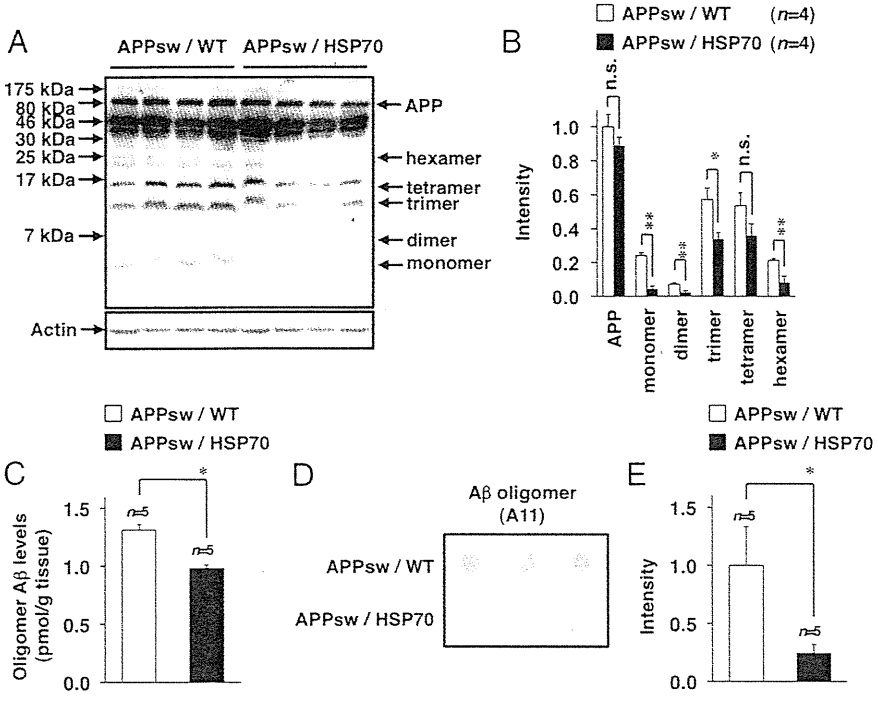


Figure 5. Effects of HSP70 overexpression on the levels of Aβ oligomers in the brains of transgenic mice expressing APPsw. ***A–E***, Whole-cell extracts (***A***, ***B***) or soluble fractions (***C–E***) were prepared from the brains of 12-month-old APPsw/WT and APPsw/HSP70 mice, and then subjected to immunoblotting with an antibody to Aβ (6E10) (***A***), ELISA for Aβ oligomer [APPsw/WT (n = 5) and APPsw/HSP70 mice (n = 5)] (***C***), or dot-blotting assay with an antibody against Aβ oligomer (A11) (***D***). The band intensity of APP and each form of Aβ was determined, corrected with that of actin, and expressed relative to the control [APPsw/WT (n = 4) and APPsw/HSP70 mice (n = 4)] (***B***). The dot intensity was determined and expressed relative to the control (APP in APPsw/WT mice) [APPsw/WT (n = 5) and APPsw/HSP70 mice (n = 5)] (***E***). Values are given as mean ± SEM (***B***, ***C***, ***E***). $^{**}p < 0.01$; $^{*}p < 0.05$. n.s., Not significant.

It has been suggested that inflammation stimulates the progression of the pathogenesis of AD (Wyss-Coray, 2006). However, inflammation also activates the phagocytotic activity of microglia and astrocytes, resulting in stimulation of Aβ clearance (Wyss-Coray et al., 2001). Thus, the relationship between inflammation and progression of AD is complicated, with some proinflammatory cytokines (such as TNF-α) being suggested to promote the progression of AD, whereas others (such as IL-1β and IL-6) have a negative effect in mouse models of the disease (Tesseur et al., 2006; He et al., 2007; Hoshino et al., 2007b; Shaftel et al., 2007). TGF-β1, a key cytokine regulating the response of the brain to injury and inflammation, has also been suggested to suppress the progression of AD. Therefore, we then examined the expression of cytokines that have been suggested to affect the phagocytosis of Aβ (IL-1β, IL-6, TNF-α, and TGF-β1) (Wyss-Coray et al., 2001; Tesseur et al., 2006; He et al., 2007; Hoshino et al., 2007b; Shaftel et al., 2007). As shown in Figure 7*A*, the mRNA expression of *tgf-β1* in the brain was higher in the APPsw/HSP70 mice than in APPsw/WT animals. However, no differences were observed in the mRNA expression of the other cytokines (Fig. 7*A*). Similar results were obtained at the protein level, as judged by ELISA (Fig. 7*B*). The increased mRNA expression of *tgf-β1* and protein level of TGF-β1 by overexpression of HSP70 was also observed in wild-type mice (without expression of APPsw) (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).

We also examined the effect of overexpression of HSP70 on the mRNA expression of *tgf-β1* in primary cultures of neurons, astrocytes, and microglia. As shown in Figure 7*C*, *tgf-β1* mRNA expression was higher in the neuron and astrocyte cultures prepared from APPsw/HSP70 mice than in those from APPsw/WT animals. However, no such difference was observed in the microglial cultures (Fig. 7*C*). Together, these findings suggest that the higher expression of *tgf-β1* is responsible for the lower level of Aβ seen in the brains of APPsw/HSP70 mice, compared with their APPsw/WT counterparts.

Finally, we examined the effect of overexpression of HSP70 on microglial activation by immunohistochemical analysis with an antibody against Iba1 (a marker for activated microglia). As shown in supplemental Figure 5 (available at www.jneurosci.org as supplemental material), the level of Iba1 expression was higher in APPsw/HSP70 mice than APPsw/WT, suggesting that expression of HSP70 activates microglia in APP23 mice.

Discussion

HSPs have attracted considerable attention as AD drug targets because of their unique properties as molecular chaperones (i.e., their ability to unfold and refold abnormal proteins). Other activities of HSPs *in vitro*, such as cytoprotection against Aβ neurotoxicity, suppression of inflammation, and stimulation of Aβ phagocytosis, have increased this attention. Furthermore, the ameliorative effect of HSPs has also been suggested in the case of other neurodegenerative conditions, such as polyglutamine diseases and Parkinson's disease (Adachi et al., 2003; Katsuno et al., 2005; Lo Bianco et al., 2008). Despite this, no *in vivo* evidence exists to support the idea that HSPs are protective in vertebrate AD models. Therefore, in this study, we tested this idea using transgenic mouse strains expressing APPsw and HSP70.

Because AD results in cognitive impairment, it is important to find endogenous factors that affect not only the pathological but also the functional (cognitive) phenotypes in the animal models. This notion is supported by previous reports that some endogenous factors ameliorate cognitive dysfunction in AD model mice without affecting the pathological phenotypes (such as Aβ plaque deposition) (Roberson et al., 2007; Kanninen et al., 2009). In the present study, we found that APPsw/HSP70 mice display a higher level of cognitive function (spatial learning and memory) than APPsw/WT mice, suggesting that overexpression of HSP70 ameliorates the deficits in spatial learning and memory caused by expression of APPsw. Furthermore, we demonstrated that no significant differences in spatial learning and memory exist between WT/WT and WT/HSP70 mice, suggesting that overexpression of HSP70 does not affect the cognitive ability of wild-type mice.

We also found that there are lower levels of Aβ in both the soluble and insoluble fractions of APPsw/HSP70 brains than in those prepared from APPsw/WT mice, suggesting that overexpression of HSP70 suppresses production of Aβ. However, no such differences were observed in the levels of CTFα and CTFβ, indicators of proteolysis by α- or β-secretase, respectively. Furthermore, the activities of β- and γ-secretase in the brain were also indistinguishable between the two strains of mice. These *in vivo* re-

sults suggest that overexpression of HSP70 does not affect the production of Aβ. In fact, we showed that the level of Aβ in the conditioned medium was similar between HSP70-overexpressing neurons and control neurons. Inflammatory factors, such as PGE_2 (prostaglandin E_2) and proinflammatory cytokines, are known to enhance the production of Aβ (Wyss-Coray, 2006; Hoshino et al., 2007b, 2009), and the antiinflammatory activity of HSP70 has recently been reported (Chan et al., 2004; Sun et al., 2005; Tang et al., 2007). However, since expression of proinflammatory cytokines was not affected by overexpression of HSP70 either *in vivo* or *in vitro*, the antiinflammatory activity of HSP70 does not appear to contribute to the ameliorative effect of HSP70 in AD model mice.

As described in Introduction, Aβ is cleared from the brain through enzyme-mediated degradation, phagocytosis by microglia and astrocytes, and transport into the blood and lymph nodes (Miners et al., 2008; Zlokovic, 2008; Rodríguez et al., 2009). The inability of HSP70 to affect the production of Aβ suggests that the clearance of Aβ is enhanced in transgenic mice expressing HSP70. We found that the expression of IDE, an Aβ-degrading enzyme, is enhanced by overexpression of HSP70 both *in vitro* and *in vivo*. We also demonstrated that the expression of TGF-β1 is increased by overexpression of HSP70. Given that it has been reported that TGF-β1 stimulates Aβ clearance through activation of phagocytic microglia (Wyss-Coray et al., 2001) and we here suggested that overexpression of HSP70 activates microglia, we consider that the lower level of Aβ observed in the brains of transgenic mice expressing HSP70 is attributable to the stimulation of Aβ clearance through upregulation of expression of IDE and TGF-β1. In terms of the mechanism underpinning the upregulation of TGF-β1 expression because of overexpression of HSP70, we consider a possibility that extracellular HSP70 is responsible, as it has previously been reported that extracellular HSP70 stimulates the expression of TGF-β1 (Kimura et al., 1998).

However, there is no direct evidence to show that lower levels of Aβ in mice with overexpression of HSP70 is mediated by Aβ-degrading enzyme and TGF-β1, and thus another mechanism may be involved in the phenomenon. For example, Hsp70 might increase the degradation of Aβ by stimulating autophagy or Hsp70 might directly modulate the activity of microglia to degrade Aβ.

The current findings show that the level of Aβ plaque deposition in the brain

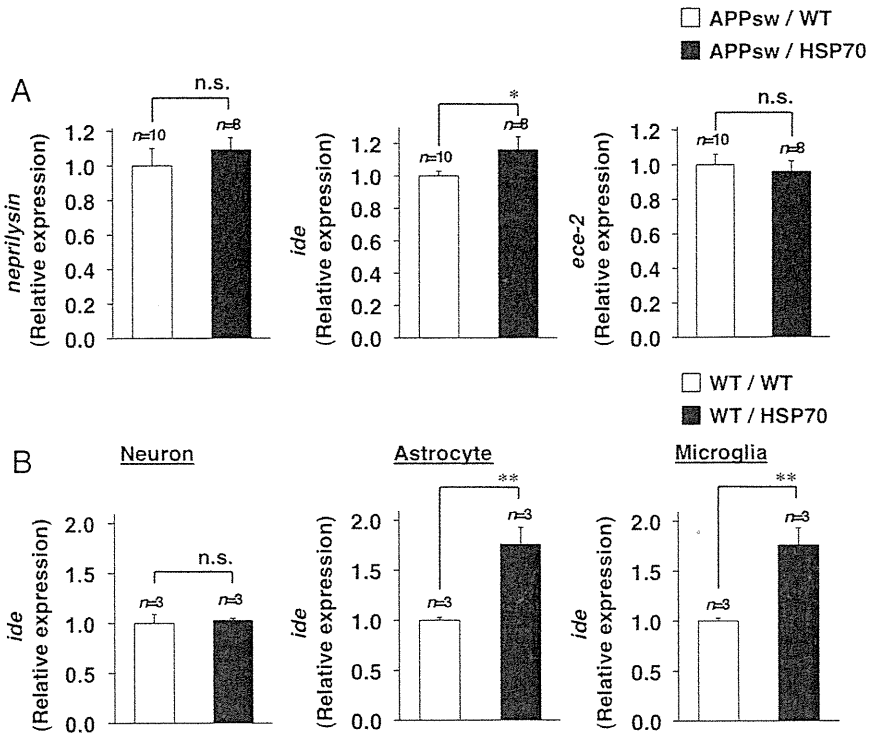


Figure 6. Effects of HSP70 overexpression on the expression of Aβ-degrading enzymes. ***A***, ***B***, Total RNA was extracted from the brains of 12-month-old APPsw/WT ($n = 10$) and APPsw/HSP70 mice ($n = 8$) (***A***) or from primary cultures of neurons, astrocytes and microglia prepared from WT/WT ($n = 3$) and WT/HSP70 mice ($n = 3$) (***B***). RNA samples were subjected to real-time RT-PCR using a specific primer for each gene. Values were normalized to *gapdh* gene expression and expressed relative to the control sample. Values are given as mean ± SEM (***A***) or SD (***B***). $^{**}p < 0.01$; $^{*}p < 0.05$; n.s., not significant.

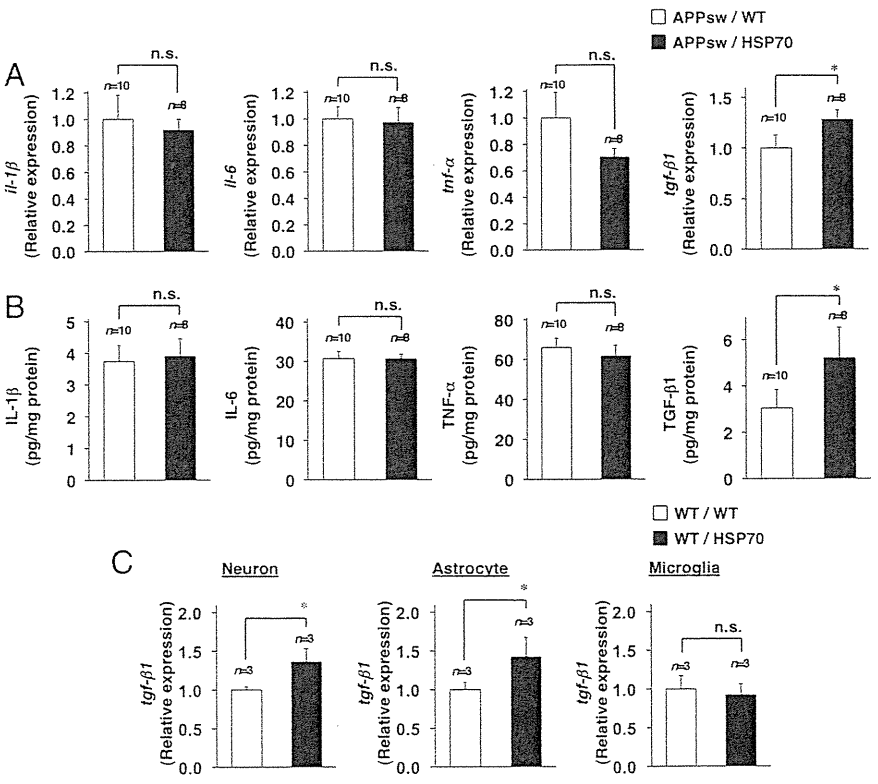


Figure 7. Effects of HSP70 overexpression on cytokine expression. ***A***, ***B***, Total RNA (***A***) or whole-cell extracts (***B***) were prepared from the brains of 12-month-old APPsw/WT ($n = 10$) and APPsw/HSP70 mice ($n = 8$). ***C***, Total RNA was also extracted from primary culture of neurons, astrocytes, and microglia prepared from WT/WT ($n = 3$) and WT/HSP70 mice ($n = 3$). RNA samples were subjected to real-time RT-PCR as described in the legend of Figure 6 (***A***, ***C***). The amounts of cytokine in the whole-cell extracts were determined by ELISA (***B***). Values are given as mean ± SEM (***A***, ***B***) or SD (***C***). $^{*}p < 0.05$. n.s., Not significant.

is reduced in APPsw/HSP70 mice compared with APPsw/WT animals. We also found that the level of Aβ oligomers is lower in the APPsw/HSP70 mouse brain. One explanation of these phenotypes is the lower level of monomeric Aβ in the APPsw/HSP70 mice than in the APPsw/WT animals. However, we believe that the antiaggregation activity of HSP70 for Aβ that was suggested by previous *in vitro* studies (Muchowski and Wacker, 2005; Evans et al., 2006; Kumar et al., 2007; Yoshiike et al., 2008) is involved in these phenotypes. We also found that APPsw/HSP70 mice have less neuronal and synaptic loss than their APPsw/WT counterparts. Again, the phenotype can be explained by the lower level of Aβ. However, we consider that the cytoprotective activity of HSP70 against Aβ-induced neuronal apoptosis that was suggested by previous *in vitro* studies (Magrané et al., 2004) is involved in the phenotype.

As outlined in Introduction, the beneficial effects of HSP70 in animal models of various diseases suggest the potential therapeutic benefit of HSP70 inducers for these conditions (Tanaka et al., 2007, 2010; Asano et al., 2009; Suemasu et al., 2009; Matsuda et al., 2010; Yamashita et al., 2010), a possibility that can now be expanded to include AD based on the results of the present study. A number of recent studies have revealed new molecules that induce HSPs (Kieran et al., 2004; Westerheide et al., 2004; Yan et al., 2004). However, the development of new candidate drugs requires them to pass through the clinical trials process, with the prospect of encountering side effects. In light of this, we have focused our attention on geranylgeranylacetone (GGA). GGA, a leading antiulcer drug on the Japanese market, has been reported to be a nontoxic HSP inducer (Hirakawa et al., 1996). GGA has also been shown to upregulate the expression of HSP70 in various tissues, including the stomach, intestine, liver, heart, eye, ear, skin, lung, and brain (Hirakawa et al., 1996; Ooie et al., 2001; Katsuno et al., 2005; Asano et al., 2009). We and another group have previously reported that GGA suppresses not only gastric lesions but also lesions of the small intestine and inflammatory bowel disease-related colitis (Ohkawara et al., 2005; Asano et al., 2009; Suemasu et al., 2009). Furthermore, we have demonstrated that GGA ameliorates the phenotype in an animal model of spinal and bulbar muscular atrophy (a polyglutamine disease) by suppressing the aggregation of pathogenic proteins (Katsuno et al., 2005). Compared with new molecules that induce HSPs, GGA has an advantage, given that its safety has already been clinically demonstrated. We therefore consider that animal and clinical studies should be performed to prove the effectiveness of GGA for preventing and treating AD.

References

Adachi H, Katsuno M, Minamiyama M, Sang C, Pagoulatos G, Angelidis C, Kusakabe M, Yoshiki A, Kobayashi Y, Doyu M, Sobue G (2003) Heat shock protein 70 chaperone overexpression ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse model by reducing nuclear-localized mutant androgen receptor protein. J Neurosci 23:2203–2211.

Asano T, Tanaka K, Yamakawa N, Adachi H, Sobue G, Goto H, Takeuchi K, Mizushima T (2009) HSP70 confers protection against indomethacin-induced lesions of the small intestine. J Pharmacol Exp Ther 330:458–467.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Chan JY, Ou CC, Wang LL, Chan SH (2004) Heat shock protein 70 confers cardiovascular protection during endotoxemia via inhibition of nuclear factor-kappaB activation and inducible nitric oxide synthase expression in the rostral ventrolateral medulla. Circulation 110:3560–3566.

Evans CG, Wisén S, Gestwicki JE (2006) Heat shock proteins 70 and 90 inhibit early stages of amyloid beta-(1–42) aggregation in vitro. J Biol Chem 281:33182–33191.

Farmery MR, Tjernberg LO, Pursglove SE, Bergman A, Winblad B, Näslund J (2003) Partial purification and characterization of gamma-secretase from post-mortem human brain. J Biol Chem 278:24277–24284.

Fukumoto H, Cheung BS, Hyman BT, Irizarry MC (2002) Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease. Arch Neurol 59:1381–1389.

Haass C, Selkoe DJ (2007) Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol 8:101–112.

Hardy J, Selkoe DJ (2002) The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297:353–356.

He P, Zhong Z, Lindholm K, Berning L, Lee W, Lemere C, Staufenbiel M, Li R, Shen Y (2007) Deletion of tumor necrosis factor death receptor inhibits amyloid beta generation and prevents learning and memory deficits in Alzheimer's mice. J Cell Biol 178:829–841.

Hirakawa T, Rokutan K, Nikawa T, Kishi K (1996) Geranylgeranylacetone induces heat shock proteins in cultured guinea pig gastric mucosal cells and rat gastric mucosa. Gastroenterology 111:345–357.

Hoshino T, Tsutsumi S, Tomisato W, Hwang HJ, Tsuchiya T, Mizushima T (2003) Prostaglandin E2 protects gastric mucosal cells from apoptosis via EP2 and EP4 receptor activation. J Biol Chem 278:12752–12758.

Hoshino T, Nakaya T, Araki W, Suzuki K, Suzuki T, Mizushima T (2007a) Endoplasmic reticulum chaperones inhibit the production of amyloid-beta peptides. Biochem J 402:581–589.

Hoshino T, Nakaya T, Homan T, Tanaka K, Sugimoto Y, Araki W, Narita M, Narumiya S, Suzuki T, Mizushima T (2007b) Involvement of prostaglandin E2 in production of amyloid-beta peptides both in vitro and in vivo. J Biol Chem 282:32676–32688.

Hoshino T, Namba T, Takehara M, Nakaya T, Sugimoto Y, Araki W, Narumiya S, Suzuki T, Mizushima T (2009) Prostaglandin E2 stimulates the production of amyloid-beta peptides through internalization of the EP4 receptor. J Biol Chem 284:18493–18502.

Huang SM, Mouri A, Kokubo H, Nakajima R, Suemoto T, Higuchi M, Staufenbiel M, Noda Y, Yamaguchi H, Nabeshima T, Saido TC, Iwata N (2006) Neprilysin-sensitive synapse-associated amyloid-beta peptide oligomers impair neuronal plasticity and cognitive function. J Biol Chem 281:17941–17951.

Iwata N, Mizukami H, Shirotani K, Takaki Y, Muramatsu S, Lu B, Gerard NP, Gerard C, Ozawa K, Saido TC (2004) Presynaptic localization of neprilysin contributes to efficient clearance of amyloid-β peptide in mouse brain. J Neurosci 24:991–998.

Kanninen K, Heikkinen R, Malm T, Rolova T, Kuhmonen S, Leinonen H, Ylä-Herttuala S, Tanila H, Levonen AL, Koistinaho M, Koistinaho J (2009) Intrahippocampal injection of a lentiviral vector expressing Nrf2 improves spatial learning in a mouse model of Alzheimer's disease. Proc Natl Acad Sci U S A 106:16505–16510.

Katsuno M, Sang C, Adachi H, Minamiyama M, Waza M, Tanaka F, Doyu M, Sobue G (2005) Pharmacological induction of heat-shock proteins alleviates polyglutamine-mediated motor neuron disease. Proc Natl Acad Sci U S A 102:16801–16806.

Kauppinen TM, Swanson RA (2005) Poly(ADP-ribose) polymerase-1 promotes microglial activation, proliferation, and matrix metalloproteinase-9-mediated neuron death. J Immunol 174:2288–2296.

Kieran D, Kalmar B, Dick JR, Riddoch-Contreras J, Burnstock G, Greensmith L (2004) Treatment with arimoclomol, a coinducer of heat shock proteins, delays disease progression in ALS mice. Nat Med 10:402–405.

Kimura Y, Yamada K, Sakai T, Mishima K, Nishimura H, Matsumoto Y, Singh M, Yoshikai Y (1998) The regulatory role of heat shock protein 70-reactive CD4+ T cells during rat listeriosis. Int Immunol 10:117–130.

Kobayashi K, Noda Y, Matsushita N, Nishii K, Sawada H, Nagatsu T, Nakahara D, Fukabori R, Yasoshima Y, Yamamoto T, Miura M, Kano M, Mamiya T, Miyamoto Y, Nabeshima T (2000) Modest neuropsychological deficits caused by reduced noradrenaline metabolism in mice heterozygous for a mutated tyrosine hydroxylase gene. J Neurosci 20:2418–2426.

Kumar P, Ambasta RK, Veereshwarayya V, Rosen KM, Kosik KS, Band H, Mestril R, Patterson C, Querfurth HW (2007) CHIP and HSPs interact with beta-APP in a proteasome-dependent manner and influence Abeta metabolism. Hum Mol Genet 16:848–864.

Lo Bianco C, Shorter J, Régulier E, Lashuel H, Iwatsubo T, Lindquist S,

Aebischer P (2008) Hsp104 antagonizes alpha-synuclein aggregation and reduces dopaminergic degeneration in a rat model of Parkinson disease. J Clin Invest 118:3087–3097.

Magrané J, Smith RC, Walsh K, Querfurth HW (2004) Heat shock protein 70 participates in the neuroprotective response to intracellularly expressed β-amyloid in neurons. J Neurosci 24:1700–1706.

Matsuda M, Hoshino T, Yamashita Y, Tanaka K, Maji D, Sato K, Adachi H, Sobue G, Ihn H, Funasaka Y, Mizushima T (2010) Prevention of UVB radiation-induced epidermal damage by expression of heat shock protein 70. J Biol Chem 285:5848–5858.

Mattson MP (2004) Pathways towards and away from Alzheimer's disease. Nature 430:631–639.

Mima S, Tsutsumi S, Ushijima H, Takeda M, Fukuda I, Yokomizo K, Suzuki K, Sano K, Nakanishi T, Tomisato W, Tsuchiya T, Mizushima T (2005) Induction of claudin-4 by nonsteroidal anti-inflammatory drugs and its contribution to their chemopreventive effect. Cancer Res 65:1868–1876.

Miners JS, Baig S, Palmer J, Palmer LE, Kehoe PG, Love S (2008) Abeta-degrading enzymes in Alzheimer's disease. Brain Pathol 18:240–252.

Morimoto RI, Santoro MG (1998) Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. Nat Biotechnol 16:833–838.

Muchowski PJ, Wacker JL (2005) Modulation of neurodegeneration by molecular chaperones. Nat Rev Neurosci 6:11–22.

Ohkawara T, Nishihira J, Takeda H, Miyashita K, Kato K, Kato M, Sugiyama T, Asaka M (2005) Geranylgeranylacetone protects mice from dextran sulfate sodium-induced colitis. Scand J Gastroenterol 40:1049–1057.

Ooie T, Takahashi N, Saikawa T, Nawata T, Arikawa M, Yamanaka K, Hara M, Shimada T, Sakata T (2001) Single oral dose of geranylgeranylacetone induces heat-shock protein 72 and renders protection against ischemia/reperfusion injury in rat heart. Circulation 104:1837–1843.

Perez N, Sugar J, Charya S, Johnson G, Merril C, Bierer L, Perl D, Haroutunian V, Wallace W (1991) Increased synthesis and accumulation of heat shock 70 proteins in Alzheimer's disease. Brain Res Mol Brain Res 11:249–254.

Plumier JC, Ross BM, Currie RW, Angelidis CE, Kazlaris H, Kollias G, Pagoulatos GN (1995) Transgenic mice expressing the human heat shock protein 70 have improved post-ischemic myocardial recovery. J Clin Invest 95:1854–1860.

Plumier JC, Krueger AM, Currie RW, Kontoyiannis D, Kollias G, Pagoulatos GN (1997) Transgenic mice expressing the human inducible Hsp70 have hippocampal neurons resistant to ischemic injury. Cell Stress Chaperones 2:162–167.

Roberson ED, Scearce-Levie K, Palop JJ, Yan F, Cheng IH, Wu T, Gerstein H, Yu GQ, Mucke L (2007) Reducing endogenous tau ameliorates amyloid beta-induced deficits in an Alzheimer's disease mouse model. Science 316:750–754.

Rodríguez JJ, Olabarria M, Chvatal A, Verkhratsky A (2009) Astroglia in dementia and Alzheimer's disease. Cell Death Differ 16:378–385.

Saito Y, Sano Y, Vassar R, Gandy S, Nakaya T, Yamamoto T, Suzuki T (2008) X11 proteins regulate the translocation of amyloid beta-protein precursor (APP) into detergent-resistant membrane and suppress the amyloidogenic cleavage of APP by beta-site-cleaving enzyme in brain. J Biol Chem 283:35763–35771.

Shaftel SS, Kyrkanides S, Olschowka JA, Miller JN, Johnson RE, O'Banion MK (2007) Sustained hippocampal IL-1 beta overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. J Clin Invest 117:1595–1604.

Sturchler-Pierrat C, Abramowski D, Duke M, Wiederhold KH, Mistl C, Rothacher S, Ledermann B, Bürki K, Frey P, Paganetti PA, Waridel C, Calhoun ME, Jucker M, Probst A, Staufenbiel M, Sommer B (1997) Two amyloid precursor protein transgenic mouse models with Alzheimer disease-like pathology. Proc Natl Acad Sci U S A 94:13287–13292.

Suemasu S, Tanaka K, Namba T, Ishihara T, Katsu T, Fujimoto M, Adachi H, Sobue G, Takeuchi K, Nakai A, Mizushima T (2009) A role for HSP70 in protecting against indomethacin-induced gastric lesions. J Biol Chem 284:19705–19715.

Sun D, Chen D, Du B, Pan J (2005) Heat shock response inhibits NF-kappaB activation and cytokine production in murine Kupffer cells. J Surg Res 129:114–121.

Tanaka K, Namba T, Arai Y, Fujimoto M, Adachi H, Sobue G, Takeuchi K, Nakai A, Mizushima T (2007) Genetic evidence for a protective role for heat shock factor 1 and heat shock protein 70 against colitis. J Biol Chem 282:23240–23252.

Tanaka K, Ishihara T, Azuma A, Kudoh S, Ebina M, Nukiwa T, Sugiyama Y, Tasaka Y, Namba T, Ishihara T, Sato K, Mizushima Y, Mizushima T (2010) Therapeutic effect of lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 298:L348–L360.

Tang D, Kang R, Xiao W, Wang H, Calderwood SK, Xiao X (2007) The anti-inflammatory effects of heat shock protein 72 involve inhibition of high-mobility-group box 1 release and proinflammatory function in macrophages. J Immunol 179:1236–1244.

Tesseur I, Zou K, Esposito L, Bard F, Berber E, Can JV, Lin AH, Crews L, Tremblay P, Mathews P, Mucke L, Masliah E, Wyss-Coray T (2006) Deficiency in neuronal TGF-beta signaling promotes neurodegeneration and Alzheimer's pathology. J Clin Invest 116:3060–3069.

Tomita S, Kirino Y, Suzuki T (1998a) Cleavage of Alzheimer's amyloid precursor protein (APP) by secretases occurs after O-glycosylation of APP in the protein secretory pathway. Identification of intracellular compartments in which APP cleavage occurs without using toxic agents that interfere with protein metabolism. J Biol Chem 273:6277–6284.

Tomita S, Kirino Y, Suzuki T (1998b) A basic amino acid in the cytoplasmic domain of Alzheimer's beta-amyloid precursor protein (APP) is essential for cleavage of APP at the alpha-site. J Biol Chem 273:19304–19310.

Van Dam D, D'Hooge R, Staufenbiel M, Van Ginneken C, Van Meir F, De Deyn PP (2003) Age-dependent cognitive decline in the APP23 model precedes amyloid deposition. Eur J Neurosci 17:388–396.

Wang J, Ho L, Chen L, Zhao Z, Zhao W, Qian X, Humala N, Seror I, Bartholomew S, Rosendorff C, Pasinetti GM (2007) Valsartan lowers brain beta-amyloid protein levels and improves spatial learning in a mouse model of Alzheimer disease. J Clin Invest 117:3393–3402.

Westerheide SD, Bosman JD, Mbadugha BN, Kawahara TL, Matsumoto G, Kim S, Gu W, Devlin JP, Silverman RB, Morimoto RI (2004) Celastrols as inducers of the heat shock response and cytoprotection. J Biol Chem 279:56053–56060.

Wyss-Coray T (2006) Inflammation in Alzheimer disease: driving force, bystander or beneficial response? Nat Med 12:1005–1015.

Wyss-Coray T, Lin C, Yan F, Yu GQ, Rohde M, McConlogue L, Masliah E, Mucke L (2001) TGF-beta1 promotes microglial amyloid-beta clearance and reduces plaque burden in transgenic mice. Nat Med 7:612–618.

Yamashita Y, Hoshino T, Matsuda M, Kobayashi C, Tominaga A, Nakamura Y, Nakashima K, Yokomizo K, Ikeda T, Mineda K, Maji D, Niwano Y, Mizushima T (2010) HSP70 inducers from Chinese herbs and their effect on melanin production. Exp Dermatol 19:e340–e342.

Yan D, Saito K, Ohmi Y, Fujie N, Ohtsuka K (2004) Paeoniflorin, a novel heat shock protein-inducing compound. Cell Stress Chaperones 9:378–389.

Yoshiike Y, Minai R, Matsuo Y, Chen YR, Kimura T, Takashima A (2008) Amyloid oligomer conformation in a group of natively folded proteins. PLoS One 3:e3235.

Zlokovic BV (2008) The blood-brain barrier in health and chronic neurodegenerative disorders. Neuron 57:178–201.

Biochemical Pharmacology 81 (2011) 1124–1135

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

Masaya Takehara, Tatsuya Hoshino, Takushi Namba, Naoki Yamakawa, Tohru Mizushima *

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, 5-1, Oe-honmachi, Kumamoto 862-0973, Japan

ARTICLE INFO

Article history:
Received 13 December 2010
Accepted 22 February 2011
Available online 1 March 2011

Keywords:
Cancer stem cells
Differentiation
Acetaminophen
Anti-cancer drug

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.

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,

* Corresponding author. Tel.: +81 96 371 4323; fax: +81 96 371 4323.
E-mail addresses: mt0125@gpo.kumamoto-u.ac.jp (M. Takehara), thoshino@gpo.kumamoto-u.ac.jp (T. Hoshino), takushi@kumamoto-u.ac.jp (T. Namba), bananao@gpo.kumamoto-u.ac.jp (N. Yamakawa), mizu@gpo.kumamoto-u.ac.jp (T. Mizushima).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.02.012

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 3β (GSK3β). 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-bromoindirubin-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 PGE_2 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 (Crlj: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 μg/ml streptomycin in a humidified atmosphere of 95% air with 5% CO_2 at 37 °C.

Determination of PGE_2 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 trypsine 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: width^2 × length × 0.5.

2.11. Immunohistochemical analysis

Tumor xenografts were embedded in OCT compound (Sakura Finetechnical 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 ± 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

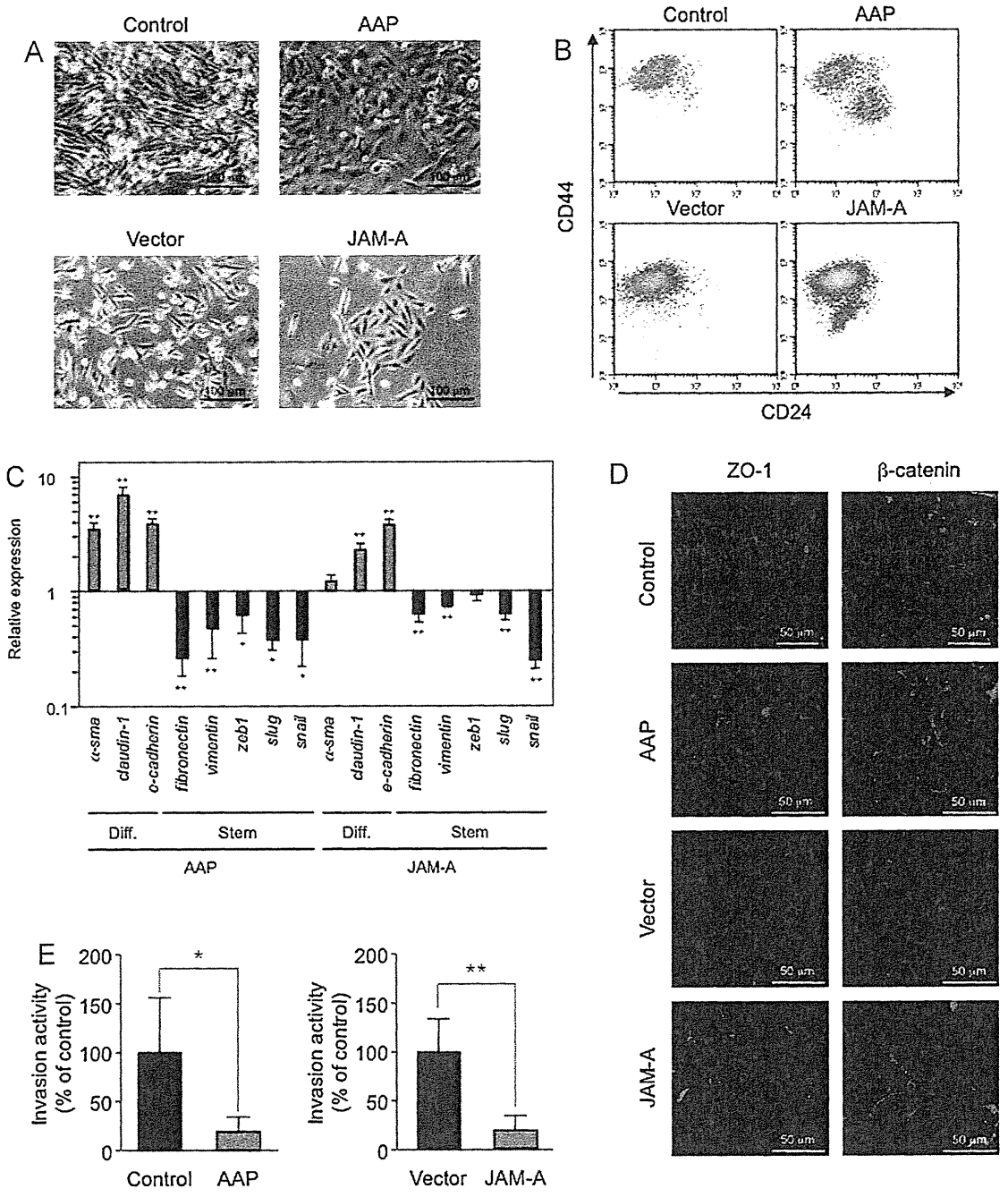


Fig. 1. AAP-induced differentiation of MDA-MB-231 cells. MDA-MB-231 cells treated with (AAP) and without (Control) 1 mM AAP for 4 days or cells stably transfected with the expression plasmid for JAM-A (JAM-A) or empty vector (Vector) were analyzed (A–E). Cell morphology was examined by phase-contrast 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 ± 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 over-expression of JAM-A (Fig. 1E). By use of HPLC analysis, we confirmed that 98.9 ± 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

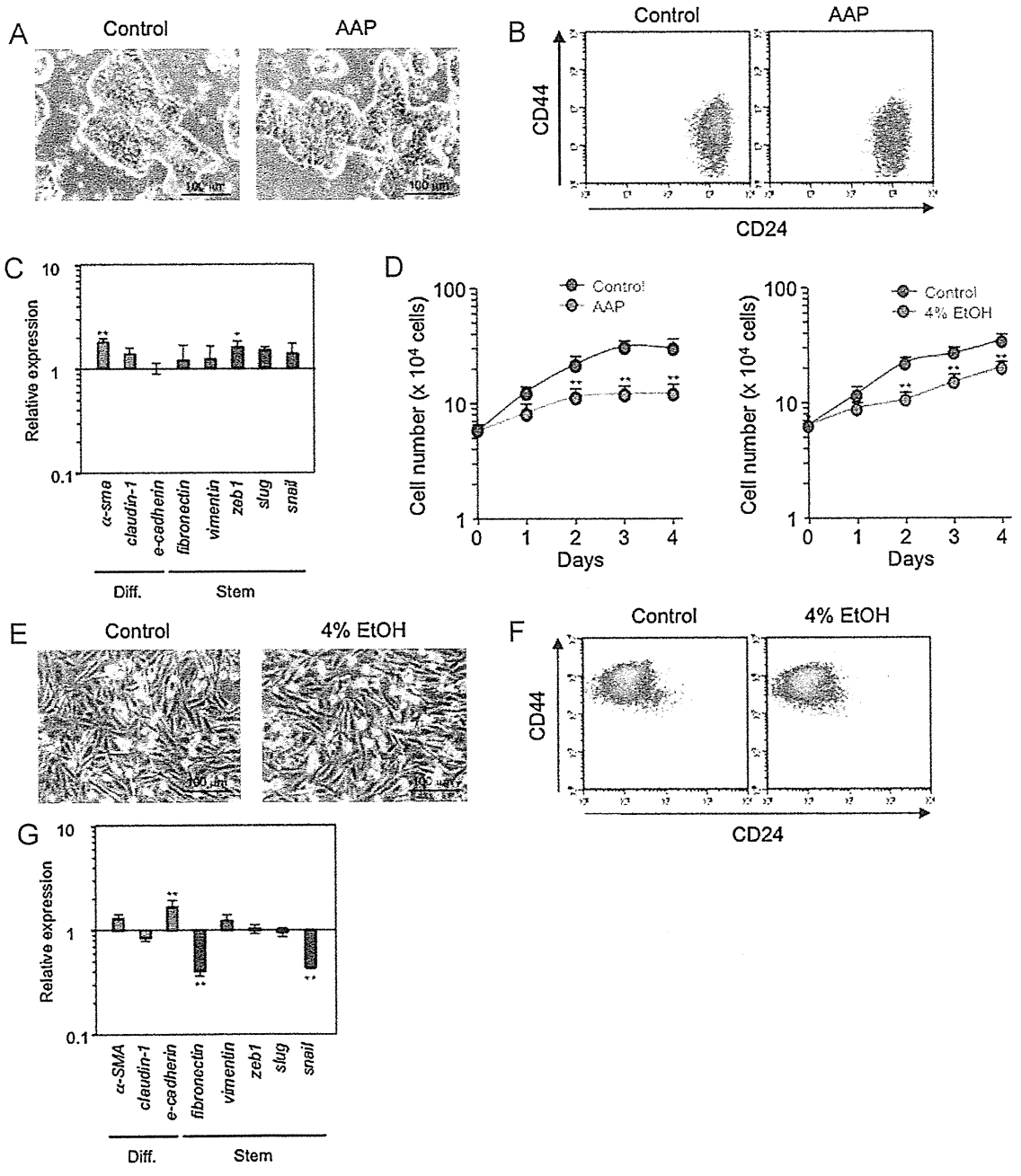


Fig. 2. Specificity of AAP-induced differentiation of MDA-MB-231 cells. MCF-7 (A–C) or MDA-MB-231 (D–G) cells were treated for 4 days with (AAP) or without (Control) 1 mM AAP (A–D) or 4% ethanol (EtOH) (D–G). Cell morphology (A and E), cell surface expression of CD24 and CD44 (B and F) and the mRNA expression (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 ± 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

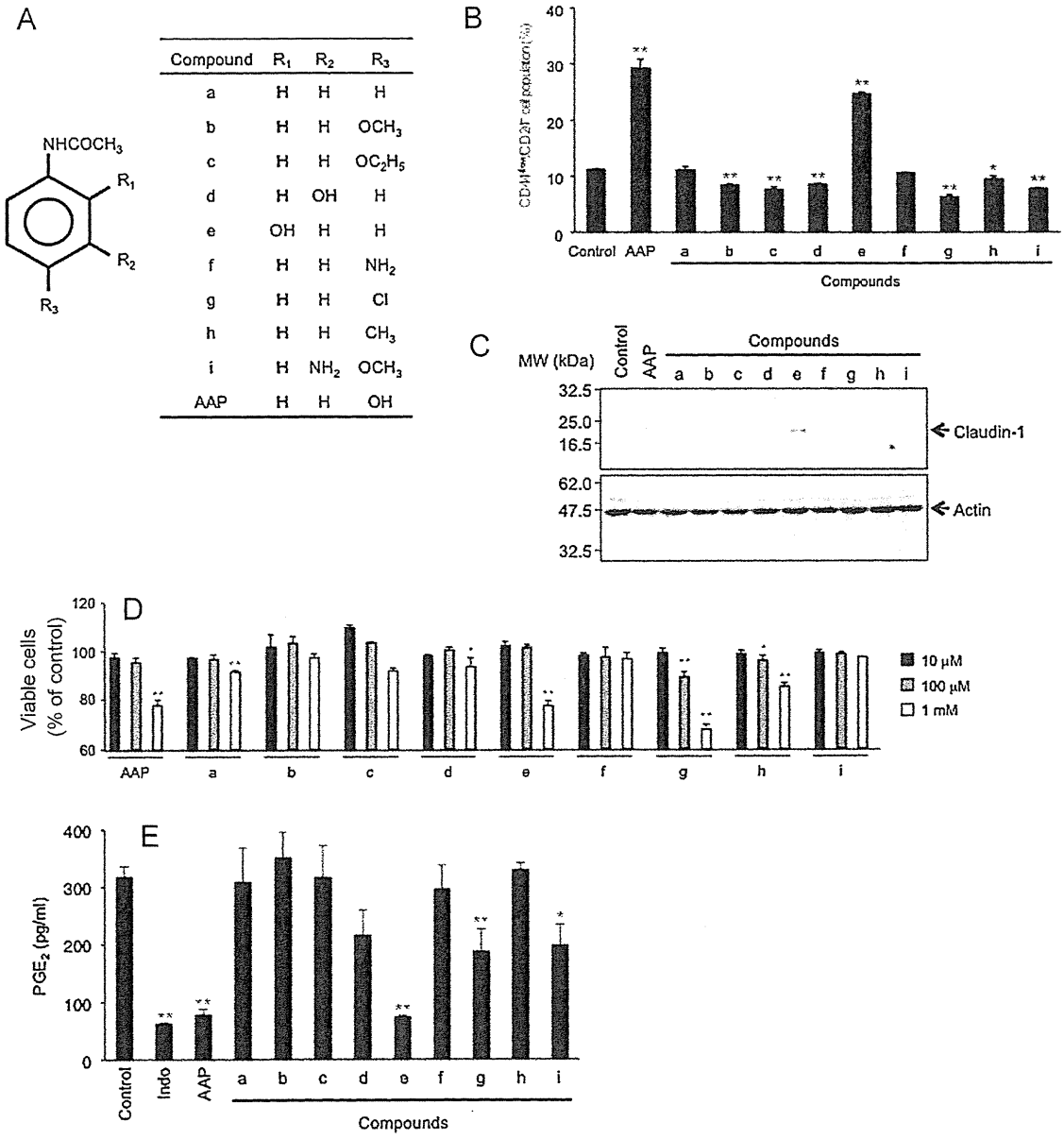


Fig. 3. Structure–function relationship of AAP for induction of differentiation of MDA-MB-231 cells. Chemical structures of AAP and its derivatives (compounds a–i) are shown (A). MDA-MB-231 cells were treated with or without (Control) 1 mM (B, C, E) or the indicated concentrations (D) of AAP and its 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 PGE_2 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 PGE_2 in the culture medium. As shown in Fig. 3E, treatment of cells with 1 mM AAP decreased the PGE_2 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-β1 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-β1* 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

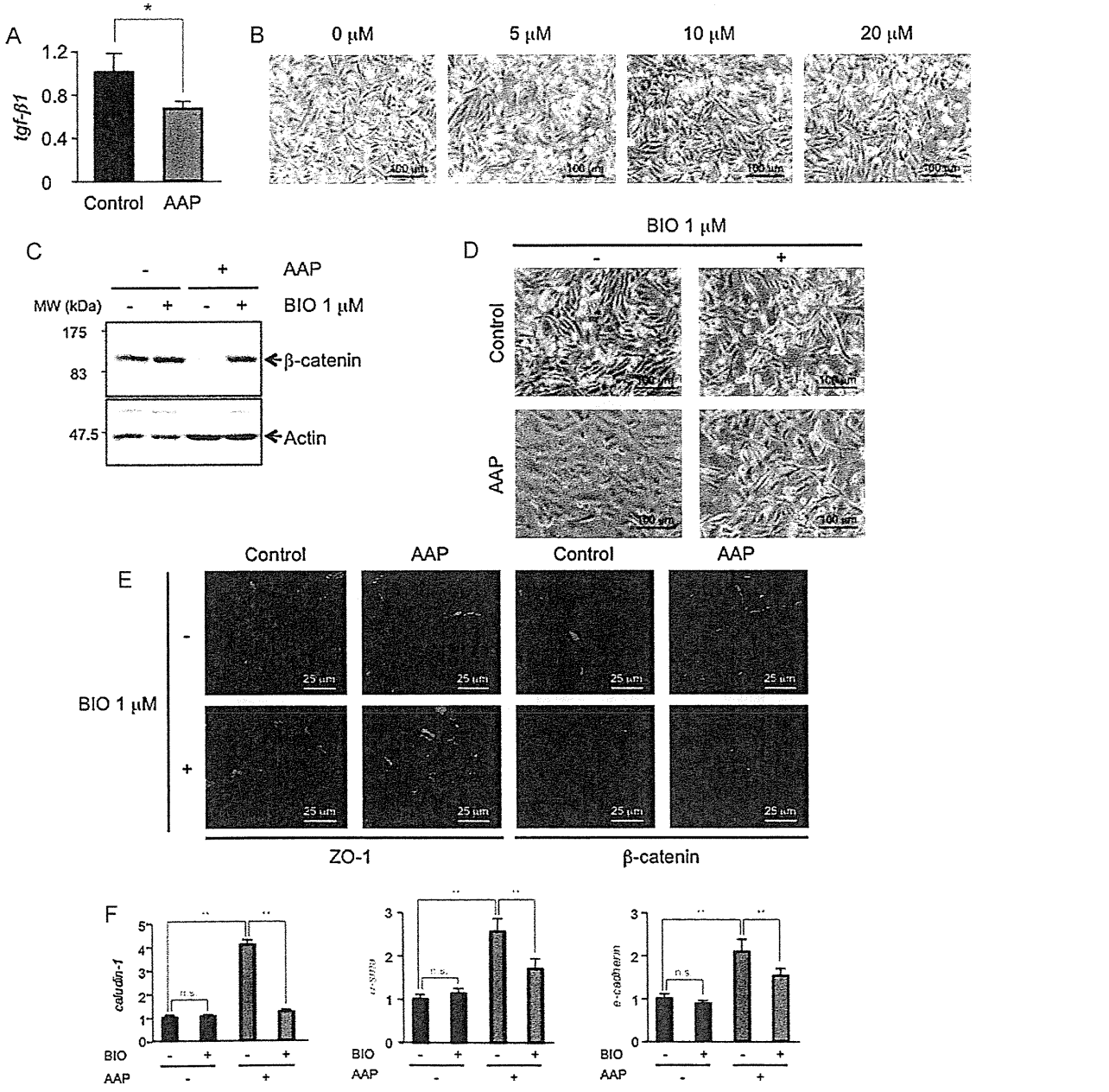


Fig. 4. Involvement of the Wnt/β-catenin canonical signaling pathway in AAP-induced differentiation of MDA-MB-231 cells. MDA-MB-231 cells were treated for 4 days with (AAP) or without (Control) 1 mM AAP in the presence or absence of 1 μM BIO (A, C–F). MDA-MB-231 cells were treated for 4 days with the 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 ± 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 GSK3β [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 doxorubicine 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

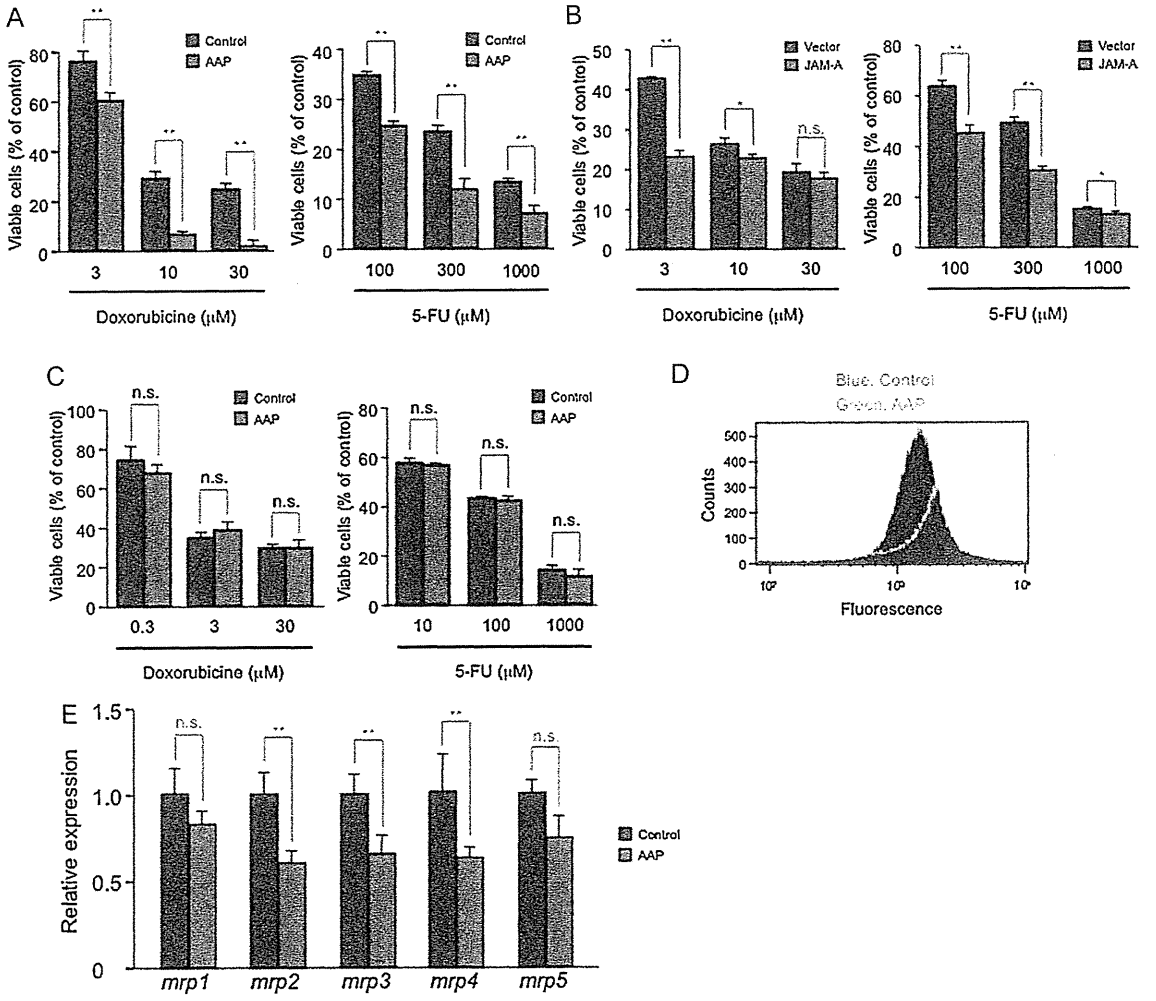


Fig. 5. AAP-induced increase in susceptibility of MDA-MB-231 cells to anti-cancer drugs. MDA-MB-231 (A, D, E) or MCF-7 (C) cells were treated with (AAP) or without (Control) 1 mM AAP for 4 days. MDA-MB-231 cells stably transfected with the expression plasmid for JAM-A (JAM-A) or vector (Vector) were 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 ± 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-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

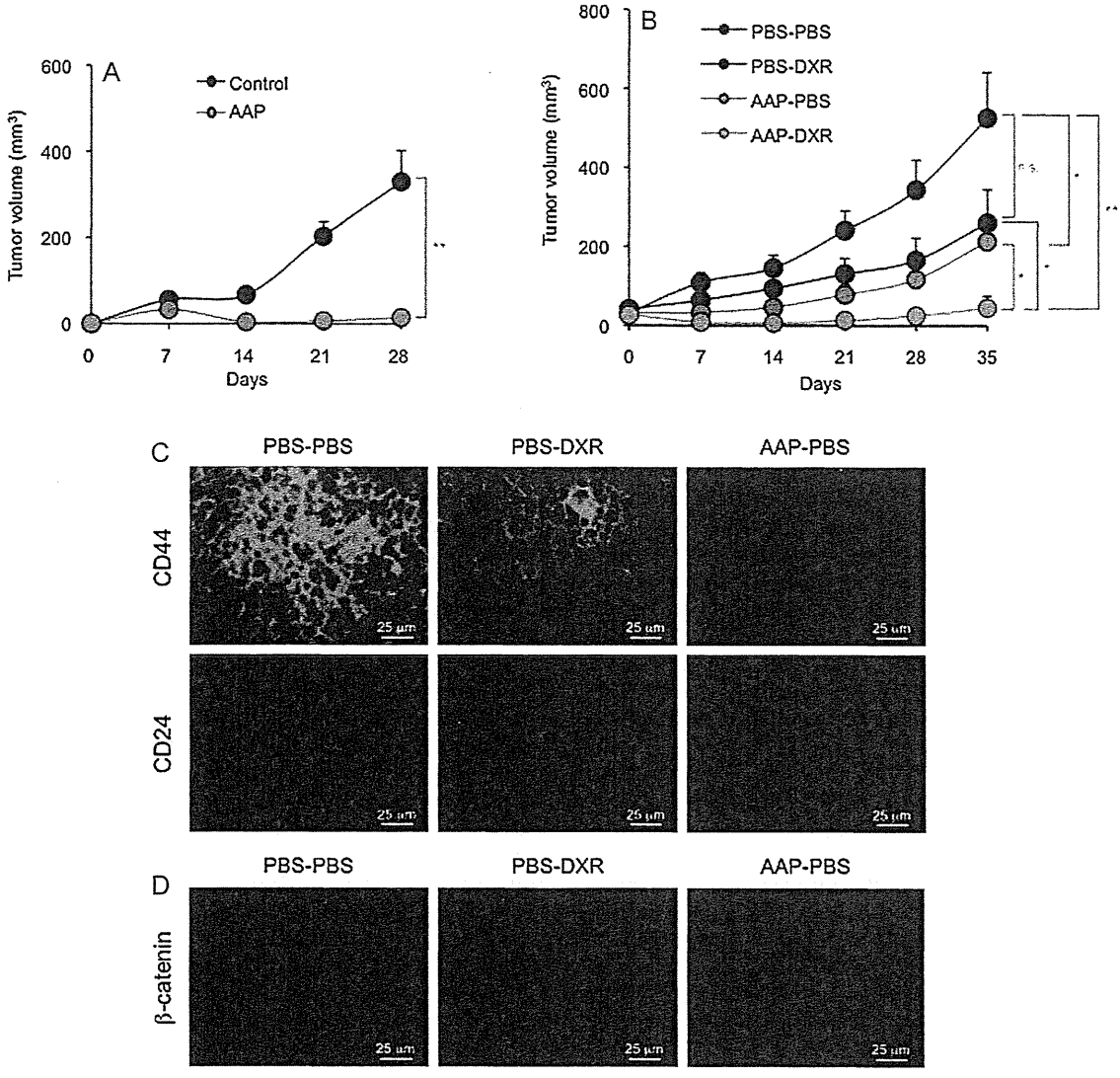
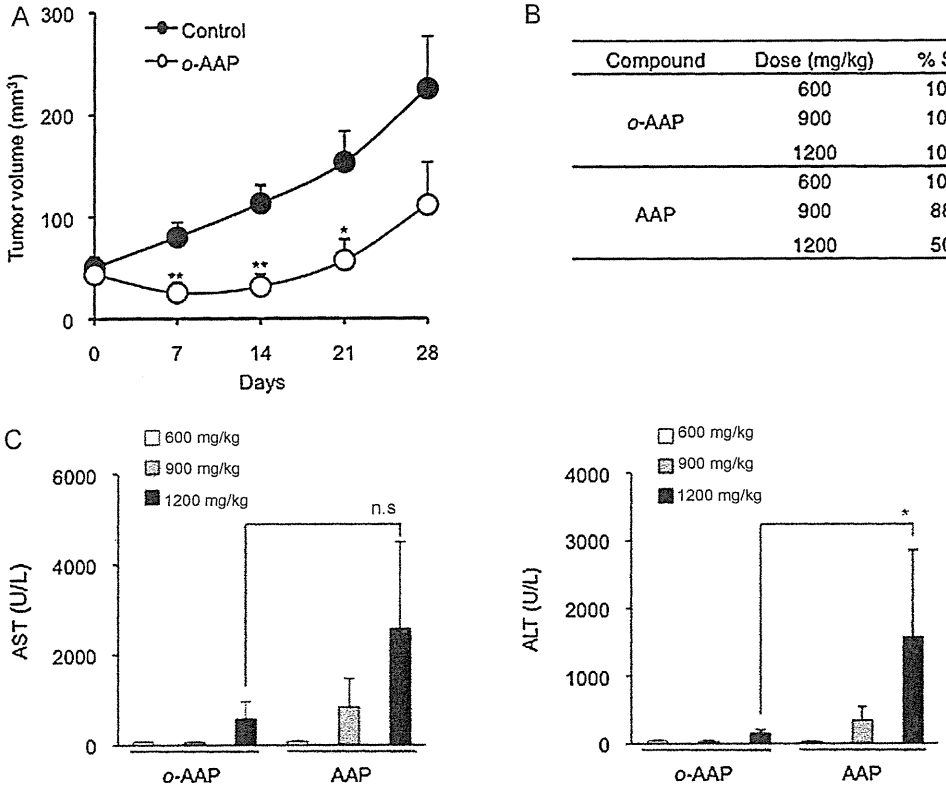


Fig. 6. Effect of AAP on the growth of tumor xenografts in nude mice. MDA-MB-231 cells treated with (AAP) and without (Control) 1 mM AAP for 4 days were inoculated subcutaneously into the right hind footpad of each nude mouse (1×10^7 cells/mouse) at day 0 (A). MDA-MB-231 cells were inoculated subcutaneously 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 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 (1×10^7 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.

Acknowledgments

Grants-in-Aid of Scientific Research from the Ministry of Health, Labour and Welfare of Japan, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Grants-in-Aid of the Japan Science and Technology Agency.

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.

References

[1] Golub TR. Genome-wide views of cancer. N Engl J Med 2001;344:601–2.

[2] Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 2008;8:755–68.
[3] Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells – an integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744–9.
[4] Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 1997;3:730–7.
[5] Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 2003;100:3983–8.
[6] Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, et al. Identification of a cancer stem cell in human brain tumors. Cancer Res 2003;63:5821–8.
[7] Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, et al. Identification and expansion of human colon-cancer-initiating cells. Nature 2007;445:111–5.
[8] Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, et al. Identification of pancreatic cancer stem cells. Cancer Res 2007;67:1030–7.
[9] Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 2005;65:10946–51.
[10] Croker AK, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, et al. High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability. J Cell Mol Med 2009;13:2236–52.
[11] Sheridan C, Kishimoto H, Fuchs RK, Mehrotra S, Bhat-Nakshatri P, Turner CH, et al. CD44+/CD24− breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. Breast Cancer Res 2006;8:R59.
[12] Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, et al. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res 2005;65:5506–11.
[13] Arai F, Hirao A, Ohmura M, Sato H, Matsuoka S, Takubo K, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 2004;118:149–61.
[14] Dean M. ABC transporters, drug resistance, and cancer stem cells. J Mammary Gland Biol Neoplasia 2009;14:3–9.
[15] Wu C, Alman BA. Side population cells in human cancers. Cancer Lett 2008;268:1–9.
[16] Dave B, Chang J. Treatment resistance in stem cells and breast cancer. J Mammary Gland Biol Neoplasia 2009;14:79–82.
[17] Phillips TM, McBride WH, Pajonk F. The response of CD24(−/low)/CD44+ breast cancer-initiating cells to radiation. J Natl Cancer Inst 2006;98:1777–85.
[18] Li X, Lewis MT, Huang J, Gutierrez C, Osborne CK, Wu MF, et al. Intrinsic resistance of tumorigenic breast cancer cells to chemotherapy. J Natl Cancer Inst 2008;100:672–9.
[19] Shafee N, Smith CR, Wei S, Kim Y, Mills GB, Hortobagyi GN, et al. Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. Cancer Res 2008;68:3243–50.
[20] Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, et al. Let-7 regulates self renewal and tumorigenicity of breast cancer cells. Cell 2007;131:1109–23.
[21] Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell 2009;138:645–59.
[22] Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res 2009;69:7507–11.
[23] Lobo NA, Shimono Y, Qian D, Clarke MF. The biology of cancer stem cells. Annu Rev Cell Dev Biol 2007;23:675–99.
[24] Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259–73.
[25] Brennan KR, Brown AM. Wnt proteins in mammary development and cancer. J Mammary Gland Biol Neoplasia 2004;9:119–31.
[26] Korkaya H, Paulson A, Charafe-Jauffret E, Ginestier C, Brown M, Dutcher J, et al. Regulation of mammary stem/progenitor cells by PTEN/Akt/beta-catenin signaling. PLoS Biol 2009;7:e1000121.
[27] Fodde R, Brabletz T. Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol 2007;19:150–8.
[28] Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 2008;133:704–15.
[29] Sabbah M, Emami S, Redeuilh G, Julien S, Prevost G, Zimber A, et al. Molecular signature and therapeutic perspective of the epithelial-to-mesenchymal transitions in epithelial cancers. Drug Resist Updat 2008;11:123–51.
[30] Li Y, Welm B, Podsypanina K, Huang S, Chamorro M, Zhang X, et al. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci USA 2003;100:15853–8.
[31] Liu BY, McDermott SP, Khwaja SS, Alexander CM. The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci USA 2004;101:4158–63.
[32] James LP, Mayeux PR, Hinson JA. Acetaminophen-induced hepatotoxicity. Drug Metab Dispos 2003;31:1499–506.
[33] Flower RJ, Vane JR. Inhibition of prostaglandin synthetase in brain explains the anti-pyretic activity of paracetamol (4-acetamidophenol). Nature 1972;240:410–1.
[34] Lanz R, Polster P, Brune K. Antipyretic analgesics inhibit prostaglandin release from astrocytes and macrophages similarly. Eur J Pharmacol 1986;130:105–9.
[35] Swierkosz TA, Jordan L, McBride M, McGough K, Devlin J, Botting RM. Actions of paracetamol on cyclooxygenases in tissue and cell homogenates of mouse and rabbit. Med Sci Monit 2002;8:BR496–503.
[36] Hinz B, Cheremina O, Brune K. Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. FASEB J 2008;22:383–90.
[37] Yamakawa N, Suemasu S, Kimoto A, Arai Y, Ishihara T, Yokomizo K, et al. Low direct cytotoxicity of loxoprofen on gastric mucosal cells. Biol Pharm Bull 2010;33:398–403.
[38] Takehara M, Nishimura T, Mima S, Hoshino T, Mizushima T. Effect of claudin expression on paracellular permeability, migration and invasion of colonic cancer cells. Biol Pharm Bull 2009;32:825–31.
[39] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[40] Ludwig JA, Szakacs G, Martin SE, Chu BF, Cardarelli C, Sauna ZE, et al. Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. Cancer Res 2006;66:4808–15.
[41] Naik MU, Naik TU, Suckow AT, Duncan MK, Naik UP. Attenuation of junctional adhesion molecule-A is a contributing factor for breast cancer cell invasion. Cancer Res 2008;68:2194–203.
[42] Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M, et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. Cell Stem Cell 2007;1:555–67.
[43] Dontu G, Abdallah WM, Foley JM, Jackson KW, Clarke MF, Kawamura MJ, et al. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev 2003;17:1253–70.
[44] Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nat Med 2004;10:55–63.
[45] Borst P, Evers R, Kool M, Wijnholds J. A family of drug transporters: the multidrug resistance-associated proteins. J Natl Cancer Inst 2000;92:1295–302.
[46] Ferreira MJ, Gyemant N, Madureira AM, Tanaka M, Koos K, Didziapetris R, et al. The effects of jatrophane derivatives on the reversion of MDR1- and MRP-mediated multidrug resistance in the MDA-MB-231 (HTB-26) cell line. Anticancer Res 2005;25:4173–8.
[47] Legrand O, Simonin G, Perrot JY, Zittoun R, Marie JP. Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients. Blood 1998;91:4480–8.
[48] Vazquez-Martin A, Oliveras-Ferraros C, Cufi S, Del Barco S, Martin-Castillo B, Menendez JA. Metformin regulates breast cancer stem cell ontogeny by transcriptional regulation of the epithelial–mesenchymal transition (EMT) status. Cell Cycle 2010;9:3807–14.
[49] North TE, Goessling W, Walkley CR, Lengerke C, Kopani KR, Lord AM, et al. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. Nature 2007;447:1007–11.
[50] Vermeulen L, De Sousa EMF, van der Heijden M, Cameron K, de Jong JH, Borovski T, et al. Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 2010;12:468–76.
[51] Wang Y, Krivtsov AV, Sinha AU, North TE, Goessling W, Feng Z, et al. The Wnt/beta-catenin pathway is required for the development of leukemia stem cells in AML. Science 2010;327:1650–3.
[52] Nusse R. Wnt signaling and stem cell control. Cell Res 2008;18:523–7.
[53] Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D. Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 1999;55:929–37.
[54] Pratt S, Shepard RL, Kandasamy RA, Johnston PA, Perry 3rd W, Dantzig AH. The multidrug resistance protein 5 (ABCC5) confers resistance to 5-fluorouracil and transports its monophosphorylated metabolites. Mol Cancer Ther 2005;4:855–63.
[55] Hjelle JJ, Brzeznicka EA, Klaassen CD. Comparison of the effects of sodium sulfate and N-acetylcysteine on the hepatotoxicity of acetaminophen in mice. J Pharmacol Exp Ther 1986;236:526–34.

J. Biochem. 2011;149(5):499–505 doi:10.1093/jb/mvr032

JB THE JOURNAL OF BIOCHEMISTRY

JB Review

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

Received January 28, 2011; accepted February 24, 2011; published online March 24, 2011

Tohru Mizushima*†

Graduate School of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan

*Tohru Mizushima, Department of Analytical Chemistry, Faculty of Pharmacy, Keio University, 1-5-30 Shibakoen, Minato-ku, Tokyo 105-8512, Japan. Tel/Fax: +81-3-5400-2628, email: mizushima-th@pha.keio.ac.jp

†Present address: Tohru Mizushima, Keio University, Faculty of Pharmacy, Tokyo 105-8512, Japan

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

Featured Article

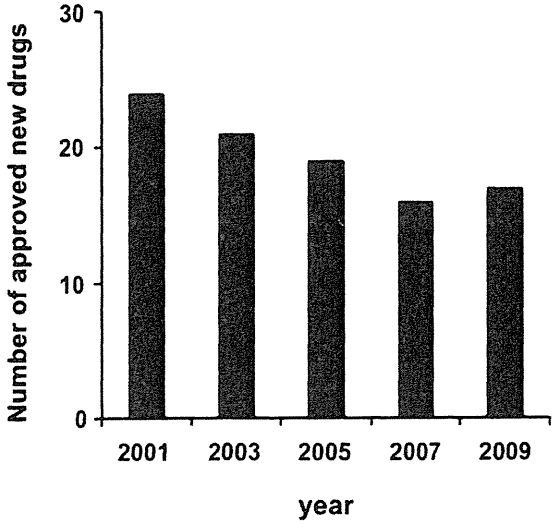


Fig. 1 Decrease in the number of new drugs approved by FDA.

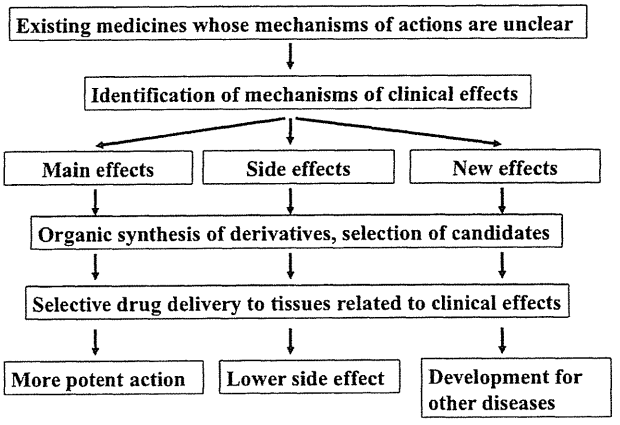


Fig. 2 Drug re-profiling strategy.

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

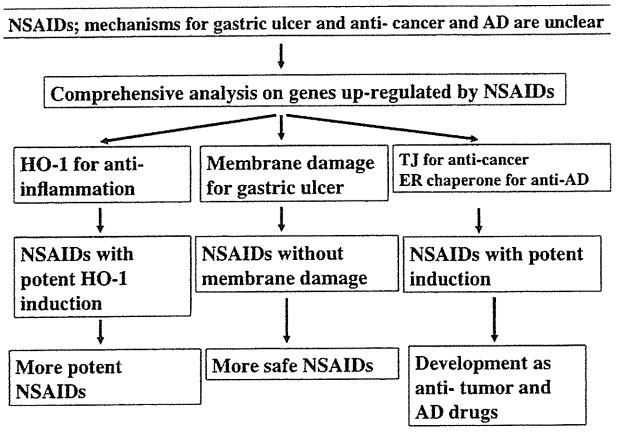


Fig. 3 Drug re-profiling study for NSAIDs.

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