Enhancement of All-trans Retinoic Acid-Induced HL-60 Cell Differentiation by Thalidomide and Its Metabolites

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Received January 13, 2005; accepted January 20, 2005; published online January 24, 2005

Thalidomide (Thal: 1) and its two metabolites, 5-hydroxythalidomide (5-HT: 2) and N-hydroxythalidomide (N-HT: 3), showed an enhancing effect on all-trans retinoic acid (ATRA)-induced HL-60 cell differentiation. 5-HT and N-HT showed tubulin polymerization-inhibiting activity, but thalidomide did not.

Key words thalidomide; hydroxythalidomide; retinoic acid; differentiation; tubulin

Thalidomide (Thal: 1), a sedative drug which was once withdrawn from the market because of its teratogenicity, has been established to be effective for the treatment of various diseases, including leprosy, multiple myeloma (MM), AIDS, and various cancers.1–5 The anti-MM activity of Thal (1) is of particular interest, because Thal (1) overcomes the resistance of human MM cells to conventional drug therapy.6 Although various pharmacological effects elicited by Thal (1), including tumor necrosis factor (TNF)-α production-regulating activity, anti-angiogenic activity and cyclooxygenase (COX)-inhibiting activity,1–5,7,8 have been reported, the mechanism of its anti-MM activity is unclear. During our structural development studies of Thal (1), we found that one of its major metabolites, 5-hydroxythalidomide (5-HT: 2), possesses tubulin polymerization-inhibiting activity, resulting in G1 arrest and apoptosis of myeloma cells.5,9 We also previously reported that tubulin polymerization inhibitors, including colchicine, vinblastine, rhizoxin, maytansine, and etisiloxin, and tubulin depolymerization inhibitors, including taxol, enhance the chemically induced differentiation of human leukemia cell lines.10 Though the relationship between cell differentiation induction and tubulin polymerization/depolymerization inhibition is not clear, enhancement of chemically induced cell differentiation seems to be one of general features of tubulin disruptors.10 These findings led us to suspect that the enhancing effect of Thal (1) and/or its metabolites plays a role in the anti-MM activity of the drug, at least in part. Thal (1) is known to be metabolically unstable, and many metabolites have been identified, including 5-HT (2) and N-hydroxythalidomide (N-HT: 3). In this paper, we deal with the enhancing effect on all-trans retinoic acid (ATRA)-induced HL-60 cell differentiation and the tubulin polymerization-inhibiting activity of Thal (1), 5-HT (2) and N-HT (3).

Thal (1) and 5-HT (2) were prepared as described previously.9,10 N-HT (3) was prepared by the method described by Robin et al.11 Briefly, N-phthaloylglutamic acid anhydride was condensed with O-benzylhydroxylamine in the presence of dicyclohexylcarbodiimide (DCC) to give N-benzylthalidomide, which was deprotected by hydrogenation (H2 gas, Pd/C) to afford N-HT (3). The structures of the synthesized compounds were confirmed by NMR and mass spectroscopy and elemental analysis.

First, we investigated the effect of the compounds on tubulin polymerization using the method previously described.9 Briefly, microtubulin was prepared from porcine brain and tubulin polymerization was followed by turbidity measurements at 37 °C in microtubule assembly buffer containing 0.5 mM MgCl2 and 1 mM GTP (pH 6.5).5 As already reported, Thal (1) showed no effect on tubulin polymerization, and 5-HT (2) showed tubulin polymerization-inhibiting activity with the IC50 value of approximately 50 μM (Fig. 2).9 N-HT (3) was revealed to possess tubulin polymerization-inhibiting activity with similar potency with that of 5-HT (2), as shown in Fig. 2.

Next, we investigated the effect of these compounds on ATRA-induced HL-60 cell differentiation. ATRA was selected because it is used clinically in differentiation-inducing therapy of leukemia, especially for the treatment of acute promyelocytic leukemia (APL), and is an endogenous active form of vitamin A that is generally present in normal serum. Measurement of HL-60 cell differentiation was performed as described previously.10 Briefly, HL-60 cells were incubated in RPMI1640 medium in the presence or absence of a test compound with 2 nm ATRA for 3 d. Treated HL-60 cells were mixed with phosphate-buffered saline (PBS) containing 0.2% nitroblue tetrazolium (NBT) and 20 μM TPA in a 1 : 1 (v/v) ratio and incubated at 37 °C for 20 min. NBT positivity was measured by counting 200–300 cells and the results were expressed as the percentage of NBT-positive cells (Figs. 3a–c). The cell differentiation was also confirmed morphologically by microscopy after Wright-Giemsa staining (Figs. 3d–h). Of course, the percentage values differed from experiment to experiment, but the results were basically reproducible and a typical set of data is presented in Fig. 3. Under our experimental conditions, the percentages of NBT-positive cells when HL-60 cells were treated with 2 nm ATRA for 3 d were typically approximately 2.5% (almost negligible, because non-treated HL-60 cells generally contain 1—2% NBT-positive cells), 15% and 40%, respectively. Thal (1), 5-HT (2) and N-HT (3) showed no HL-60 cell differentiation-inducing activity by themselves in the concentration range examined (Fig. 3).

As expected, 5-HT (2) and N-HT (3), both of which possess tubulin polymerization-inhibiting activity (Fig. 2), showed an enhancing effect on the ATRA-induced HL-60 cell differentiation in a dose-dependent manner (Fig. 3, panels b and c). As mentioned above, HL-60 cell differentiation-inducing activity of ATRA was scarcely detected at the concentration of 2 nm. But addition of 100 μM 5-HT (2) or N-HT

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(3) increased the amount of NBT-positive cells to 22% and 32%, respectively (Fig. 3), which exceeds the HL-60 cell differentiation-inducing effect elicited by 5 nM ATRA (15% NBT-positive cells, vide supra). Differentiation-enhancing activity could be observed at 1 μM 5-HT (2) and N-HT (3). Although differentiation-inducing activity of 5-HT (2) and N-HT (3) had been expected on the basis of their tubulin polymerization-inhibiting activity, the much higher activity of N-HT (3) than that of 5-HT (2) cannot be interpreted in terms of their similar potency of tubulin polymerization-inhibiting activity (Fig. 2). Moreover, unexpectedly, Thal (1), which does not possess tubulin polymerization-inhibiting activity, also showed an enhancing effect on ATRA-induced HL-60 cell differentiation (Fig. 3, panel a). The results indicate that the tested compounds elicit differentiation-enhancing activity through a mechanism other than tubulin polymerization-inhibiting activity, even though this activity might play a role to some extent in the cases of 5-HT (2) and N-HT (3).

Another possible mechanism of enhancement of ATRA-induced cell differentiation is activation of nuclear receptor RXR. RXR agonists have been established to act as synergists of ATRA.13) However, Thal (1), 5-HT (2) and N-HT (3) did not activate RXRs as far as investigated by a reporter gene assay method.

In conclusion, we found that Thal (1) and its two metabolites, 5-HT (2) and N-HT (3), possess an enhancing effect on ATRA-induced HL-60 cell differentiation. Although differentiation-enhancing activity has been demonstrated to be a general feature of tubulin disruptors,10) only 5-HT (2) and N-HT (3) were found to possess tubulin polymerization-inhibiting activity. The lack of tubulin polymerization activity of Thal (1), as well as its differentiation-enhancing activity, suggest that these compounds enhance differentiation through some other mechanism than tubulin polymerization-inhibiting activity. Further investigation is in progress.

Our results have some implications for thalidomide, i.e., (i) the anti-MM activity of Thal (1) might be partly explained by the tubulin polymerization-inhibiting activity of its metabolites, 5-HT (2) and N-HT (3), and the cell differentiation-enhancing activity of Thal (1), 5-HT (2) and N-HT (3), (ii) application of thalidomide to ATRA differentiation-inducing therapy should make it possible to lower the necessary dose of ATRA, and (iii) Thal (1) therapy might result in cell differentiation induction, because normal serum contains nanomolar order of ATRA, at which concentration Thal (1), 5-HT (2) and N-HT (3) would increase ATRA-induced cell differentiation to the level induced by pharmacological doses of ATRA.

Acknowledgements

The work described in this paper was partially supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Society for the Promotion of Science.

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