OSU-03012, a Novel Celecoxib Derivative, Is Cytotoxic to Myeloma Cells and Acts through Multiple Mechanisms
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Abstract Purpose: OSU-03012 is a novel celecoxib derivative, without cyclooxygenase-2 inhibitory activity, capable of inducing apoptosis in various cancer cells types, and is being developed as an anticancer drug. We investigated the in vitro activity of OSU-03012 in multiple myeloma (MM) cells.

Experimental Design: U266, ARH-77, IM-9, and RPMI-8226, and primary myeloma cells were exposed to OSU-03012 for 6, 24, or 72 h. Cytotoxicity, caspase activation, apoptosis, and effects on intracellular signaling pathways were assessed.

Results: OSU-03012 was cytotoxic to MM cells with mean LC50 3.69 ± 0.23 and 6.25 ± 0.86 μmol/L and at 24 h for primary MM cells and cell lines, respectively. As a known PDK-1 inhibitor, OSU-03012 inhibited the PI3K/Akt pathway with downstream effects on BAD, GSK-3β, FoxO1a, p70S6K, and MDM-2. However, transfection of MM cells with constitutively active Akt failed to protect against cell death, indicating activity against other pathways is important. Phospho (p)-signal transducers and activators of transcription 3 and p-MAP/ERK kinase 1/2 were down-regulated, suggesting that OSU-03012 also inhibited the Janus-activated kinase 2/signal transducer and activator of transcription 3 and mitogen-activated protein kinase pathways. Although expression of Bcl-2 proteins was unchanged, OSU-03012 also down-regulated survivin and X-linked inhibitor of apoptosis (XIAP), and also induced G2 cell cycle arrest with associated reductions in cyclins A and B. Finally, although OSU-03012 induced cleavage of caspases 3, 8 and 9, caspase inhibition did not prevent cell death.

Conclusions: We conclude that OSU-03012 has potent activity against MM cells and acts via different mechanisms in addition to phosphoinositide-3-kinase/Akt pathway inhibition. These studies provide rationale for the clinical investigation of OSU-03012 in MM.

Multiple myeloma (MM) is a clonal disorder affecting terminally differentiated B cells, with the accumulation of plasma cells in the bone marrow. In spite of recent improvements in outcome, treatment of MM remains essentially palliative. Although conventional cytotoxic chemotherapy prolongs survival in symptomatic patients, the prognosis of treated patients with MM remains poor (1). High-dose chemotherapy and stem cell transplantation has further improved progression-free survival and overall survival, but almost all patients relapse following transplantation (2, 3). Furthermore, whereas recently approved drugs such as thalidomide, bortezomib, and lenalidomide improve response rates and delay progression compared with more conventional cytotoxic agents (4–6), relapses still invariably occur, indicating the need for continued investigation of novel agents in this disease.

The selective cyclooxygenase-2 (COX-2) inhibitor, celecoxib, is a potent nonsteroidal anti-inflammatory drug that has been shown to mediate apoptosis in a number of cancer lines by blocking phosphoinositide-dependent kinase-1 (PDK-1)/Akt signaling independently of COX-2 inhibition (7–10). In Jurkat cells, celecoxib induced apoptosis through a caspase-dependent mechanism (12), and in prostate cancer cells, it mediated cell death mainly through inhibition of the PDK-1/Akt signaling pathway (7, 9, 10). The concentration of celecoxib required to induce apoptosis ranged from 25 to 100 μmol/L, which is difficult to reach in vivo (7). Based on these findings, celecoxib was structurally modified to dissociate COX-2 inhibitory and apoptosis-inducing activities, producing a novel class of derivatives with high potency in deactivating Akt through PDK-1 inhibition (13), with the ability of inducing apoptosis in the 1- to 10-μmol/L range in prostate cancer cells (13). One derivative, OSU-03012, has been shown to induce rapid apoptosis in primary chronic lymphocytic leukemia (CLL) cells, glioblastoma, pancreatic, and breast cancer cells lines.
Materials and Methods

**Myeloma cells, culture conditions, and reagents.** The MM cell lines U266, RPMI 8226, ARH-77, and IM-9 cell lines were purchased from American Type Culture Collection. Cell lines were cultured in RPMI 1640 (Life Technologies, Invitrogen Company) and supplemented with 10% ARH-77 and IM-9) or 15% (U266 and RPMI 8226) heat-inactivated fetal bovine serum (FBS; Life Technologies, Invitrogen Company). 100 units/ml penicillin, 10 μg/ml streptomycin, and 2 mmol/L L-glutamine (Life Technologies, Invitrogen Company). Primary MM cells were purified from bone marrow aspirates obtained from informed consent from three patients with MM at the time of diagnostic aspirations in accordance with the Declaration of Helsinki. Approval was obtained from the Institutional Review Board of diagnostic aspirations in accordance with the Declaration of Helsinki. Myeloma cells, culture conditions, and reagents.

**Cell viability analysis by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.** Cell viability was analyzed by CellTiter 96 Nonradioactive Cell Proliferation Assay (Promega). Myeloma cells were plated in 96-well flat-bottomed plates in a 100-μL total volume at densities of 2 × 10^4 cells per well for cell lines and 5 × 10^4 cells per well for primary MM cells. Triplicate wells were treated with 10% PBS-supplemented RPMI 1640 containing 1, 5, 10, or 50 μmol/L OSI-03012. The plates were incubated at 37°C in 5% CO₂ for 24 or 72 h. After 24- or 72-h treatment, 15 μL of the kit’s Dye (tetrazolium salt) solution was added to each well, and the cells were incubated at 37°C for 4 h. A total of 100 μL of stop solution were then added into each well, and the plates were incubated for 1 h. Absorbance was measured at 570 nm in a Synergy HT (BioTek) multidetection plate reader. Media only treated cells served as the indicator of 100% cell viability.

**Apoptosis and flow-cytometric analysis.** Following incubation with the treatment drugs, cells were stained with annexin V-FITC and propidium iodide (PI) according to the manufacturer’s directions (BD PharMingen) and analyzed by flow cytometry. To assess mitochondrial integrity following drug treatment, we assessed mitochondria membrane potential using the Dual Sensor-MitoCasp Kit from Cell Technology Inc. Cells were stained and washed according to the manufacturer’s protocol. In experiments assessing caspase-dependent apoptosis, 100 μmol/L Z-VAD-fmk or 20 μmol/L QVD-OPH, was added 60 min before the addition of OSI-03012. To assess for casp9-dependent cell death, 1 or 2.5 μmol/L CA-074 Me, 1 or 2.5 μmol/L zFA-fmk, or 100 μmol/L peptatin A was added 60 min before the addition of OSI-03012. Flow cytometry analysis was done using a Beckman-Coulter EPICS XL cytometer. Fluorosphores were excited at 488 nm. Fluorescence was measured using channel FL1 for annexin V-FITC and Caspase Detection Reagent (Cell Technology Inc.), channel FL2 for mitochondria membrane potential dye (Cell Technology Inc.), and FL3 for PI. Data were analyzed with the System II software package (Beckman-Coulter). At least 10,000 cells were counted for each treated sample. Each sample was run in duplicate or triplicate.

**Cell cycle analysis.** Cell cycle distribution was determined by staining DNA with PI (Sigma). Briefly, cells were incubated without OSI-03012 or with OSI-03012 for 24 h and then harvested, washed in PBS, and counted. About 1 × 10^6 cells were collected and fixed in 70% ethanol. Cell pellets were stained with PI with simultaneous RNase treatment at 37°C for 30 min. The percentage of cells in the different phases of the cell cycle was measured with FACS Calibur flow cytometer (Becton Dickinson) and analyzed by using MoFlo LT 3.0 software (Verity software House Inc.).

**Western blot analysis.** Cells were treated with OSI-03012 for 6 or 24 h and washed with ice-cold PBS and resuspended in lysis buffer containing the phosphatase inhibitors sodium orthovanadate (1 mMol/L and microcystin (1 μmol/L; both from Sigma). Phosphatase inhibitors were used in the lysis preparation to avoid any degradation that could occur with dephosphorylation of proteins in the sample. Soluble cell lysates were collected after centrifugation at 13,000 rpm for 10 min. Equivalent amounts of proteins (50 μg) from each lystate were resolved in 4% to 20% SDS-PAGE. Protein was transferred to 0.2-μm nitrocellulose membranes (Schleicher & Schuell), and the blot was probed with primary antibody specific for the following proteins at the indicated dilutions: phospho–Akt Ser473 (1:200), Akt (1:1,000), caspase-3 (1:1,000), caspase-8 (1:500) and caspase-9 (1:500), poly (ADP-ribose) polymerase (PARP; 1:1,000), Survivin (1:500), XIAP (1:1,000), phospho–MAPK kinase 1/2 (p-MEK1/2 Ser21/22; 1:200) and MEK1/2 (1:500), Bax (1:500), Bir (1:500), phospho–FoxO1a Ser256 (1:200), FoxO1a (1:1,000), phospho–GSK-3α/β (Ser21/27; 1:250), GSK-3β (1:200), phospho-MDM2 (Ser166; 1:500), Cyclin A (1:1,000), B1 (1:1,000), and D1 (1:1,000), p21 (1:500), p27 (1:500), phospho-Stat3 (Tyr705; 1:200), phospho-Stat3 (Ser277; 1:200), phospho-p70S6K (Thr389; 1:750), phospho-BAD (Ser136; 1:200), and BAD (1:1,000; Cell Signaling Technology); phospho–Akt-Thr308 (1:300), p70S6K (1:200), Bcl-2 (1:1,000), Mcl-1 (1:1,000), Stat3 (1:200), MDM2 (1:500), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:100,000), and a-tubulin (1:2,000; Santa Cruz Biotechnology); and Novus Biologicals). Following incubation with antibody, the proteins were detected with chemiluminescent substrate (GeneCopoeia). Standard Western blot analysis. Densitometric analysis of the data was done using GraphPad Prism 4 or Excel software with standard methods.
OSU-03012 induces MM cell death mainly through caspase-independent and cathepsin-independent pathways. Apoptosis may occur through caspase-dependent or caspase-independent mechanisms. We evaluated the activation caspase-3, caspase-8, caspase-9 and PARP cleavage after treatment with OSU-03012. As shown in Fig. 3A for U266 cells, caspase-3, caspase-8, caspase-9, and PARP were strongly cleaved following 6 and 24 h exposure to 10 μmol/L OSU-03012 compared with control samples not exposed to the drug. Similar results were observed for the other cell lines tested (data not shown). As was shown in Fig. 2B, the activation of caspasess following OSU-03012 exposure was associated with a loss of membrane potential. In spite of caspase cleavage, however, up to 100 μmol/L of the pan-caspase inhibitor Z-VAD-fmk did not protect OSU-03012-mediated cell death in any of the cells tested (Fig. 3B). Similarly, whereas 20 μmol/L of the caspase inhibitor Q-VD-OPH strongly prevented OSU-03012–induced PARP cleavage, it did not inhibit the OSU-03012–induced cell death as shown for U266 cells (Fig. 3B). These observations suggest that in spite of the activation of caspasess, OSU-03012–induced apoptosis of MM cells is mediated mainly by caspase-independent mechanisms.

Recent data have highlighted a major role of lysosomal cathepsin proteases in cell death, particularly in the setting where caspase activity is suppressed (26, 27). We evaluated the contribution of cathepsin B, cathepsin L, and cathepsin D to the cytotoxicity of OSU-03012 in MM by evaluating whether cathepsin inhibitors can prevent OSU-03012–induced cell death. As shown in Fig. 3C, none of the selective cathepsin B inhibitor CA-074 Me, the cathepsin B/L inhibitor zFA-fmk, or the selective inhibitor of cathepsin D, pepstatin A, protected against the cytotoxic effects of OSU-03012, as determined by annexin V–FITC/PI staining (Fig. 3C). The lack of protection against cell death by inhibitors of cathepsin B, cathepsin L, and cathepsin D suggests that cathepsins do not mediate cell death triggered by OSU-03012 in MM cells.

Change in anti- and proapoptotic proteins in MM cells following treatment with OSU-03012. The relative levels of antiapoptotic and proapoptotic proteins have been shown to be important for the survival of MM cell and their resistance to chemotherapeutic agents (28–30). We therefore assessed the change in expression of Bcl-2 family members and other antiapoptotic proteins following exposure of MM cells to OSU-03012. As shown in Fig. 4 for U266 cells, the levels of the antiapoptotic proteins Bcl-2 and Mcl-1 protein did not change significantly after 6 or 24 h of exposure to OSU-03012. Similarly, the expression of the proapoptotic protein BAX did not change at 6 h, but marginally decreased to levels below baseline by 24 h of drug exposure (Fig. 4). Furthermore, there was minimal change in the expression of the three major isoforms of BIM (BIM-EL, BIM-L, and BIM-S; Fig. 4). These data showing either absence or only minor changes in the
expression levels of Bcl-2 family members suggest that they are not likely to play a significant role in OSU-03012–induced apoptosis of MM cells.

The inhibitor of apoptosis (IAP) family of antiapoptotic proteins also regulates programmed cell death (31, 32). The expression of two members of IAP family proteins, survivin and XIAP (cross-linked inhibitor of apoptosis), were examined in U266 cells exposed to OSU-03012. As shown in Fig. 4, treatment of U266 cells with 10 μmol/L OSU-03012 for 6 or 24 h resulted in decreased expression of survivin. The expression of XIAP, however, was unaffected at 6 h but seemed to decline 24 h treatment with OSU-03012 (Fig. 4).

**OSU-03012 inhibits Akt activation and PI3K/Akt pathway downstream events, although constitutively active Akt does not prevent OSU-03012–induced cell death.** We investigated the ability of OSU-03012 to inhibit phosphorylation of Akt in MM cells. Immunoblotting for the detection of Thr308 and Ser473 p-Akt and total Akt in U266 cells was done following exposure to OSU-03012 for 6 and 24 h. Figure 5A shows that 10 μmol/L OSU-03012 inhibited both Ser473 and Thr308 phosphorylation, with no change in total Akt at 6 h. The ratios of p-Akt/Akt (normalized to actin) were determined by quantifying band intensities of immunoblots using densitometry. The ratio of p-Akt(Thr308)/Akt and p-Akt(Ser473)/Akt reduced to 0.49 and 0.39, respectively, with an even more profound drop at 24 h (0.14 and 0.33, respectively). Similar results were also observed in ARH-77 and IM-9 cell lines, and to a lower extent, in primary MM cells from one patient (Fig. 5A).

Because Akt represents a key signaling component in cell survival and apoptosis by activating downstream factors, including BAD (33, 34), forkhead transcription factors (21), GSK-3β (22), and MDM-2 (35, 36), we evaluated the change in the expression of these proteins. Figure 5B shows that p-GSK-3β(Ser21), p-FoxO1a (Ser256), and p-MDM-2(Ser166) levels were all significantly down-regulated with OSU-03012 treatment compared with vehicle control at 6 or 24 h. The levels of p-BAD (Ser136) and BAD were not decreased at 6 h, but declined at 24 h treatment with OSU-03012 (Fig. 5B). Similarly, a decline in p-p70S6K (Thr389) or p70S6K could not be shown at 6 h, but a decrease was observed after 24 h exposure to OSU-03012 (Fig. 5B).

To further investigate the role of Akt inhibition in OSU-03012–mediated cell death, we assessed the potential protective effect of transient expression of the constitutively active form of Akt (CA-Akt) on drug-induced MM cell death. Western blot analysis confirmed that CA-Akt was overexpressed after transient transfection into IM-9 cells at 24 and 96 h (Fig. 5C). The transiently transfected IM-9 cells were exposed to 1 to 10 μmol/L OSU-03012 in 10% FBS-containing medium for 24 or 72 h to examine for a protective effect against OSU-03012–induced cell death and compared with control IM-9 cells transfected with an empty pcDNA vector. As shown in Fig. 5C, CA-Akt overexpression failed to provide significant protection against OSU-03012–induced death, suggesting that the drug may have important effects on additional intracellular signaling pathways in MM cells.
OSU-03012 down-regulates STAT3 and MEK 1/2 expression in MM cells. STAT3 is constitutively activated in primary MM cells and U266, resulting in proliferative and antiapoptotic responses (37). Maximal activation of STAT3 requires phosphorylation on both tyrosine and serine residues (38). We investigated STAT3 protein expression in MM cells following exposure to OSU-03012. After U266 cells were incubated for 6 or 24 h with 10 μmol/L OSU-03012, the expression of both phospho-STAT3 (p-STAT3)-Tyr705 and p-STAT3-Ser727, as well as total STAT3 protein, were reduced (Fig. 5D). In addition, the expression of phospho-MEK1/2 (Ser217/221) and MEK1/2 were decreased at 24 h with 10 μmol/L OSU-03012 but not at 6 h (Fig. 5D). Similar results were obtained using primary MM cells, although a reduction in phospho-MEK1/2 (Ser217/221) was obvious earlier at 6 h compared with cell lines (Fig. 5D). These changes indicate that OSU-03012 acts on additional pathways other than PI3K/Akt in MM cells.

OSU-03012 down-regulates cyclins A, B1, and D1 expression and induces G2 arrest in MM cells. The effect of OSU-03012 on the expression of cyclin A, cyclin B1, cyclin D1, p21, and p27 was evaluated. As shown in Fig. 6A, 10 μmol/L OSU-03012 markedly down-regulates cyclin D1 at 6 and 24 h. Although the levels of cyclin A, cyclin B1, p21, and p27 were unchanged following 6 h of exposure to OSU-03012, those of cyclins A and B1 and p27, but not that of p21, were reduced at 24 h. These changes were accompanied by cell cycle arrest. As shown in Fig. 6B, after 24-h exposure to OSU-03012, G2 phase arrest was observed only at the higher concentration (10 μmol/L) of the drug.

Discussion

In this study, we have shown that OSU-03012 has potent cytotoxic activity against MM cell lines as well as primary MM cells and is likely to mediate cell death in MM cells by acting on multiple pathways other than by preventing Akt activation.
In addition to inhibiting PDK-1 and Akt activation, OSU-03012 down-regulates the antiapoptotic proteins, XIAP and survivin, cyclin D1, cyclin A, and cyclin B and inhibits MEK and STAT3 activation. Furthermore, whereas caspases of the extrinsic and mitochondrial pathways are activated, induction of cell death seems to follow a predominantly caspase-independent mechanism.

The effects of celecoxib and its derivative 2,5-dimethylcelecoxib (2,5-DMC), devoid of COX-2 inhibition, were studied by Kardosh et al. (39) in MM cell lines. Both drugs induced cell death through multitarget mechanisms independent of COX-2, including down-regulation of cyclin A, cyclin B, and p27; activation of caspase-3 and PARP; degradation of survivin; and a decrease in p-MEK1/2, but with no effect on p-Akt or Akt levels (39). Although 2,5-DMC interfered with the PDK-1/Akt signaling pathway in PC-3 prostate cancer cells (40), similar changes were not observed in MM cells (39), suggesting that differences are likely to exist between the different celecoxib derivatives in their mechanism of action against different neoplastic cell types. Our data are consistent with this supposition. Although OSU-03012 also seems to inhibit Akt phosphorylation in MM cells as previously shown in prostate cancer cells (13), its activity against other signaling pathways is likely to be at least as important for causing MM cell death. Indeed, transfection of constitutively active Akt into MM cells failed to protect against OSU-03012–induced cell death, unlike in PC-3 prostate cancer cells where protection was observed (13).
Although the PI3K/Akt pathway is known to be constitutively activated in many primary myeloma cells and cell lines (41) and has been shown to play a key role in the survival and proliferation of MM cells in response to interleukin-6 (IL-6) and insulin-like growth factor-I (IGF-I) (19, 20), signaling through the JAK/STAT and MAPK pathways also contribute significantly to the survival and proliferation of MM (37, 42). Our data indicate that OSU-03012 also inhibits phosphorylation of MEK1/2 and causes significant down-regulation of STAT3, effects not previously described for this drug in other cell types. OSU-03012 down-regulated the expression of both p-STAT3-Tyr705 and p-STAT3-Ser727, both of which are required for maximal activation of STAT3 (38). Phosphorylation of STAT3 at Ser727 is known to be mediated by mTOR (43), one of the downstream effectors in the PI3K/Akt pathway, and its down-regulation therefore likely reflects the effect of inhibition of Akt activation by OSU-03012. However, phosphorylation of STAT3 at Tyr705 is mediated by members of the JAK/Tyk family of tyrosine kinases, and its down-regulation in MM cells suggests that OSU-03012 also has inhibitory activity on the JAK/STAT pathway.

OSU-03012 also induced G2 cell cycle arrest in MM cells. Arrest of the cell cycle in G2 by OSU-03012 was associated with the down-regulation of cyclins A and B, known to be required for the progression of DNA synthesis and G2-M transition (44).
Although the mechanism by which inhibition of cyclins A and B and, therefore, G2 arrest by OSU-03012 remains uncertain, this may be related to a possible effect on the MAPK signaling pathway evidenced by the down-regulation of p-MEK1/2 and MEK1/2. In this respect, the selective COX-2 inhibitor, etodolac, was recently reported to induce G2 arrest in human hepatocellular carcinoma cell lines through the inhibition of expression of cyclins A and B due to an effect on the MAPK signaling pathway, without inhibition of the PDK-1/Akt pathway (45). Of note, however, although cyclin D1 was significantly down-regulated, no significant G1-S phase arrest was observed.

OSU-03012 also modulated the expression of antiapoptotic proteins of the IAP family, XIAP and survivin, without an effect on Bcl-2 apoptosis family member proteins. Increased expression of the IAP family proteins, including XIAP and survivin, correlate with poor outcome and chemotherapy-induced drug resistance in MM (46). The mechanism by which XIAP and survivin are reduced by OSU-03012 remains uncertain, although it is known that Akt activates these prosurvival genes (47, 48). In addition, inhibition of STAT3, also observed with OSU-03012, has also been reported to decrease survivin expression (49). Therefore, the down-regulation of the IAP family proteins by OSU-03012 may also be related to its effect on reducing Akt activation and STAT3 inhibition. Given the apparent absence of an effect of OSU-03012 on other antiapoptotic and proapoptotic proteins of the Bcl-2 family, it is likely that XIAP and survivin are important targets for modulating apoptosis by this drug in MM. Furthermore, the down-regulation of survivin by OSU-03012 may contribute to the observed G2-M arrest because survivin expression has been implicated in this phase of cell cycle progression in other cell types (48, 50).

In CLL cells, OSU-03012 activated PARP cleavage, caspase-3 and caspase-9, but not caspase-8 cleavage, indicating that the drug acted at least in part through the intrinsic pathway of apoptosis to promote cell death of CLL cells (14). However, Z-VAD-fmk did not prevent OSU-03012–mediated cell death. The data in CLL suggest that although OSU-03012 induced signaling in the mitochondrial pathway of apoptosis, it also activated alternative cell death pathways that are caspase independent. In our study of MM cells, OSU-03012 also induced caspase cleavage and strong PARP cleavage. However, neither of the caspase inhibitors Z-VAD-fmk or Q-VD-OPH were protected from OSU-03012–mediated cell death, although Q-VD-OPH strongly blocked drug-induced PARP cleavage. Our data confirm that OSU-03012 induces cell death in MM cells mainly through a caspase-independent pathway.

Cathepsin B has recently been reported to play an important role in OSU-03012–induced killing of glioma cells (15). Inhibition of cathepsin B suppressed the lethality of OSU-03012 in human glioma cells, whereas inhibition of caspases did not (15). In the current study, however, we could not confirm the dependence on cathepsins for OSU-03012–mediated cell death of MM cells. Selective cathepsin inhibitors did not abrogate cell death induced by OSU-03012, suggesting that cathepsins are not essential for the cell death triggered by OSU-03012 in MM. This is consistent with the notion that celecoxib and various celecoxib derivatives use different mechanisms to induce cell death. Furthermore, OSU-03012 likely promotes cell death through different pathways in various types of cancer cells.

In conclusion, OSU-03012 shows potent in vitro cytotoxic activity against MM cells. While confirming its previously reported ability to inhibit Akt phosphorylation, we propose that this is unlikely to be the sole mechanism contributing to cytotoxicity against MM cells. Although the precise mechanism of OSU-03012–induced cell death remains uncertain, OSU-03012 seems to target multiple pathways in MM, including the inhibition of the JAK/STAT and MAPK pathways, the down-regulation of inhibitors of apoptosis, and proteins involved in G2-M cell cycle progression. However, as the survival and proliferation of MM depend on multiple pathways, such pleiotropic effects of OSU-03012 likely contribute to its efficacy and potential for synergy with many other agents. Our data provide a rationale for clinical investigation of OSU-03012 in MM.

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