



Acetaminophen-induced differentiation of human breast cancer stem cells and inhibition of tumor xenograft growth in mice

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ABSTRACT

It is now believed that cancer stem cells (CSCs) that are resistant to chemotherapy due to their undifferentiated nature drive tumor growth, metastasis and relapse, so development of drugs that induce differentiation of CSCs should have a profound impact on cancer eradication. In this study, we screened medicines that are already in clinical use for drugs that induce differentiation of CSCs. We used MDA-MB-231, a human breast cancer cell line that contains cancer stem cell-like cells. We found that acetaminophen, an anti-inflammatory, antipyretic and analgesic drug, induces differentiation of MDA-MB-231 cells. Differentiation was assessed by observing alterations in cell shape and expression of differentiated and undifferentiated cell markers, a decrease in cell invasion activity and an increase in susceptibility to anti-tumor drugs. This increased susceptibility seems to involve suppression of expression of multidrug efflux pumps. We also suggest that this induction of differentiation is mediated by inhibition of a Wnt/ β -catenin canonical signaling pathway. Treatment of MDA-MB-231 cells with acetaminophen *in vitro* resulted in the loss of their tumorigenic ability in nude mice. Furthermore, administration of acetaminophen inhibited the growth of tumor xenografts of MDA-MB-231 cells in both the presence and absence of simultaneous administration of doxorubicine, a typical anti-tumor drug for breast cancer. Analysis with various acetaminophen derivatives revealed that *o*-acetamidophenol has a similar differentiation-inducing activity and a similar inhibitory effect on tumor xenograft growth. These results suggest that acetaminophen may be beneficial for breast cancer chemotherapy by inducing the differentiation of CSCs.

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1. Introduction

Despite the monoclonal origins of cancers, they are composed of heterogeneous populations of cells with different proliferative, differentiative and tumorigenic properties [1]. To explain this, the cancer stem cell (CSC) hypothesis is proposed: within a given tumor there is a small population of cells that have the capacity to behave like stem cells; in other words they are able to self-renew and are pluripotent, and thus they give rise to heterogeneous tumor phenotypes [2,3]. The existence of CSCs was first proven in the context of acute myelogenous leukemia and subsequently verified in breast, brain, prostate, colon and pancreatic cancers [4–9]. Such studies have also identified an expression profile of cell surface markers that is characteristic of CSCs in each tissue and organ. For example, it has been reported that breast cancer cells

that express a high level of clusters of differentiation (CD)-44 and a low or undetectable level of CD24 ($CD44^+/CD24^{-/low}$) have CSC properties [5].

Mortality from cancers remains high due to their resistance to chemo- and radiotherapy, metastasis and relapse. It is now believed that CSCs play important roles in these events. For example, $CD44^+/CD24^{-/low}$ breast cancer cells have higher levels of tumorigenic and metastatic activity *in vivo* and higher levels of invasion, migration, proliferation and anchorage-independent colony formation, than relatively differentiated cells ($CD44^{-/low}/CD24^+$) [5,10–12]. CSCs are also resistant to chemo- and radiotherapy [13–17]. Therefore, chemotherapy kills the bulk of tumor cells but is not so effective at killing CSCs, which survive to regenerate new tumors (relapse) after a period of latency [18,19]. Supporting this notion, it has recently been reported that chemotherapy of primary breast cancer patients increases the level of $CD44^+/CD24^{-/low}$ cells in cancer core biopsies [18,20]. It has also been reported that the level of $CD44^+/CD24^{-/low}$ cells in breast tumors correlates with the poor efficacy of chemo- and radiotherapy [16,18]. Therefore, drugs that specifically and effectively kill CSCs would be beneficial for the treatment of cancers and recently such compounds have been reported [18,21,22]. Alternatively,

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drugs that induce differentiation of CSCs may also be therapeutically important because such drugs would convert CSCs to be more susceptible to chemotherapy and less active in metastasis. However, chemicals that induce differentiation of CSCs have not yet been reported.

In order to modulate the stem cell-like properties of CSCs, it is important to understand the molecular mechanisms which maintain these properties. Recent studies suggest that these mechanisms may be common both to CSCs and normal stem cells [23]. In breast CSCs and mammary gland stem cells, various signaling pathways, such as the Wnt/ β -catenin canonical pathway and the transforming growth factor- β (TGF- β) pathway, play important roles in the maintenance of stem cell-like properties [24–28]. In the Wnt/ β -catenin canonical signaling pathway, the binding of Wnt ligands to their receptors inhibits the activity of a multiprotein complex that includes glycogen synthase kinase 3 β (GSK3 β). This complex phosphorylates β -catenin to target it for ubiquitination and proteolysis. Therefore, when Wnt signal transduction is activated, β -catenin accumulates in the cytosol and some part of this protein translocates to the nucleus. In the nucleus, β -catenin binds to T-cell factor/lymphoid enhancing factor 1 (Tcf/Lef1) family proteins to regulate the transcription of specific genes, including those important for the maintenance of stem cell-like properties (such as *snail*) [25,28,29]. On the other hand, aberrant activation of the Wnt/ β -catenin canonical pathway is one of the most frequent signaling abnormalities known in human cancers and it has also been reported that β -catenin is abnormally stabilized in over 50% of breast carcinomas [25]. These data suggests that the Wnt/ β -catenin canonical pathway plays an important role in the maintenance of the stem cell-like properties of breast CSCs. In fact, recently, it has been reported in breast CSCs and mammary gland stem cells that activation or inhibition of the Wnt/ β -catenin canonical pathway has positive or negative effects on maintenance of their properties of self-renewal and pluripotency [26,30,31]. Therefore, compounds that inhibit this pathway may be beneficial for cancer chemotherapy.

Acetaminophen (AAP) is one of the most widely used over-the-counter anti-inflammatory, antipyretic and analgesic drugs available worldwide. The advantage of this drug is that it has less gastrointestinal toxicity than other anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (NSAIDs). On the other hand, the disadvantage of this drug is that it induces hepatotoxicity, causing hepatic centrilobular necrosis [32]. Although discovered more than 100 years ago and used extensively for a long period, the mode of action for its anti-inflammatory, antipyretic and analgesic effects is still unclear. The anti-inflammatory action of NSAIDs is mediated via their inhibitory effect on cyclooxygenase (COX) activity and the synthesis of prostaglandins (PGs), which have a strong capacity to induce inflammation. Although the anti-inflammatory, antipyretic and analgesic actions of AAP resemble those of NSAIDs, in the past it has been believed that this drug acts centrally and is a weak inhibitor of COX [33]; however, this idea is not supported by later studies [34,35]. It was recently suggested that this weak inhibition of COX by AAP is responsible for its anti-inflammatory effect [36]. It seems that COX-inhibition and decreases in the level of PGs play some roles in the anti-inflammatory, antipyretic and analgesic effects of AAP.

In this study, we screened compounds that induce differentiation of CSCs. We used a human breast cancer cell line, MDA-MB-231 cells, which were recently reported to contain mainly (about 80%) CD44⁺/CD24^{-low} cells (cancer stem cell-like cells) [10,11]. Also, it has been reported that the CD44⁺/CD24^{-low} subpopulation of MDA-MB-231 cells has higher levels of growth, anchorage-independent colony formation, adhesion, migration and invasion *in vitro*, and tumorigenicity *in vivo*, than its CD44^{-low}/CD24⁺ subpopulation [10]. As for the chemical library for screening, we

originally prepared a chemical library containing about 250 medicines already in clinical use. We found that AAP induces differentiation of MDA-MB-231 cells through inhibition of the Wnt/ β -catenin canonical signaling pathway. We also found that treatment of MDA-MB-231 cells with AAP *in vitro* resulted in the loss of their tumorigenic ability in nude mice and that administration of AAP inhibited the growth of tumor xenografts of MDA-MB-231 cells both in the presence and absence of the simultaneous administration of doxorubicine. Analysis of various acetaminophen derivatives revealed that *o*-acetamidophenol has similar differentiation-inducing activity and inhibitory effects on tumor xenograft growth, compared to acetaminophen. These results suggest that AAP could be effective for breast cancer therapy through the induction of differentiation of CSCs.

2. Materials and methods

2.1. Chemicals and animals

Dulbecco's modified Eagle's medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal bovine serum (FBS), G418, LY364947, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), crystal violet, BSA and 6-bromindirubin-3'-oxime (BIO) were purchased from Sigma (St. Louis, MO), and lipofectamine (TM2000) and pcDNA3.1(–) were from Invitrogen (Carlsbad, CA). The RNeasy kit was obtained from Qiagen (Valencia, CA), the first-strand cDNA synthesis kit came from TAKARA Bio (Ohtsu, Japan) and iQ SYBR Green Supermix was from Bio-Rad (Hercules, CA). Calcein acet-oxymethyl ester (calcein-AM) and Hepes were from DOJINDO (Kumamoto, Japan). Matrigel was from BD Biosciences (San Jose, CA) and 24-well transwells were from Costar (Lowell, MA). Transaminase C II-test_{Wako}, methanol, paraformaldehyde and streptomycin were from Wako Pure Chemical Industries (Tokyo, Japan). The enzyme immunoassay (EIA) kit for PGE₂ was obtained from Cayman (Ann Arbor, MI). An antibody against claudin-1 was from Zymed (San Francisco, CA) and an antibody against actin was from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate (FITC)-conjugated anti-CD44 (clone G44-26) antibody, phycoerythrin (PE)-conjugated anti-CD24 antibody (clone ML5) and an antibody against β -catenin were obtained from BD Biosciences (San Jose, CA). An antibody against CD24 (clone SN3) was from Lab Vision (Fremont, CA). Alexa Fluor 594 goat anti-mouse IgG was obtained from Invitrogen (Carlsbad, CA). Mounting medium for immunohistochemical analysis (VECTASHIELD) was from Vector Laboratories (Burlingame, CA). Penicillin was from MEIJI SEIKA KAISHA, LTD. (Tokyo, Japan). Female ICR wild-type mice and nude mice (CrJ:CD1-Foxn1^{nu} mice) (4–6 weeks old) were from Charles River (Kanagawa, Japan). 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, and were approved by the Animal Care Committee of Kumamoto University.

2.2. Cell culture and plasmid construction

MDA-MB-231 and MCF-7 (breast cancer cell lines) cells were obtained from ATCC (Manassas, VA). The cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C.

Determination of PGE₂ levels in culture media was done by EIA as previously described [37]. We used a MTT assay for determining viable cell numbers, as described previously [37]. Briefly, cells were incubated for 2 h with MTT solution at a final concentration of 0.5 mg/ml. Isopropanol and hydrochloric acid were added to the

culture medium at final concentrations of 50% and 20 mM, respectively. The optical density of each sample at 570 nm was determined spectrophotometrically using a reference wavelength of 630 nm.

Full-length human cDNA for junctional adhesion molecule A (JAM-A) was prepared by PCR and cloned into pcDNA3.1(–) to create a plasmid for its overexpression. Transfection of MDA-MB-231 cells with the plasmid was carried out using Lipofectamine (TM2000) according to the manufacturer's protocol. The stable transfectants overexpressing JAM-A were selected by real-time RT-PCR analysis. Positive clones were maintained in the presence of 400 µg/ml G418.

2.3. Real-time RT-PCR analysis

Total RNA was extracted using an RNeasy kit according to the manufacturer's protocol. Samples (1 µg RNA) were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions. Synthesized cDNA was used in real-time RT-PCR (Chromo 4 instrument; Bio-Rad, Hercules, CA) experiments using iQ SYBR GREEN Supermix, and analyzed with Opticon Monitor Software according to the manufacturer's instructions. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or reverse transcriptase-free controls. To normalize the amount of total RNA present in each reaction, *actin* cDNA was used as an internal standard. Primer sequences are available upon request.

2.4. Immunoblotting analysis

Whole cell extracts were prepared as described previously [38]. The protein concentrations of the samples were determined by the Bradford method [39]. Samples were applied to 10% polyacrylamide gels containing SDS, subjected to electrophoresis, and the proteins then immunoblotted with each antibody.

2.5. Analysis of expression of cell surface markers by fluorescence activated cell sorting (FACS)

Cells (5×10^5) were incubated with FITC-conjugated anti-CD44 antibody and PE-conjugated anti-CD24 antibody. Samples were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Acquisition of events was stopped at 30,000.

2.6. Calcein-AM accumulation assay

The drug efflux activity in cells was estimated by a calcein-AM accumulation assay as described previously [40], with some modifications. Cells (1×10^6) treated with trypsin were suspended in DMEM containing 2% FBS, 10 mM Hepes and 1 µM calcein-AM and incubated for 10 min at 37 °C. After centrifugation and re-suspension in PBS, the green fluorescence intensity of each sample was measured using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Acquisition of events was stopped at 30,000.

2.7. Cell invasion assay

Cell invasion activity was measured by a transwell matrigel invasion assay as described previously [38], with some modifications. Serum-free DMEM containing 5 mg/ml matrigel was applied to the upper chamber of a 24-well transwell and incubated at 37 °C for 4 h. The cell suspension was applied to the matrigel and the lower chamber was filled with DMEM containing 10% FBS. The plate was incubated at 37 °C for 24 h. Cells were removed from the upper surface of the membrane and the lower surface of the

membrane was stained for 10 min with 0.5% crystal violet in 25% methanol, rinsed with distilled water and air-dried overnight. The crystal violet was then extracted with 0.1 M sodium citrate in 50% ethanol and the absorbance was measured at 585 nm.

2.8. Immunostaining

MDA-MB-231 cells were grown in a Lab-Tek II chamber slide system (Nalge Nunc International, Rochester, NY). Cells were fixed in 1% paraformaldehyde for 20 min and blocked in PBS containing 3% BSA for 30 min. The samples were then incubated with each primary antibody. After washing, samples were incubated with the respective secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR). Images were captured on a confocal laser-scanning fluorescence microscope (Olympus FV500, Olympus, Tokyo, Japan).

2.9. Evaluation of liver injury

Liver injury was evaluated by measuring the catalytic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma by use of a transaminase C II-test_{Wako} according to the manufacturer's instructions.

2.10. Assay for tumor xenograft growth

Cells (1×10^7 , suspension in 0.2 ml of serum-free DMEM) were subcutaneously inoculated into the right hind footpad of each nude mouse. Tumors were measured weekly using calipers and their volumes were calculated using the following standard formula: $\text{width}^2 \times \text{length} \times 0.5$.

2.11. Immunohistochemical analysis

Tumor xenografts were embedded in OCT compound (Sakura Finetech Co., Tokyo, Japan) and cryosectioned. Sections were blocked with 3% goat serum for 15 min, incubated for 12 h with each primary antibody in the presence of 2.5% BSA, and finally incubated for 3 h with Alexa Fluor 594 goat anti-mouse IgG (except for detection of CD44). Samples were mounted with VECTASHIELD and inspected using fluorescence microscopy (Olympus BX51, Olympus, Tokyo, Japan).

2.12. Statistical analysis

All values are expressed as the mean \pm S.D. or S.E.M. Two-way analysis of variance (ANOVA) followed by the Tukey test was used to evaluate differences between more than three groups. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. Identification of AAP as a drug inducing differentiation of MDA-MB-231 cells

At first, from about 250 medicines already in clinical use (supplemental Table S1), we screened for drugs that induce differentiation of MDA-MB-231 cells. It has been reported that undifferentiated (CD44⁺/CD24^{low}) or differentiated (CD44^{low}/CD24⁺) breast cancer cells show a dispersed spindle-shaped mesenchymal cell structure or a cobble-stone-like epithelial monolayer structure, respectively [24,28]. Thus, we searched for drugs that induced morphological change of MDA-MB-231 cells (from a mesenchymal cell structure to a cobble-stone-like structure) after treatment for 4 days and found that AAP induces such morphological change (Fig. 1A). This morphological change was

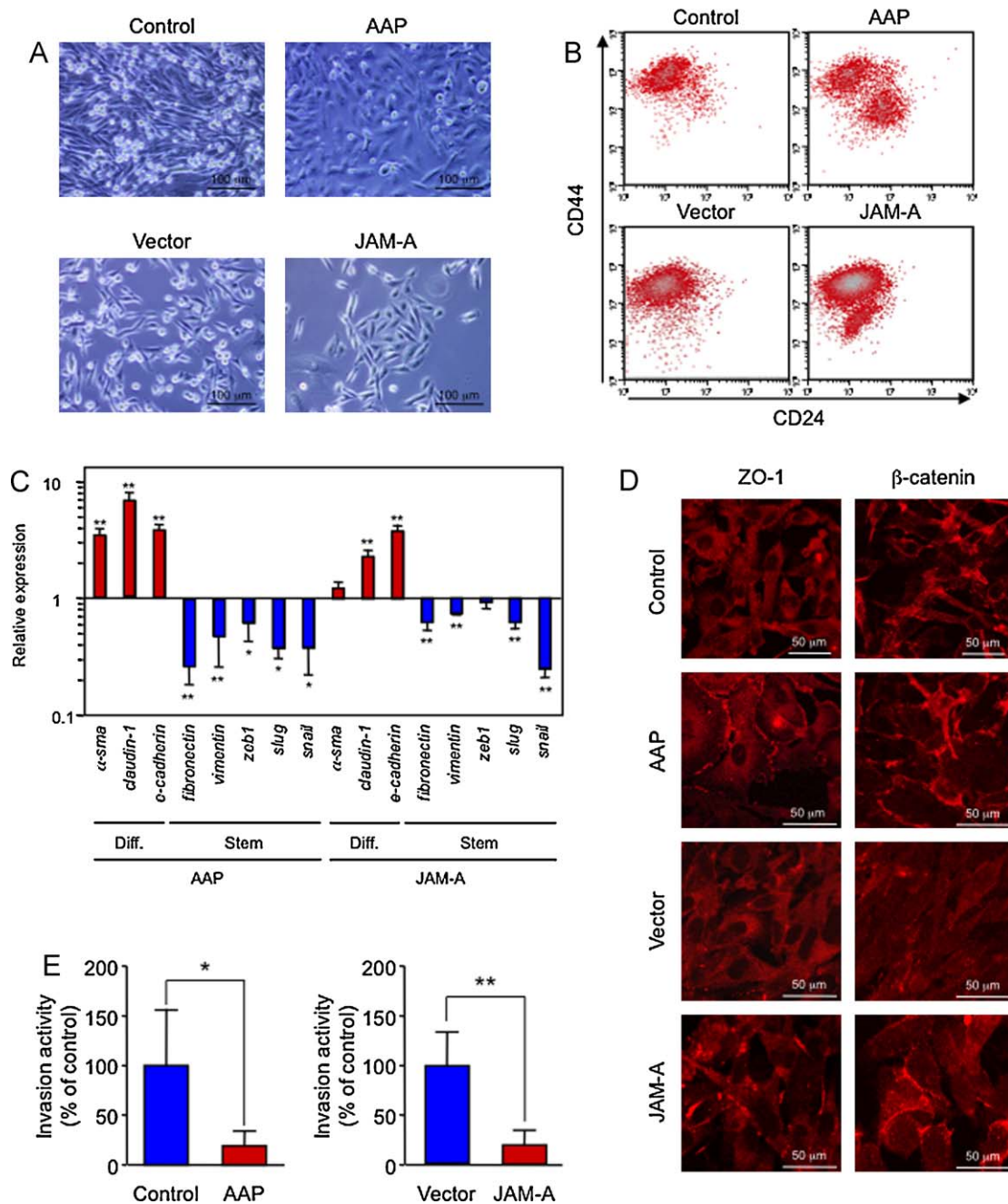


Fig. 1. AAP-induced differentiation of MDA-MB-231 cells. MDA-MB-231 cells treated with (AAP) and without (Control) 1 mM AAP for 4 days or cells stably transfected with the expression plasmid for JAM-A (JAM-A) or empty vector (Vector) were analyzed (A–E). Cell morphology was examined by phase-contrast microscopic observation (A). Cell surface expression of CD24 and CD44 was analyzed by FACS as described in Section 2 (B). Total RNA was extracted and subjected to real-time RT-PCR using a specific primer set for each gene (Diff., markers for differentiated cells; Stem, markers for stem cells). Values were normalized to the *actin* gene, expressed relative to the control (C). Expression of ZO-1 and β -catenin was monitored by an immunostaining assay (D). Cell invasion activity was measured on matrigel-coated transwells as described in Section 2 and is expressed relative to the control (E). Values shown are mean \pm S.D. ($n = 3$ –7). * $P < 0.05$; ** $P < 0.01$.

irreversible: the cell shape was maintained after incubation in AAP-free medium for at least 2 days (data not shown). It has been reported that overexpression of JAM-A, a tight junction protein, in MDA-MB-231 cells induces such morphological change and inhibits cell invasion activity [41], suggesting that overexpression of JAM-A induces differentiation of MDA-MB-231 cells. We confirmed that overexpression of JAM-A induces such morphological changes (Fig. 1A) and we then used JAM-A-overexpressing MDA-MB-231 cells as a positive control in the following experiments. As described above, MDA-MB-231 cells was reported to contain a main subpopulation of CD44⁺/CD24^{-low} cells and a minor subpopulation of CD44^{-low}/CD24⁺ cells [10,11]; we confirmed this by flow

cytometry analysis of the surface expression of CD44 and CD24 (Fig. 1B). Furthermore, we found that either treatment with AAP or overexpression of JAM-A in MDA-MB-231 cells decreases the number of CD44⁺/CD24^{-low} cells and increases the number of CD44^{-low}/CD24⁺ cells (Fig. 1B).

It was reported that the subpopulation of CD44⁺/CD24^{-low}/high aldehyde dehydrogenase (ALDH)⁺ has much high tumorigenicity *in vivo* than CD44⁺/CD24^{-low}/ALDH⁻ [42]. As shown in supplemental Fig. S1, treatment of cells with AAP decreased the number of ALDH⁺ cells.

We also examined the mRNA expression of markers for differentiated cells (α -smooth muscle actin (α -SMA), claudin-1

and E-cadherin) and stem cells (fibronectin, vimentin, zinc finger E-box binding homeobox 1 (ZEB-1), Slug and Snail), referring to data in previous reports [24,28,43]. As shown in Fig. 1C, treatment with AAP and overexpression of JAM-A in MDA-MB-231 cells up-regulated or down-regulated mRNA expression of markers for differentiated cell or stem cell-like cells, respectively. It was recently reported that zonula occludens-1 (ZO-1), which localizes cell–cell contacts in differentiated epithelial cells, localizes broadly in the cytosol of MDA-MB-231 cells, and overexpression of JAM-A causes translocation of the protein to cell–cell contacts [41]. By immunostaining analysis, we found that not only ZO-1 but also β -catenin translocates to the cell–cell contacts by overexpression of JAM-A, and found that a similar translocation is observed in cells treated with AAP (Fig. 1D). Furthermore, the invasion activity of MDA-MB-231 cells, judged by a transwell matrigel invasion assay,

was significantly suppressed by treatment with AAP or overexpression of JAM-A (Fig. 1E). By use of HPLC analysis, we confirmed that $98.9 \pm 3.74\%$ of AAP in the medium was not metabolized or degraded after incubation for 4 days. Thus, the results in Fig. 1 strongly suggest that AAP induces differentiation of MDA-MB-231 cells. The results also suggest that overexpression of JAM-A in MDA-MB-231 cells also induces differentiation of MDA-MB-231 cells.

We then examined the effect of AAP on a differentiated breast cancer cell line, MCF-7 [10]. Without treatment with AAP, MCF-7 cells showed a cobble-stone-like structure and most of them were CD44^{−/low}/CD24⁺ cells (Fig. 2A and B), as described previously [10]. Treatment with AAP did not affect these phenotypes (Fig. 2A and B). Furthermore, the treatment did not affect the mRNA expression of markers for differentiated and stem cells as distinctly (Fig. 2C) as

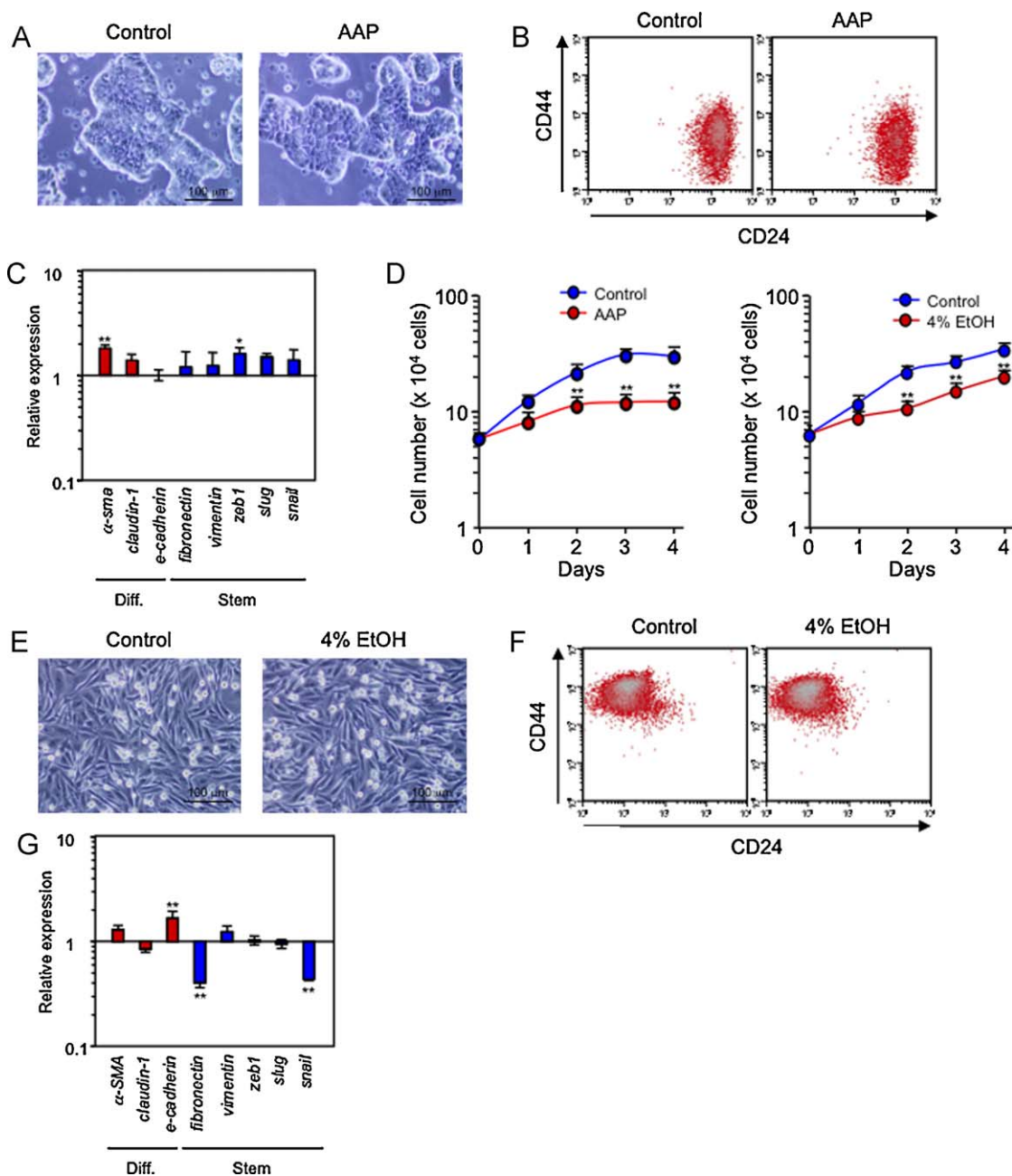


Fig. 2. Specificity of AAP-induced differentiation of MDA-MB-231 cells. MCF-7 (A–C) or MDA-MB-231 (D–G) cells were treated for 4 days with (AAP) or without (Control) 1 mM AAP (A–D) or 4% ethanol (EtOH) (D–G). Cell morphology (A and E), cell surface expression of CD24 and CD44 (B and F) and the mRNA expression (C and G) were examined as described in the legend of Fig. 1. The viable cell numbers were monitored by direct cell counting (D). Values shown are mean \pm S.D. ($n = 3–4$). * $P < 0.05$; ** $P < 0.01$.

was seen for MDA-MB-231 cells (Fig. 1C). These results suggest that AAP affects cell morphology, the expression of surface markers and the expression of differentiation-related genes specifically in undifferentiated (stem cell-like) breast cancer cells, supporting the notion that AAP induces differentiation of MDA-MB-231 cells.

Another characteristic feature of the induction of differentiation of CSCs is the inhibition of cell proliferation, and we showed that treatment with AAP suppressed the growth of MDA-MB-231 cells (Fig. 2D). Treatment of cells with 4% ethanol suppressed the growth of MDA-MB-231 cells similarly (Fig. 2D), however, this treatment did not affect cell morphology, the expression profile of CD44 and CD24, and mRNA expression of differentiation-related genes as distinctly (Fig. 2E–G) as that seen with AAP treatment (Fig. 1A–C). This finding suggests that AAP-dependent alterations of these phenotypes are not the result of cell growth inhibition.

3.2. Molecular mechanism for AAP-induced differentiation of MDA-MB-231 cells

In order to identify a structure–function relationship and molecular mechanism for AAP-induced differentiation of MDA-MB-231 cells, we examined the effects of various AAP derivatives (Fig. 3A) on the differentiation of MDA-MB-231 cells. As shown in Fig. 3B and C, of these AAP derivatives, compound e (*o*-acetamidophenol) increased the ratio of CD44^{low}/CD24⁺ cells to total cells and induced the expression of claudin-1 to a similar extent as AAP, suggesting that *o*-acetamidophenol induces differentiation of MDA-MB-231 cells. Examination of the effect of each AAP derivative on cell growth revealed that not only AAP and *o*-acetamidophenol, but also some other derivatives (such as compound g) inhibit growth (Fig. 3D), confirming the idea that

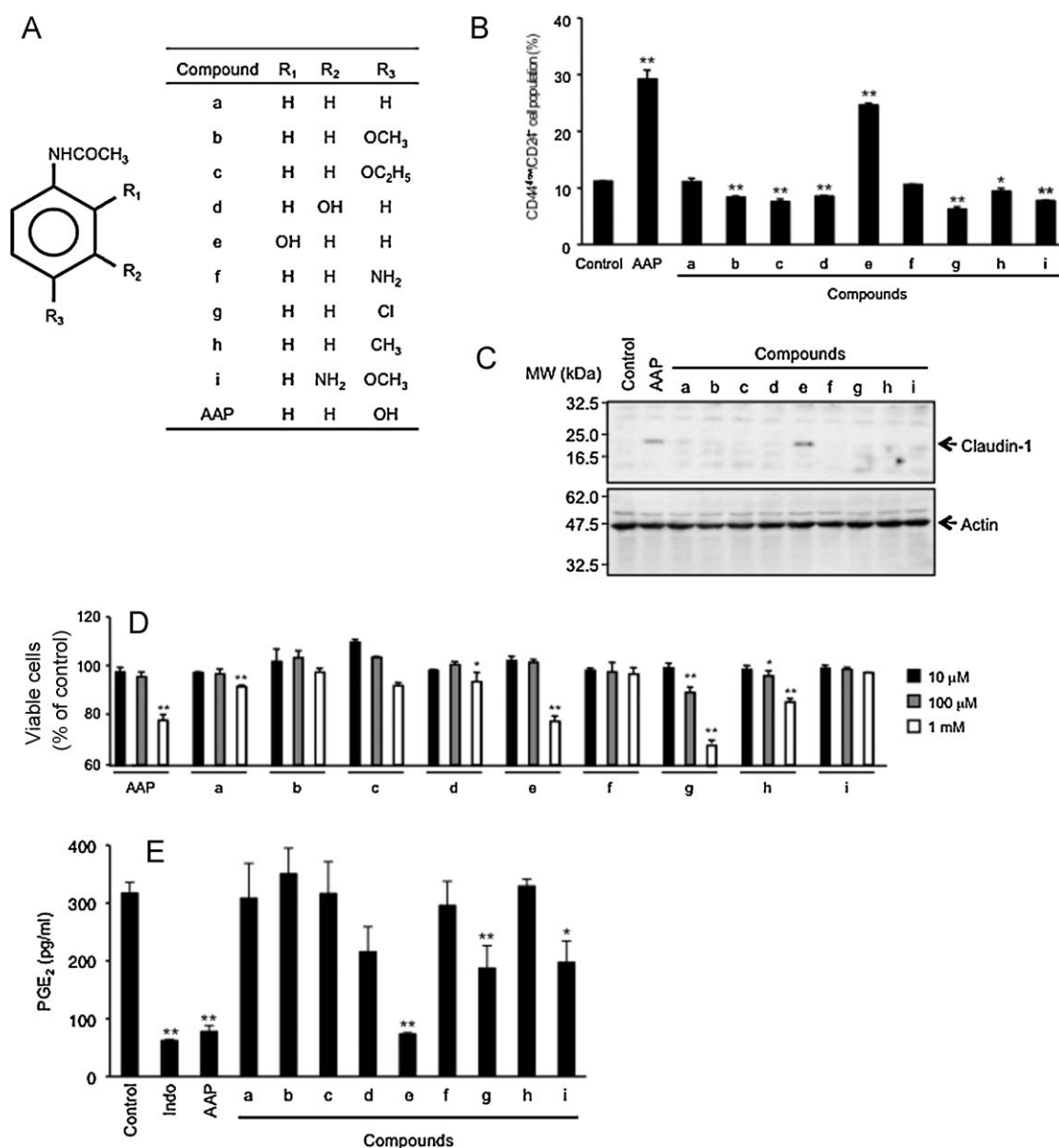


Fig. 3. Structure–function relationship of AAP for induction of differentiation of MDA-MB-231 cells. Chemical structures of AAP and its derivatives (compounds a–i) are shown (A). MDA-MB-231 cells were treated with or without (Control) 1 mM (B, C, E) or the indicated concentrations (D) of AAP and its derivatives or 0.1 mM indomethacin (Indo) (E) for 4 days (B–D) or 4 h (E). Cell surface expression of CD24 and CD44 was examined as described in the legend of Fig. 1 and the percentage of differentiated cells (CD44^{low}/CD24⁺) to total cells was determined (B). Whole cell extracts were analyzed by immunoblotting with an antibody against claudin-1 or actin (C). The viable cell number was determined by the MTT method (D). Cells were further incubated with 50 μM arachidonic acid for 20 min and the amount of PGE₂ in the culture medium was determined by EIA (E). Values are mean ± S.D. (n = 3). *P < 0.05; **P < 0.01.

AAP-dependent alterations of differentiation-related phenotypes do not result from cell growth inhibition.

Using these AAP derivatives, we next examined the relationship between the anti-inflammatory activity and differentiation-inducing activity of AAP. The anti-inflammatory activity of each AAP derivative was estimated by its ability to decrease the level of PGE₂ in the culture medium. As shown in Fig. 3E, treatment of cells with 1 mM AAP decreased the PGE₂ level to a similar extent as 0.1 mM indomethacin. Furthermore, a similar decrease was observed with 1 mM *o*-acetamidophenol but not with other AAP derivatives (Fig. 3E), suggesting a close relationship between AAP's anti-inflammatory and differentiation-inducing activities.

As described in Section 1, various signaling pathways were reported to contribute to the maintenance of the stem cell-like

properties of breast CSCs and mammary gland stem cells [24,25]. Therefore, we tried to identify the signaling pathway involved in maintaining the stem cell-like properties of MDA-MB-231 cells and AAP-dependent induction of differentiation. TGF- β 1 is an important cytokine for maintenance of stem-cell like properties in various CSCs and normal stem cells and it has been reported that inhibition of the TGF- β signaling pathway induces differentiation of some CSCs [24]. Measurement of the expression of *tgf- β 1* mRNA by real-time RT-PCR revealed that expression decreased after treatment of MDA-MB-231 cells with AAP (Fig. 4A). However, since an inhibitor of the TGF- β type I receptor (LY364947) did not affect the stem cell-like morphology of MDA-MB-231 cells (Fig. 4B), it is unlikely that the TGF- β signaling pathway contributes to the maintenance of stem

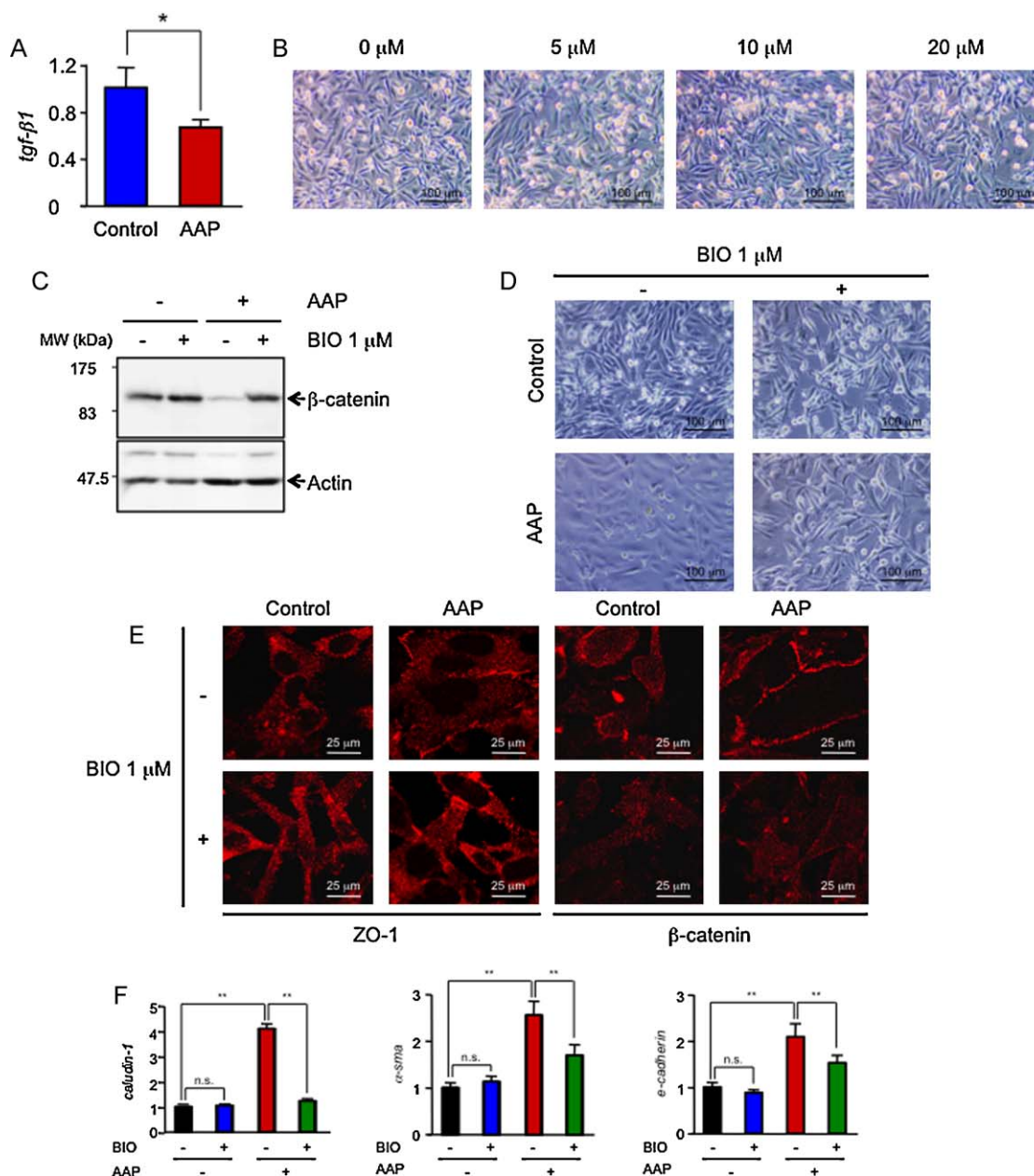


Fig. 4. Involvement of the Wnt/ β -catenin canonical signaling pathway in AAP-induced differentiation of MDA-MB-231 cells. MDA-MB-231 cells were treated for 4 days with (AAP) or without (Control) 1 mM AAP in the presence or absence of 1 μ M BIO (A, C–F). MDA-MB-231 cells were treated for 4 days with the indicated concentrations of LY364947 (B). The mRNA expression of each gene was examined and expressed as described in the legend of Fig. 1 (A and F). Cell morphology and expression of ZO-1 and β -catenin were examined as described in the legend of Fig. 1 (B, D, E). The amount of β -catenin was monitored by immunoblotting as described in the legend of Fig. 3 (C). Values shown are mean \pm S.D. ($n = 3$ –4). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

cell-like properties and AAP-dependent induction of differentiation of MDA-MB-231 cells.

Next, we examined the contribution of the Wnt/ β -catenin canonical signaling pathway. As shown in Fig. 4C, the level of β -catenin decreased clearly in cells treated with AAP and this decrease was suppressed by simultaneous treatment with BIO, a specific inhibitor of GSK3 β [44], suggesting that the Wnt/ β -catenin canonical signaling pathway is inhibited by the treatment with AAP. To examine the contribution of this inhibition to AAP-induced differentiation of MDA-MB-231 cells, we examined the effect of BIO on AAP-dependent alterations of phenotypes related to differentiation. Simultaneous treatment of cells with BIO suppressed AAP-dependent morphological change, translocation of ZO-1 and β -catenin to cell–cell contacts and up-regulation of mRNA expression of markers for differentiated cells (Fig. 4D–F). These results suggest that AAP induces differentiation of MDA-MB-231 cells through inhibition of the Wnt/ β -catenin canonical signaling pathway.

3.3. An AAP-induced increase in susceptibility of MDA-MB-231 cells to anti-cancer drugs in vitro

High expression of ATP-binding cassette (ABC) transporters, such as multidrug resistance-associated proteins (MRPs) and

multidrug resistance-1 (MDR1), which efflux intracellular anti-cancer drugs has been observed in various CSCs, and is responsible for their phenotypic resistance to chemotherapy [14,15,45]. Overexpression of MRPs (but not MDR1) in MDA-MB-231 cells and its contribution to multidrug resistance of the cells has been reported [46]. Therefore, the results described above suggest that treatment of MDA-MB-231 cells with AAP makes them more susceptible to anti-cancer drugs. In fact, pre-treatment of MDA-MB-231 cells with AAP made them more susceptible to doxorubicin or 5-fluorouracil (5-FU) (Fig. 5A). We also found that overexpression of JAM-A made MDA-MB-231 cells more susceptible to these anti-cancer drugs (Fig. 5B). On the other hand, pre-treatment of MCF-7 cells with AAP did not affect their sensitivity to these anti-cancer drugs (Fig. 5C), suggesting that AAP makes MDA-MB-231 cells more susceptible to anti-cancer drugs through induction of differentiation. We next examined the effect of treatment of MDA-MB-231 cells with AAP on the drug efflux activity by the calcein-AM accumulation assay. Due to its hydrophobicity calcein-AM is incorporated non-specifically into cells through cytoplasmic membranes and is then converted to a fluorescent molecule, calcein, in cells. Thus, an increase in the level of calcein in cells reflects a reduction in the drug efflux activity of cells [47]. As shown in Fig. 5D, a higher level of accumulation of

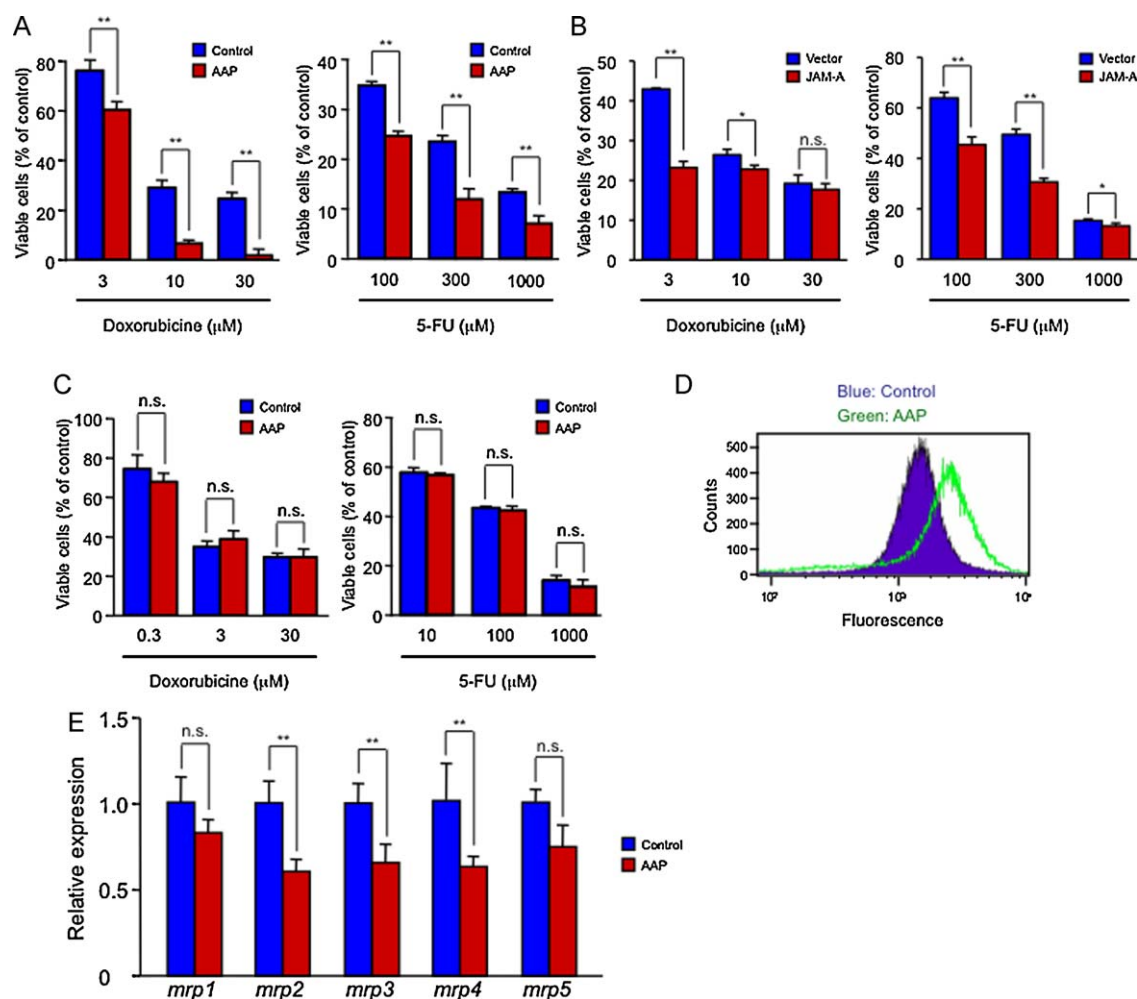


Fig. 5. AAP-induced increase in susceptibility of MDA-MB-231 cells to anti-cancer drugs. MDA-MB-231 (A, D, E) or MCF-7 (C) cells were treated with (AAP) or without (Control) 1 mM AAP for 4 days. MDA-MB-231 cells stably transfected with the expression plasmid for JAM-A (JAM-A) or vector (Vector) were cultured for 4 days (B). After collecting cells and re-plating in 24-well plates (6×10^4 cells for doxorubicine or 3×10^4 cells for 5-FU), cells were further incubated with the indicated concentrations of doxorubicine for 3 days or 5-FU for 5 days in the absence of AAP and cell viability was determined by MTT method (A–C). Cells were incubated with calcein-AM and the amounts of calcein in cells were monitored by FACS analysis as described in Section 2 (D). The mRNA expression of each gene was examined and expressed as described in the legend of Fig. 1 (E). Values shown are mean \pm S.D. ($n = 3$ –4). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

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-231 cells.

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

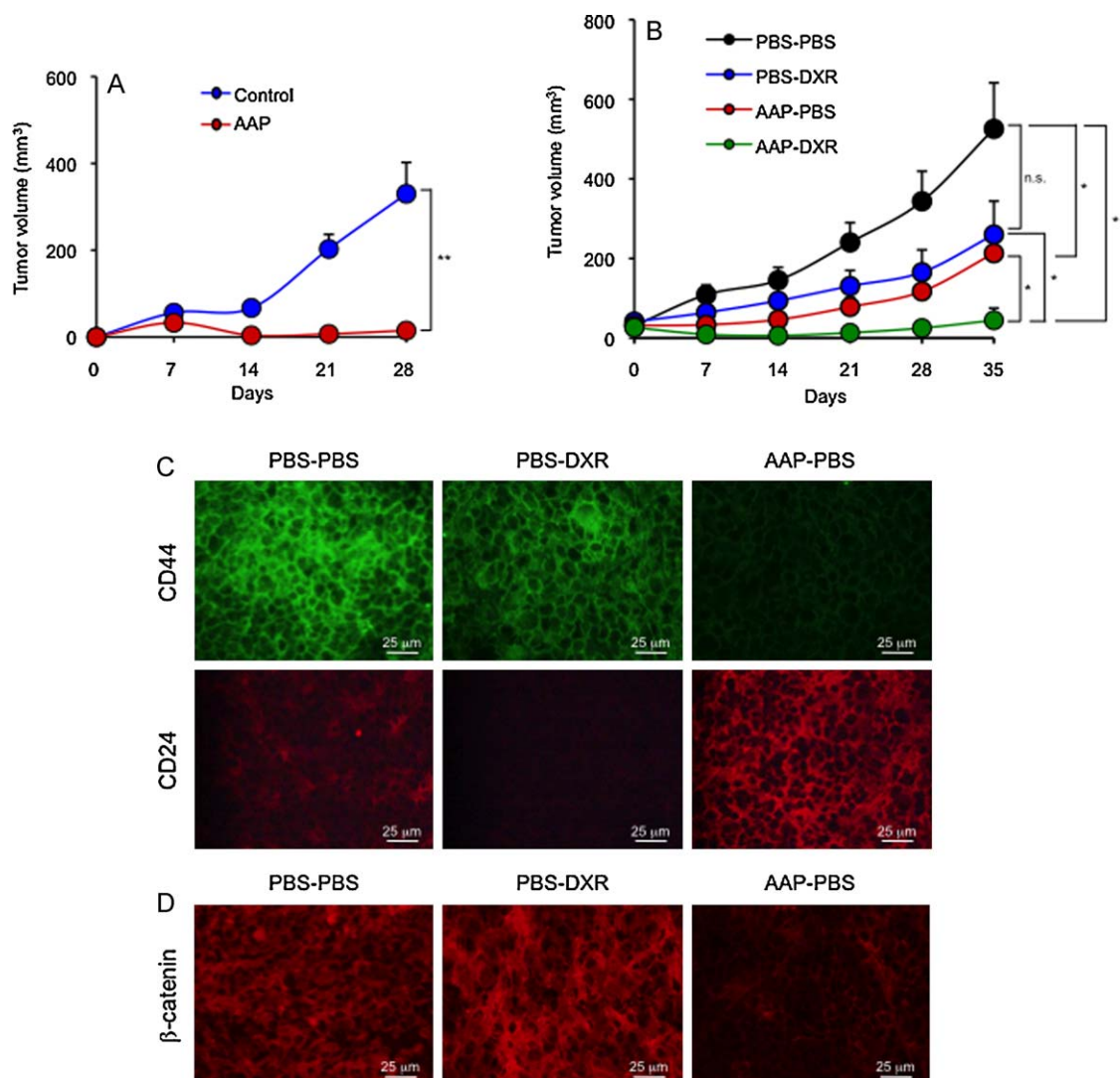


Fig. 6. Effect of AAP on the growth of tumor xenografts in nude mice. MDA-MB-231 cells treated with (AAP) and without (Control) 1 mM AAP for 4 days were inoculated subcutaneously into the right hind footpad of each nude mouse (1×10^7 cells/mouse) at day 0 (A). MDA-MB-231 cells were inoculated subcutaneously into the right hind footpad of each nude mouse (1×10^7 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 doxorubicine (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 \pm S.E.M. ($n = 6-8$). * $P < 0.05$; ** $P < 0.01$; n.s., not significant.

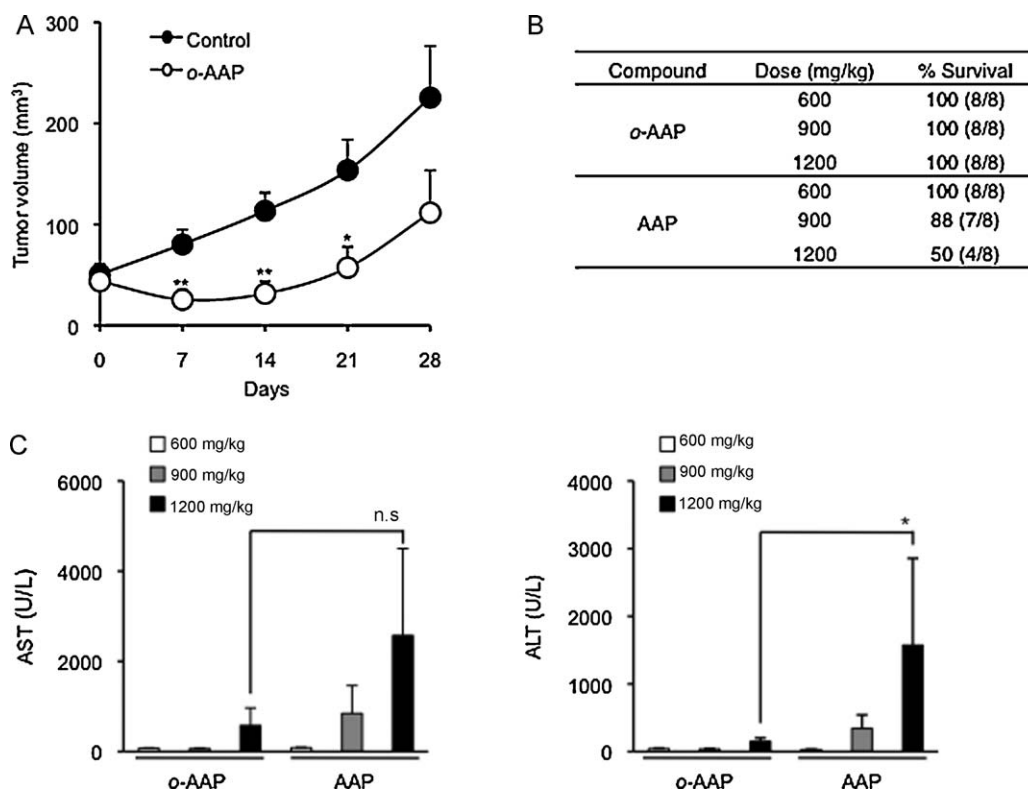


Fig. 7. Hepatotoxicity and anti-cancer effect of *o*-acetamidophenol. MDA-MB-231 cells were inoculated subcutaneously into the right hind footpad of each nude mouse (1×10^7 cells/mouse). After 2 weeks (day 0), daily oral administration of *o*-acetamidophenol (*o*-AAP) or methylcellulose (Control) were 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 \pm 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-epithelial-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 GSK3 β , 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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