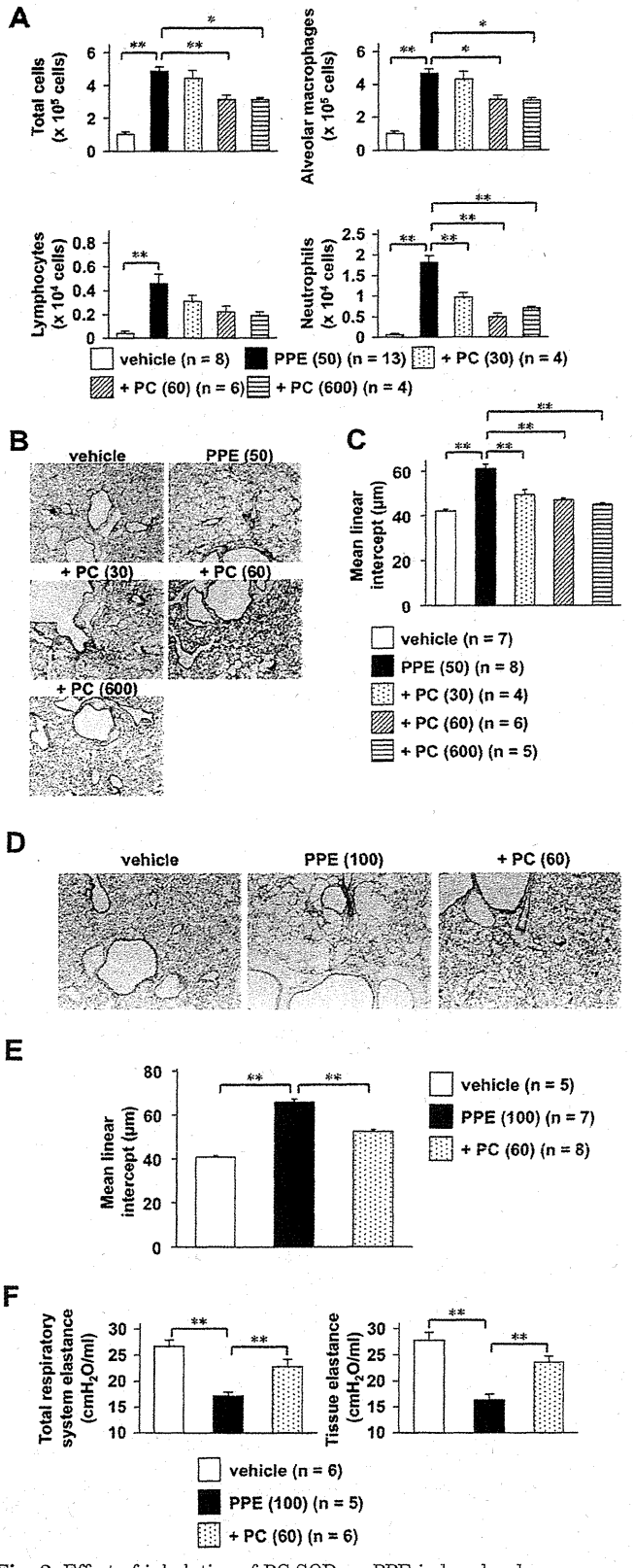


**Fig. 2.** Effect of inhalation of PC-SOD on PPE-induced pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 or 100 μg/mouse) once at day 0 inhaled the indicated doses of PC-SOD (30, 60, or 600 kU/chamber) once daily for 3 days (days 0–2) (A–C) or 14 days (days 0–13) (D–F). Inflammatory response (A), airspace size (B–E), and lung mechanics (F) were assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of three independent experiments.

TABLE 1

Effect of inhalation of unmodified SOD on PPE-induced pulmonary emphysema

Mice were treated with a single dose of PPE (50 μg/mouse) at day 0 and inhaled unmodified SOD (U-SOD; 600 kU/chamber) once daily for 3 days (days 0–2). Inflammatory response and the mean linear intercept were assessed as described in the legend of Fig. 1. Values are mean ± S.E.M.

| | PPE (50) ($n$ = 8) | + U-SOD (600 kU/chamber) ($n$ = 4) |
|---|---|---|
| Total cells, $\times 10^5$ | 4.9 ± 0.33 | 4.9 ± 0.35 |
| Alveolar macrophages, $\times 10^5$ | 4.7 ± 0.36 | 4.7 ± 0.33 |
| Lymphocytes, $\times 10^4$ | 0.40 ± 0.07 | 0.35 ± 0.06 |
| Neutrophils, $\times 10^4$ | 1.6 ± 0.15 | 1.3 ± 0.19 |
| Mean linear intercept, μm | 58.2 ± 1.30 | 57.7 ± 0.37 |

presses PPE-induced decreases in total respiratory system elastance and tissue elastance (Fig. 2F), suggesting that inhalation of PC-SOD ameliorates PPE-induced lung dysfunction. We confirmed that inhalation of PC-SOD alone did not induce pulmonary emphysema and dysfunction (Supplemental Fig. 1).

To consider the clinical relevance, it is important to examine the effect of the drug on predeveloped lesions in an animal model (Fig. 3). Thus, we examined the effect of inhalation of PC-SOD on predeveloped pulmonary emphysema. Once-daily inhalation of PC-SOD was started 3 days after the administration of PPE, and pulmonary emphysema and function were assessed at day 10. Inhalation of PC-SOD caused suppression of pulmonary emphysema at day 10, suggesting that the inhalation of PC-SOD is effective for predeveloped lesions.

The inhalation of PC-SOD also suppressed the PPE-induced alterations in lung mechanics at day 10 (Fig. 3C), suggesting that inhalation of PC-SOD suppresses the PPE-induced lung dysfunction, even when it is administered after the PPE.

**Mechanism for the Ameliorative Effects of PC-SOD on PPE-Induced Pulmonary Emphysema.** To confirm that inhaled PC-SOD decreases the pulmonary level of superoxide anion, we performed an immunohistochemical analysis to monitor the pulmonary level of 8-OHdG, the damaged nucleotide produced by various ROS, including the superoxide anion (Freeman et al., 2009). As shown in Fig. 4A, the pulmonary level of 8-OHdG was significantly increased by PPE administration, and this increase was clearly suppressed by inhalation of PC-SOD, suggesting that production of ROS in the lung was suppressed by inhalation of PC-SOD. We also used ESR analysis to monitor the production of superoxide anion in cells in BALF. The ESR spectrum was consistent with a previously reported DPhPMPO-OOH spectrum (a hyperfine coupling constant of $a^N$ = 1.24 mT, $a^H_\beta$ = 1.16 mT, $a^P$ = 3.95 mT) (Karakawa et al., 2008). As shown in Fig. 4, B and C, the peak of a radical spin adduct of the ESR spectrum corresponding to the amount of superoxide anion (DPhPMPO-OOH adduct) was higher for cells prepared from PPE-administered mice than for cells from control mice. Inhalation of PC-SOD lowered this peak, suggesting that inhaled PC-SOD suppresses PPE-induced production of superoxide anions in the lung.

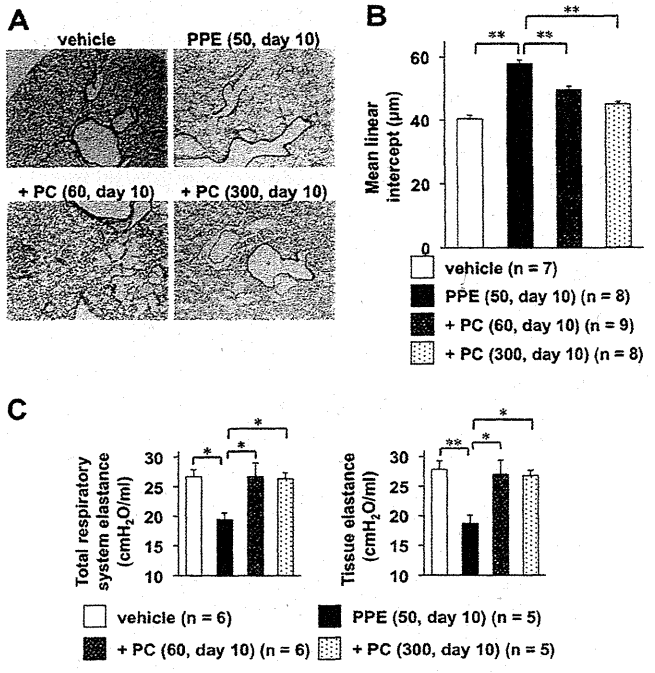


**Fig. 3.** Effect of PC-SOD on predeveloped pulmonary emphysema. Mice treated with (except vehicle) or without (vehicle) PPE (50 μg/mouse) once at day 0 inhaled the indicated doses of PC-SOD (kU/chamber) once daily from days 3 to 9. Airspace size (A and B) and lung mechanics (C) were assessed at day 10 as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments.

As described in Introduction, pulmonary cell apoptosis plays an important role in the pathogenesis of COPD and PPE-induced pulmonary emphysema. We examined the effect of inhalation of PC-SOD on PPE-induced pulmonary cell death by using the TUNEL assay. TUNEL-positive cells (indicative of cell death) increased in response to administration of PPE, and this increase was suppressed by simultaneous inhalation of PC-SOD (Fig. 4, D and E), suggesting that PC-SOD protects pulmonary cells from PPE-induced cell death, and this effect is involved in the ameliorative effects of inhalation of PC-SOD against PPE-induced pulmonary emphysema.

To examine the effect of inhalation of PC-SOD on the PPE-dependent imbalance in proteases and antiproteases, we first examined the activity of MMPs, MMP-2 and MMP-9, using gelatin zymography. The band intensities of MMP-2 and MMP-9, indicative of MMP-2 and MMP-9 activities, were higher for lung tissues prepared from PPE-administered mice than for those from control mice, and this increase was suppressed in mice that had inhaled PC-SOD (Fig. 5, A and B). We also examined the serum level of α1-antitrypsin by ELISA and found that the level of α1-antitrypsin was decreased by PPE administration and partially recovered by simultaneous inhalation of PC-SOD (Fig. 5C). These results suggest that inhalation of PC-SOD improves the PPE-dependent protease/antiprotease imbalance and this effect is involved in the ameliorative effects of inhalation of PC-SOD against PPE-induced pulmonary emphysema.

We also examined the effect of inhalation of PC-SOD on the mRNA expression of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and chemokines (MIP-2, MCP-1, and KC) in

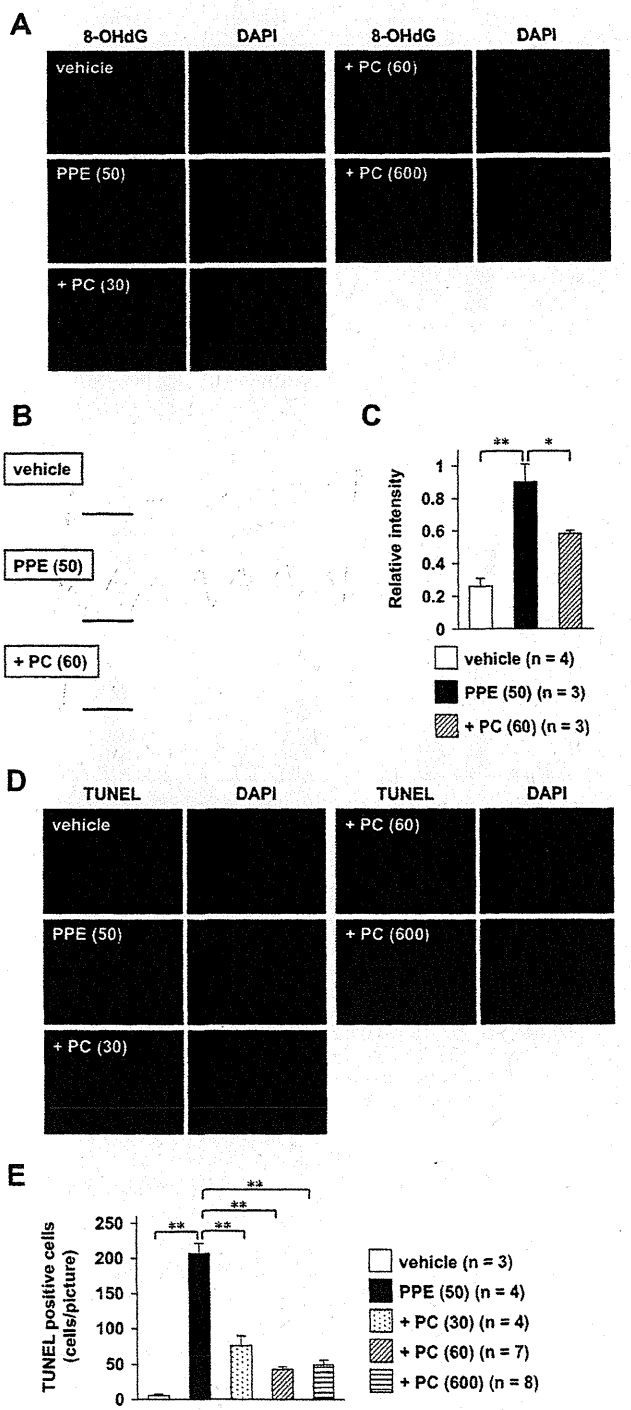


**Fig. 4.** Effect of PC-SOD on the PPE-induced increase in the level of 8-OHdG, production of superoxide anions, and pulmonary cell death. Mice treated with PPE inhaled PC-SOD (kU/chamber) for 3 days (days 0–2) (A, D, and E) or 1 day (day 0) (B and C) as described in the legend of Fig. 2. A, D, and E, sections of pulmonary tissue were prepared at day 3. A, sections were subjected to immunohistochemical analysis with an antibody against 8-OHdG or DAPI staining. B, cells in BALF were collected at day 1, incubated with a spin trap agent (DPhPMPO), and subjected to radical adduct ESR spectrum analysis to determine the amount of superoxide anion present. C, the intensity of the ESR signal of the superoxide anion adduct (DPhPMPO–OOH adduct shown by the bar in B) was determined. D, sections were subjected to TUNEL assay or DAPI staining. E, the number of TUNEL-positive cells was counted. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments.

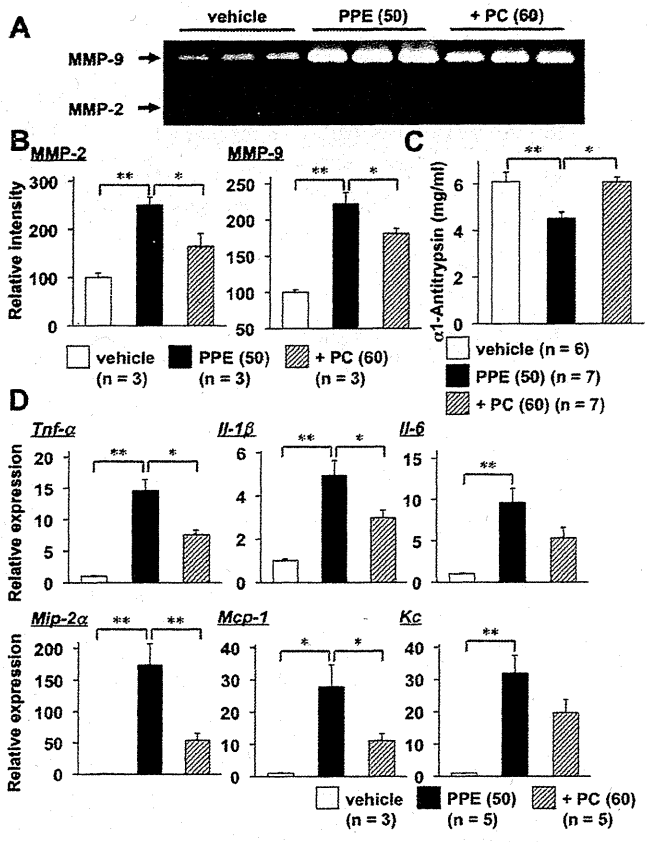


**Fig. 5.** Effect of PC-SOD on PPE-induced MMP activation, decrease in the serum level of α1-antitrypsin, and alteration in gene expression. Mice treated with PPE inhaled PC-SOD (kU/chamber) for 3 days (days 0–2) (A and B) or for 1 day (day 0) (C and D) as described in the legend of Fig. 2. A, pulmonary tissues were obtained at day 3, and MMP-2 and MMP-9 activities were measured as described under *Materials and Methods* . B, the intensity of the bands corresponding to the latent forms of MMP-2 and MMP-9 was determined and expressed relative to the control samples (vehicle). C, the serum level of α1-antitrypsin was determined by ELISA at day 1. Total RNA was extracted and subjected to real-time RT-PCR using a specific primer for each gene. D, values normalized to the *gapdh* gene are expressed relative to the control samples (vehicle). Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments.

lung tissues. The mRNA expression of all of these proinflammatory cytokines and chemokines was induced by PPE administration, and in most cases this induction was suppressed by inhalation of PC-SOD. We also measured the amounts of these proinflammatory cytokines and chemokines in BALF by ELISA and confirmed the data of mRNA expression (Supplemental Fig. 2). These results suggest that inhalation of PC-SOD suppresses PPE-induced expression of proinflammatory cytokines and chemokines in the lung and this effect is involved in the ameliorative effects of PC-SOD inhalation on the PPE-induced pulmonary inflammatory response and resulting emphysema.

**Effect of PC-SOD on CS-Induced Inflammatory Response.** PPE-induced pulmonary emphysema is a convenient and reproducible model of COPD; thus, this model has been used frequently for the evaluation of drugs for COPD. However, it is believed that the CS-induced pulmonary emphysema model is more relevant as an animal model of COPD, because it induces the disease using the same stimulus rather than just replicating one of the mechanisms of the disease. Thus, we examined the effect of PC-SOD on CS-induced pulmonary emphysema. Mice were assessed for a pulmonary inflammatory response at 3 days after exposure to CS. We found that this treatment induced an inflammatory response, as was the case for treatment with elastase (Fig. 6A). As shown in Fig. 6A, intravenously administered PC-SOD ameliorated the CS-induced increase in the total number of leukocytes and individual numbers of alveolar macrophages, lymphocytes, and neutrophils in the BALF, suggesting that intravenous administration of PC-SOD ameliorates CS-induced pulmonary inflammation. As shown in Fig. 6B, inhalation of PC-SOD also ameliorated the CS-induced inflammatory response.

We also examined the effect of PC-SOD on CS-induced pulmonary emphysema and dysfunction. Exposure of mice to CS for 4 weeks caused emphysematous lung damage and the increase in the mean linear intercept and this emphysema was suppressed by simultaneous inhalation of PC-SOD (Supplemental Fig. 3, A and B). We also found that exposure of mice to CS for 4 weeks caused decreases in total respiratory system elastance and tissue elastance, and this decrease was suppressed by simultaneous inhalation of PC-SOD (Supplemental Fig. 3C). These results suggest that inhalation of PC-SOD is effective for the treatment of CS-related pulmonary inflammation, emphysema, and lung dysfunction, including COPD.

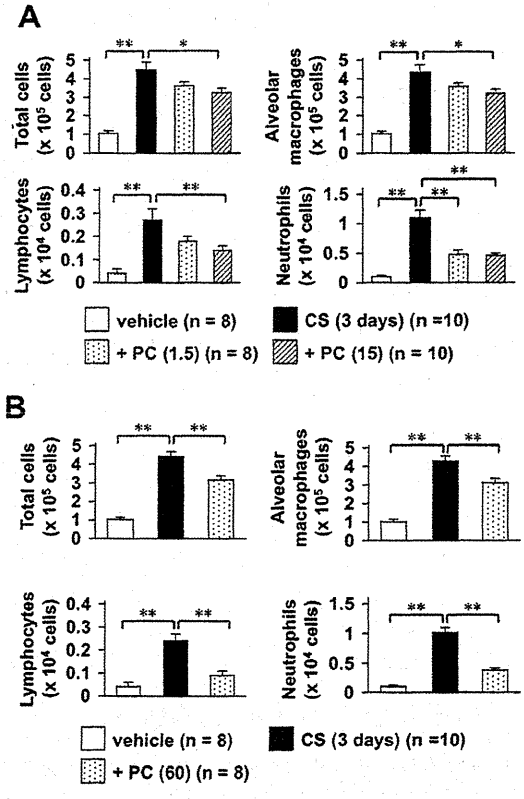


**Fig. 6.** Effect of PC-SOD on CS-induced inflammatory response. Mice were exposed to CS for 3 days as described under *Materials and Methods*. Mice were also intravenously administered PC-SOD (kU/kg) (A) or inhaled with PC-SOD (kU/chamber) (B) as described in the legends of Figs. 1 and 2. Inflammatory response was assessed as described in the legend of Fig. 1. Values are mean ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Data are representative of two independent experiments.

## Discussion

In this study, we used PC-SOD, a derivative of SOD with higher stability in plasma and a higher affinity for tissue, which shows greater therapeutic effects than SOD in animal models of various inflammatory diseases, such as IPF, colitis, focal cerebral ischemic injury, and spinal cord injury-induced motor dysfunction (Hori et al., 1997; Tamagawa et al., 2000; Ishihara et al., 2009; Tanaka et al., 2010). We have clearly shown that PC-SOD ameliorates pulmonary emphysema. This result indicates the therapeutic potential of SOD against COPD-related pulmonary emphysema and is consistent with previous results that show transgenic mice expressing SOD bear a phenotype of resistance to pulmonary emphysema (Foronjy et al., 2006; Petrache et al., 2008). In a phase I clinical study, intravenously administered PC-SOD (40–160 mg) had a terminal half-life of more than 24 h with good safety and tolerability (Broeyer et al., 2008; Suzuki et al., 2008a). Published results of a phase II clinical study have shown that intravenously administered PC-SOD (40 or 80 mg) significantly improves the symptoms of ulcerative colitis patients, which involves ROS (Suzuki et al., 2008b). A phase II clinical study has shown that intravenously administered PC-SOD (40 or 80 mg) is therapeutically effective against IPF as judged by monitoring the serum level of marker proteins (lactate dehydrogenase and surfactant protein-A). Because the safety and efficacy of PC-SOD were shown in not only the animal model but also in clinical studies the application of PC-SOD for COPD is realistic.

Here, we have shown that not only intravenous administration but also inhalation of PC-SOD ameliorates pulmonary emphysema. We believe that inhalation is a clinically more valuable route of administration than the intravenous route for two reasons. First, PC-SOD administered by inhalation does not have a bell-shaped dose-response profile. Bell-shaped dose-response curves are of clinical concern because they may reflect the presence of side effects. The lack of a bell-shaped dose-response profile upon inhalation has also been observed for bleomycin-induced pulmonary fibrosis (Tanaka et al., 2010). Because the efficacy of intravenous administration of higher doses of PC-SOD on bleomycin-induced pulmonary fibrosis was restored by simultaneous administration of catalase, which converts hydrogen peroxide to water and oxygen, the ineffectiveness of high doses of PC-SOD is probably caused by the accumulation of hydrogen peroxide (Tanaka et al., 2010). However, the reason inhalation of PC-SOD does not show the bell-shaped dose-response profile remains unknown. Second, patients treated with PC-SOD administered by inhalation would have a higher QOL than those treated intravenously. Although a phase II clinical study has shown that intravenously administered PC-SOD (40 or 80 mg) is effective for both ulcerative colitis (Suzuki et al., 2008b) and IPF, the main obstacle against proceeding into the next stage of clinical study is the poor QOL for patients undergoing the current clinical protocol of PC-SOD administration (daily intravenous infusion for 4 weeks). Furthermore, in a phase II clinical study for IPF, the plasma levels of markers (lactate dehydrogenase and surfactant protein A) but not forced vital capacity were modified by intravenous administration of PC-SOD, suggesting that a longer period of treatment with PC-SOD is required to improve forced vital capacity in patients with IPF. However, daily intravenous infusion for a longer period is not practical. Therefore, we propose that inhalation of PC-SOD for a longer period may be effective not only for IPF but also for COPD and would maintain the QOL of patients. The therapeutic potential of inhalation of PC-SOD for the treatment of COPD is also supported by observations made in this study: inhalation of PC-SOD ameliorated not only PPE-induced pathological alterations but also PPE-induced functional changes, and inhalation of PC-SOD was effective even for predeveloped pulmonary emphysema (stimulation of spontaneous restoration from pulmonary emphysema and suppression of progression of pulmonary dysfunction). Drugs for COPD should suppress both the inflammatory response and emphysematous lung destruction. Because ROS, especially superoxide anions, are suggested to induce both an inflammatory response and emphysematous lung destruction (Mak, 2008), PC-SOD was predicted to suppress both of these events. In fact, we showed that inhalation of PC-SOD suppresses a PPE-induced increase in leukocytes in BALF and the expression of proinflammatory cytokines and chemokines. We also showed that inhalation of PC-SOD suppresses PPE-induced emphysematous lung destruction. Both apoptosis and protease/antiprotease imbalance seem to be involved in emphysematous lung destruction associated with COPD (Demedts et al., 2006; Rabe et al., 2007; Petrache et al., 2008). We have shown that inhalation of PC-SOD suppresses PPE-induced pulmonary cell death and protease/antiprotease imbalance (activation of MMPs and decrease in the level of $\alpha$1-antitrypsin). We recently reported that PC-SOD protects cultured lung epithelial cells from menadione (a superoxide anion-releasing drug)-induced cell death (Tanaka et al., 2010). It has also been reported that oxidative molecules activate MMPs and suppress the expression of $\alpha$1-antitrypsin (Desrochers and Weiss, 1988; Greenlee et al., 2007; Mak, 2008; Wan et al., 2008). Thus, it seems that a PC-SOD-dependent decrease in the level of superoxide anions is responsible for the inhibitory effect of PC-SOD on PPE-induced pulmonary cell death and the protease/antiprotease imbalance.

One of the current standard clinical protocols for treatment of patients with COPD is administration of a long-acting $\beta_2$-agonist or anticholinergic along with corticosteroid inhalation. This combination regime reduces the annual rate of exacerbation and improves health status and spirometric values, although it does not improve the mortality rate with statistical significance (Calverley et al., 2007). $\beta_2$-Agonists and anticholinergics are effective in improving the airflow limitation associated with COPD (Rabe et al., 2007). On the other hand, some reports have suggested that treatment with corticosteroids does not clearly modulate the inflammatory response in patients with COPD or in a CS-induced pulmonary emphysema animal model (Rabe et al., 2007; Fox and Fitzgerald, 2009). Based on these previous observations and those in this study that inhalation of PC-SOD is effective against the CS-induced inflammatory response, we consider that a combination regime of administration of a long-acting $\beta_2$-agonist (or anticholinergics) along with inhalation of PC-SOD, instead of corticosteroids, may be therapeutically beneficial for patients with COPD.

**Authorship Contributions**

*Participated in research design:* K.-I. Tanaka and Mizushima.
*Conducted experiments:* K.-I. Tanaka, Y. Tanaka, and Miyazaki.

*Contributed new reagents or analytic tools:* Namba, Sato, and Aoshiba.

*Performed data analysis:* K.-I. Tanaka and Sato.

*Wrote or contributed to the writing of the manuscript:* K.-I. Tanaka, Aoshiba, Azuma, and Mizushima.

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