AMF-26, a Novel Inhibitor of the Golgi System, Targeting ADP-ribosylation Factor 1 (Arf1) with Potential for Cancer Therapy

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Background: Golgi is a potential target for cancer treatment, but no inhibitor became an anticancer drug.

Results: Using a unique bioinformatics approach, we identified a novel Golgi inhibitor, AMF-26, targeting Arf1 activation and possessing potent antitumor activity.

Conclusion: AMF-26 is a promising new anticancer drug lead.

Significance: Our data indicate that Arf1 activation is a promising target for cancer treatment.

ADP-ribosylation factor 1 (Arf1) plays a major role in mediating vesicular transport. Brefeldin A (BFA), a known inhibitor of the Arf1-guanine nucleotide exchange factor (GEF) interaction, is highly cytotoxic. Therefore, interaction of Arf1 with ArfGEF is an attractive target for cancer treatment. However, BFA and its derivatives have not progressed beyond the pre-clinical stage of drug development because of their poor bioavailability. Here, we aimed to identify novel inhibitors of the Arf1-ArfGEF interaction that display potent antitumor activity in vivo but with a chemical structure distinct from that of BFA. We exploited a panel of 39 cell lines (termed JFCR39) coupled with a drug sensitivity data base and COMPARE algorithm, resulting in the identification of a possible novel Arf1-ArfGEF inhibitor AMF-26, which differed structurally from BFA. By using a pulldown assay with GGA3-conjugated beads, we demonstrated that AMF-26 inhibited Arf1 activation. Subsequently, AMF-26 induced Golgi disruption, apoptosis, and cell growth inhibition. Computer modeling/molecular dynamics (MD) simulation suggested that AMF-26 bound to the contact surface of the Arf1-Sec7 domain where BFA binds. AMF-26 affected membrane traffic, including the cis-Golgi and trans-Golgi networks, and the endosomal systems. Furthermore, using AMF-26 and its derivatives, we demonstrated that there was a significant correlation between cell growth inhibition and Golgi disruption. In addition, orally administrated AMF-26 (83 mg/kg of body weight; 5 days) induced complete regression of human breast cancer BSY-1 xenografts in vivo, suggesting that AMF-26 is a novel anticancer drug candidatethat inhibits the Golgi system, targeting Arf1 activation.

Protein-protein interactions (PPIs) play an important role in many biological processes, such as growth, cell survival, and intercellular signal transduction (1, 2). Therefore, inhibitors of specific PPIs can act as novel therapeutic agents including anticancer drugs. Indeed, several small molecule-PPI inhibitors have been developed, such as Nutlin-3 for HDM2-p53 interaction (3), and ABT-737, a Bcl-2 family protein inhibitor (4). Nonetheless, the number of promising PPI inhibitors for cancer treatment remains limited.

In this study, we focused on the activation of ADP-ribosylation factors (Arfs) by its guanine nucleotide exchange factors (GEFs). Arfs are members of the Ras superfamily of small GTPases that play a major role in mediating vesicular transport in the secretory and endocytic pathways (5). The cellular activity of Arfs is stimulated by the Sec7 domain of GEFs that promote the exchange of inactive GDP-bound forms to active GTP-bound forms (6). Arf1, the best characterized Arf, localizes primarily to the Golgi apparatus and regulates both anterograde and retrograde vesicular traffic (7). The first identified small molecule PPI that targets the interaction between Arf-ArfGEF was brefeldin A (BFA, Fig. 1A), a lactone isolated from fungi, which inhibits the activation of Arf1 by a subset of its GEFs (8, 9). Treatment of BFA causes rapid but reversible disruption of the Golgi apparatus leading to the vesiculation of the cis-Golgi and trans-Golgi network (TGN) (10, 11). Furthermore, BFA showed tumor growth inhibition in vitro (12) and in vivo at an early stage (13). Therefore, inhibitors of Arf1-ArfGEF interaction are valuable tools for studying membrane traffic as well as anticancer drug candidates. However, BFA and its deriv-

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atives have not progressed beyond the pre-clinical stage of drug development (13, 14).

We previously established a panel of 39 cell lines (termed JFCR39) coupled with our own drug sensitivity data base, which is comparable with that of the NCI60 panel from the National Cancer Institute (NCI) (15–19). Using the COMPARE computer algorithm (details described under “Results”), it is possible to correlate the growth inhibitory patterns of JFCR39 (termed “fingerprint”) of a test compound with those of known anticancer drugs and inhibitors (20, 21). Here, we have attempted to identify new Arf1-ArGEF inhibitors with equivalent functions to BFA but with a different chemical structure, by using COMPARE-guided in silico screening instead of structure-based screening.

This approach enabled us to discover a novel small molecule AMF-26 (Fig. 1A), an octahydronaphthalene derivative, which disrupts the Golgi system probably by inhibiting the Arf1 activation. AMF-26 shows strong growth inhibition against JFCR39 cell lines in vitro. Furthermore, it showed potent antitumor activity against xenografts of the human breast cancer cell line BSY-1 via oral administration.

EXPERIMENTAL PROCEDURES

Chemicals—AMF-26 ((2E,4E)-5-((1S,25,4aR,6R,7S,8S,8aS)-7-hydroxy-2,6,8-trimethyl-1,2,4a,5,6,7,8,8a-octahydronaphthalen-1-yl)-2-methyl-N-(pyridin-3-yl-methyl)penta-2,4-dienamide) and its derivatives were synthesized from AMF-14, a natural product from the genus Trichoderma, and kindly provided by the Research Laboratories Kyoto, Nippon Shinyaku Co., Ltd. (Kyoto, Japan). Details of the synthesis methods and the chemical properties of the final product are described under supplemental “Experimental Procedures.” BFA and nocodazole were purchased from Sigma, and bafilomycin A1 was purchased from Tocris Bioscience (Bristol, UK). For in vitro studies, these compounds were reconstituted to 10 μM in DMSO (Sigma) and stored at −20 °C. For animal experiments, AMF-26 was suspended in 0.05% Cremophor EL (Sigma-Aldrich) in water as a solid dispersion. The antibodies for immunostaining were as follows: monoclonal to anti-GFB1 (clone 25), anti-adaptin γ (clone 88), and anti-adaptin δ (clone 18) were purchased from BD Biosciences (San Jose, CA), anti-ERGIC53 (clone G1/93) was from ALEXIS Biochemicals (Farmingdale, NY), anti-Arf (clone 1D9) and anti-Arf1 (clone EP442Y) were from Abcam (Cambridge, United Kingdom), and anti-α tubulin (clone B-5-1-2) was from Sigma. Rabbit polyclonal to anti-βCOP was from Abcam, and anti-γ cleaved poly(ADP-ribose) polymerase (PARP) was from Cell Signaling Technology (Boston, MA). Fluorescent probe LysoTracker was purchased from Invitrogen. For Western blotting, horseradish peroxidase-conjugated donkey anti-rabbit or sheep anti-mouse IgG (GE Healthcare) was used as a secondary antibody. For immunofluorescence microscopy, Alexa 488-conjugated goat anti-rabbit or anti-mouse IgG ( Molecular Probes, Eugene, OR) was used as a secondary antibody.

Cell Lines—A panel of 39 human cancer cell lines (termed JFCR39, described previously (22)) was used for the in vitro experiments. BSY-1 (human breast cancer) cells were also used for in vivo studies. MDA-MB-435 (human breast cancer) cells stably expressing GFP-tagged human clathrin light chain a (MDA-MB-435/GFP-CLCa) were prepared as described previously (23). HEK293T (human embryonic kidney) cells were purchased from American Type Culture Collection (Manassas, VA). JFCR39 and MDA-MB-435/GFP-CLCa cells were cultured in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified atmosphere including 5% CO2 at 37 °C. HEK293T was cultured in DMEM (Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal bovine serum and kanamycin, at 37 °C under 5% CO2. For in vivo studies, BSY-1 cells were grown as subcutaneous tumors in nude mice.

Analysis of Cell Growth Inhibition—The inhibition of cell proliferation was assessed by measuring changes in total cellular protein in a culture of each of the JFCR39 cell lines after 48 h of drug treatment by use of a sulforhodamine B assay (24). The 50% growth inhibition (GI50) value was calculated as described previously (18, 19).

COMPARE Analysis—Based on these sets of GI50 values, fingerprints are presented in the graphic profiles of relative sensitivity within JFCR39. To analyze the correlation between the fingerprints of drug A and drug B, we exploited the COMPARE computer algorithm as described previously (18, 20, 22). The Pearson correlation coefficient between the fingerprints of drug A and drug B was calculated (n = 39).

Arf-GTP Pulldown Assay—The pulldown assay to estimate the signals of GTP-bound Arfs was performed as described previously (25, 26). Every pulldown assay was performed with the VHS and GAT domains of human GGA3, cloned, and purified according to a published protocol (25, 26) with modifications as described under supplemental “Experimental Procedures.”

We examined the guanine nucleotide exchange activity of endogenous Arfs as follows. BSY-1 cells treated with chemicals for 1 h were scraped into 0.5 ml of cold pulldown buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, and protease inhibitors mixture (Nacalai Tesque Inc., Kyoto, Japan). The cell lysate was cleared using GSH-Sepharose 4B beads. Then the lysate was gently rotated with the beads containing 50 μg of rGST-GGA3 protein for 30 min at 4 °C. Bound proteins were washed three times with cold wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl2, 1% Nonidet P-40, 10% glycerol and protease inhibitors mixture), followed by boiling for 5 min with SDS sample buffer. Proteins in the samples were separated in 10–20% Multi-Gel II mini (Cosmo Bio Co., Tokyo, Japan), followed by electroblotting onto a polyvinylidene difluoride membrane (GE Healthcare).
After immunoblotting analysis using anti-pan-Arf and Arf1-specific antibody, immunoreactive bands were identified with the ECL Plus Western blotting detection system (GE Healthcare). Band intensity was determined using the program ImageJ (National Institute of Health).

To confirm the inhibitory efficacy of Arf1 activation, HEK293T/Arf1-HA cells were used to measure exogenous Arf1-GTP. Using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions, HEK293T cells were transfected with the Arf1-HA expression vector, prepared as described under supplemental “Experimental Procedures.” For transfection, HEK293T cells were seeded at a density of 3 \times 10^6 cells/10 ml in a 10-cm plate. The transfectants were then incubated for 24 h, and subsequently treated with chemicals for 1 h. The cell lysates were assayed as described above. Because of a difference in molecular weight, the exogenous band of intact Arf1 was apparently weaker than that of the exogenous Arf1-HA.

To relieve the steric hindrances, the coordinates of AMF-26 and of the residues within 4 Å from AMF-26 were optimized to reduce the root mean square of the gradients of potential energy below 0.05 kcal mol^{-1} Å^{-1} using SYBYL. The simplex minimization method before the conjugate-gradient minimization procedure was used. The Tripos force field was employed for the molecular energy calculation. The AMBER 4.1 charges (31) were used as the atomic charges for the proteins and the Gasteiger-Hückel charges (32–35) were used as the charges for AMF-26. The cut-off distance for the non-bonded interactions was 8 Å. The distance-dependent dielectric constant of 4r was used. The initial positions of the missing atoms in the crystal structure were generated by the SYBYL.

**Molecular Dynamics Simulation for Model Refinement**—To refine the model of the Arf1-AMF-26-Sec7 domain complex, AMBER 9 (36) was used for further minimization and MD simulations on 7 nodes of an Appro 1122Hi (AMD Opteron 248; 2.20 GHz; 14 central processing units). The partial charges for AMF-26 were derived from the restraint electrostatic potential method (39) using an ab initio calculation at the HF/6–31G* level using Gaussian 03 (40). The complex was solvated in a box of 34616 TIP3P (41) water molecules. Six K^+ ions were added to neutralize the system. The system was minimized until the root mean square of the gradients of potential energy was below 0.005 kcal mol^{-1} Å^{-1}. The 3.5-ns MD simulation was carried out at constant pressure (1 atm) and temperature (310 K), under periodic boundary conditions, and with particle-mesh Ewald treatment (42) of electrostatics. SHAKE (43) was applied to all bonds involving hydrogen, and a time step of 1 fs was used. An 8-Å cutoff was used for the non-bonded interactions.

**Immunofluorescence Microscopy**—After being cultured in a 24-well glass bottom plate for 48 h, cells were treated with chemicals for various periods of time (as indicated in the relevant figure). Cells were washed with PBS, fixed with cold 3.8% paraformaldehyde in PBS (Wako Pure Chemical Industries, Osaka, Japan) for 20 min, and then washed and permeabilized with 0.4% Triton X-100 (Sigma) in PBS for 10 min at room temperature. Cells were incubated in blocking buffer containing 1% BSA and 2% normal goat serum (Dako, Glostrup, Denmark) for 30 min before overnight incubation at 4 °C with primary antibodies diluted in blocking buffer. After being washed, cells were incubated for 1 h with secondary antibodies. Cells were then washed, stained with DAPI (Molecular Probes), and mounted with fluorescent
mounting medium (Dako). The immunostained cells were imaged using a fluorescent microscope IX81 (Olympus Corp., Tokyo, Japan) with a ×100 oil, NA 1.40 objective or with a ×40, NA 0.95 objective, and MetaMorph Software (Molecular Devices, Sunnyvale, CA).

**Transferrin Recycling Assay**—Transferrin recycling was monitored by modification of a previously published assay (44). 6 × 10^4 cells were incubated in 24-well glass bottom plates for 48 h, and then in serum-free medium for 3 h to deplete endogenous transferrin. After incubation with 20 μg/ml of Alexa Fluor 488-tagged transferrin (Molecular Probes) in PBBS for 1 h, cells were treated with chemicals for 1 h. Cells were then fixed with 3.8% paraformaldehyde in PBS for 20 min at RT, washed with PBS containing 0.1% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma), and mounted with fluorescent mounting medium (Dako).

**Flow Cytometry**—Cells were harvested, washed with ice-cold PBS, and fixed in 70% ethanol. Cells were then washed twice with ice-cold PBS again, treated with RNase A (500 μg/ml; Sigma) at 37 °C for 1 h, and stained with propidium iodide (25 μg/ml; Sigma). The DNA content of the cells was analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

**Animal Experiments**—Antitumor effect of AMF-26 was tested in vivo against human BSY-1 xenografts in mice. Animal care and treatment was performed in accordance with the guidelines of the Animal Use and Care Committee of the Japanese Foundation for Cancer Research, and conformed to the NIH Guide for the Care and Use of Laboratory Animals. Female nude mice with BALB/c genetic backgrounds were purchased from Charles River Japan, Inc. (Yokohama, Japan), maintained under specific pathogen-free conditions, and provided with sterile food and water ad libitum. Each nude mouse was subcutaneously inoculated with the generated tumor fragment of size 3 × 3 × 3 mm. When the tumors reached a volume of 100–300 mm³, animals were randomly divided into control and AMF-26 groups (each group containing five mice) and daily administration of AMF-26 was then started (day 0). We examined the toxic dose of AMF-26 at a single oral administration. The maximum tolerable dose turned out to be 100 mg/kg of body weight. The experimental group of mice was orally administered a given dose of AMF-26 (83 or 100 mg/kg of body weight) on a daily basis from day 0 to 5. The control group of mice was orally administered with 0.05% Cremophor EL (Sigma) instead of AMF-26. Tumor volume of the tumor-bearing mice was measured as described previously (22). The length (L) and width (W) of the subcutaneous tumor mass of live mice were measured using calipers. Tumor weight was calculated as tumor weight = (L × W²)/2. To assess toxicity, the body weights of the tumor-bearing mice were measured.

**Statistical Analysis**—Pearson correlation coefficients were calculated for the COMPARE analysis and statistical correlation. The two-sided Mann-Whitney U test was used to assess the statistical significance of the antitumor efficacy of AMF-26 in relative tumor growth ratio on days 3, 7, 11, 14, 17, and 21. The number of samples is indicated in the description of each experiment. All statistical tests were two-sided.

**RESULTS**

**Identification of AMF-26 as a Possible Arf1-ArfGEF Inhibitor**—The JFCR39 panel is a powerful tool for the in silico screening of compounds with specific pharmacological activities but with different chemical structures. Seed compounds with desirable pharmacological activities can be used to extract candidates with a similar mode of action in the data base by COMPARE analysis. The data base of our cell line panel holds information on 4,000 compounds, including anticancer drugs and known inhibitors of various biological pathways. Employing this protocol, JFCR39 has been successful in identifying several new anticancer agents, such as a new telomerase inhibitor (FJ5002) (45), an inhibitor of topoisomerase I and II (MS-247) (19), and a novel phosphatidylinositol 3-kinase inhibitor (ZSTK474) (22, 46, 47). For instance, the novel PI3K inhibitor ZSTK474 was structurally different from the known PI3K inhibitor LY294002, although their respective fingerprints were similar. We therefore reasoned that COMPARE analysis would be a suitable method for screening structurally diverse compounds to identify those with a similar mode of action to BFA.

When BFA was used as a seed in COMPARE analysis, AMF-26 was identified to be the compound with a highest correlation coefficient (r = 0.831) in the data base based on the fingerprint profile. AMF-26 was the only compound whose r value was higher than 0.4,000 compounds. As shown in Fig. 1C, the fingerprint of AMF-26 was similar to that of BFA, which suggested that AMF-26 has a similar biological mode of action to BFA. The mean logarithm of GI₅₀ for AMF-26 was −7.33 (at 47 nM), which is comparable with that of BFA (−7.37 at 43 nM). These results indicated that AMF-26 possesses strong growth inhibitory activity as well as that of BFA.

**Disassembly of the Golgi Apparatus Caused by AMF-26—COMPARE analysis indicated that AMF-26, like BFA, may be a Golgi disruptor. Therefore, we first examined whether AMF-26 induces Golgi disassembly by immunofluorescence staining with a monoclonal antibody to Golgi brefeldin A-resistant guanine nucleotide exchange factor 1 (GBF1). GBF1 is localized to the cis-Golgi apparatus and plays a role in vesicular trafficking by activating Arf1 (48). In control cells, GBF1 was observed in the perinuclear region, forming a ribbon-like structure (Fig. 2A). However, addition of BFA caused a rapid release of GBF1 into the cytoplasm (Fig. 2B). These results were in agreement with previously published reports (48). When the cells were treated with AMF-26, GBF1 was observed to be dispersed from the perinuclear region throughout the cytoplasm (Fig. 2C), which indicated that AMF-26 disrupted the Golgi apparatus. Moreover, this observation was confirmed by live imaging using the MDA-MB-435 stably expressing GFP-conjugated clathrin light chain a (CLCa) cells (supplemental Video S1, A–C). In MDA-MB-435/GFP-CLCa cells, the intensity of fluorescence signals of GFP-CLCa were periodically increased and decreased at the TGN, suggesting that the formation of clathrin-coated pits occurs synchronously and periodically at the TGN (23). Disruption of the Golgi apparatus was concurrent with the disappearance of GFP-CLCa signals suggesting that CLCa was affected by AMF-26. Next, we examined whether the...
**FIGURE 1. Discovery of AMF-26 as a potent Golgi disruptor.** Chemical structure of (A) BFA and (B) AMF-26. C, growth inhibition against a panel of 39 human cancer cell lines. The mean graph was produced by computer processing of the 50% growth inhibition (GI50) values as described under “Experimental Procedures.” Logarithm of the GI50 value for each cell line is indicated. In the plot, columns to the right of zero indicate sensitivity of the cell line to the compound, and columns to the left indicate resistance to the compound. The x axis represents the logarithm of difference between the mean of GI50 values for 39 cell lines and the GI50 value for each cell line in the JFCR39 panel. The mean graph of AMF-26 (right column) is very similar to that of BFA (left column) (Pearson correlation coefficients; r = 0.831). MG-MID, the mean of log GI50 values for 39 cell lines; △, the logarithm of difference between the MG-MID and the log GI50 of the most sensitive cell line; Range, the logarithm of difference between the log GI50 of the most resistant cell line and the log GI50 of the most sensitive cell line. One scale represents one logarithm difference. Quantification of the GI50 value was represented as the mean of four different experiments. Br, breast; CNS, central nervous system; Co, colon; Lu, lung; Me, melanoma; Ov, ovarian; Re, renal; St, stomach; xPg, prostate.
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AMF-26-induced disruption of Golgi was a reversible process. The normal Golgi structure reappeared in the perinuclear area 4 h after washout of drugs with drug-free medium (Fig. 2D), which indicated that the effect of AMF-26 against Golgi was fully reversible like that of BFA (49).

Several tubulin polymerization inhibitors (e.g. nocodazole and vincristine) and ionophores (e.g. bafilomycin A1 and monensin) are known to disperse the Golgi apparatus (50, 51) and vincristine) and ionophores (e.g. bafilomycin A1 and monensin) are known to disperse the Golgi apparatus (50, 51) and disrupt microtubules (supplemental Fig. S1), into the cytoplasm of AMF-26- or BFA-treated cells. Scale bar, 20 μm. D, BSY-1 cells were treated with AMF-26 (1 μM) or BFA (1 μM) for 1 h, then washed with fresh medium and subsequently cultured without drugs for the indicated time. Thereafter, cells were fixed and stained with an antibody against GBF1. The images were analyzed by MetaMorph software. After the cells were cultured in medium without AMF-26 for 4 h, the normal Golgi structure reappeared in the perinuclear area. Symbols represent the following: open circle, AMF-26; cross, BFA. Error bar, S.D.

Error bar

Comparison between the BFA and AMF-26 Complexes—To support this conclusion, we performed computer modeling/MD simulations. The octahydronaphthalene ring of AMF-26 (HP1, HP2, and DA1) was superimposed onto the lactone ring of BFA (HP1, HP2, and HA1) by SUPERPOSE (Fig. 4C). During the MD trajectory, the octahydronaphthalene ring of AMF-26 resided in the initial position. The refined model of the Arf1-AMF-26-Sec7 domain complex was compared with the X-ray structure of the Arf1-BFA-Sec7 domain complex (Fig. 4, D and E, supplemental Table S2). The same hydrophobic residues (Val-53, Thr-64, Trp-66, and Tyr-81 of Arf1 and Met-194, Thr-197, and Val-204 of Sec7 domain) interacted with the octahydronaphthalene ring of AMF-26 and the lactone ring of BFA. The hydrophobic interactions correspond to the matching of hydrophobic property spheres (HP1 and HP2) by SUPERPOSE. The positively charged Arg-19 of Arf1 interacted with the hydroxyl oxygen of AMF-26 and the carbonyl oxygen of BFA. This electrostatic interaction accounted for the match-
ing of hydrogen-bond acceptors (DA1 of AMF-26 and HA1 of BFA). In addition, the hydroxyl group of AMF-26 hydrogen-bonded to the side chain of Thr-64 of Arf1. Instead of the hydrogen bond between the hydroxyl oxygen of the lactone ring of BFA and the amide nitrogen of Asp-67 of Arf1, the amide oxygen of AMF-26 hydrogen-bonded with the main chain of Glu-54 of Arf1. This computer modeling/MD simulation suggested that AMF-26 bound to the contact surface of the Arf1-Sec7 domain where BFA is known to bind.

Effects of AMF-26 on the cis-Golgi, TGN, and Recycling Endosomes—To clarify the mechanism of Golgi disruption by AMF-26, we first examined the effect of AMF-26 on localization of cis-Golgi-associated proteins, βCOPI, GBF1, and p58/ERGIC53. COPI is the most important coat protein in facilitating retrograde intracellular transport from Golgi to the endoplasmic reticulum (ER) (52). In control cells, most of the βCOPI was localized on the cis-Golgi membrane, whereas the addition of AMF-26 caused a rapid release of βCOPI into the cytoplasm (Fig. 5A). GBF1, primarily localized in cis-Golgi as shown in Fig. 2, was dispersed into the cytoplasm of cells treated with AMF-26. Furthermore, treatment with AMF-26 caused the redistribution of p58/ERGIC53, a marker for the ER-Golgi intermediate compartment (ERGIC) (53), from its normal compact localization to a dispersed cytoplasmic
localization (Fig. 5B). These effects were similar to those of BFA.

We also examined the effects of AMF-26 on proteins associated with the TGN. AMF-26 affected adaptin γ or adaptin δ, constituents of AP-1 or AP-2, respectively (54), and eventually resulted in redistribution from the perinuclear region into the cytosol (Fig. 5, C and D).

Concerning recycling endosomes, treatment of BFA is known to induce extensive formation of membrane tubules from endosomes (10, 55). Using Alexa 488-labeled transferrin, localization (Fig. 5B). These effects were similar to those of BFA.

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Concerning recycling endosomes, treatment of BFA is known to induce extensive formation of membrane tubules from endosomes (10, 55). Using Alexa 488-labeled transferrin,
we analyzed the effect of AMF-26 on the formation of tubular endosomes. AMF-26 induced enlargement and tubulation of transferrin-positive endosomes (Fig. 5E), suggesting that AMF-26 has a similar effect on early/recycling endosomes as BFA.

**Correlation between Cell Growth Inhibition and Golgi Disruption—**It is still uncertain whether the growth-inhibitory activity of BFA is related to its effects on Golgi disruption (56, 57). We examined the correlation between cell growth inhibition and Golgi disruption activity to investigate the causal relationship of these two events. Specifically, we examined the activities of AMF-26 and its five derivatives AMF-14, AMF-35, AMF-46, AMF-48, and AMF-59 (structural formula of each compound shown in Fig. 6A). In BSY-1 cells, AMF-26 exhibited the strongest activity of cell growth inhibition with a GI50 of 12 nM and that of Golgi disruption with an EC50 of 27 nM. AMF-35 displayed the weakest activities with a GI50 and EC50 of 14 and 4.9 μM, respectively, (Fig. 6, B and C). We next investigated whether the Golgi disruption activities (the logarithm of EC50) of these six compounds were related to their growth inhibitory activities (the logarithm of GI50). Our analysis revealed a statistically significant correlation (r = 0.92 and p = 0.009) between these two activities (Fig. 6D). A statistically significant correlation between the logarithm of EC50 and the logarithm of GI50 was also observed when six other cell lines were treated with AMF-26 (r = 0.93 and p = 0.02, Fig. 6E). Taken together, our results suggested that there was a causal relationship between the Golgi disruption and cell growth inhibition.

**AMF-26 Caused Apoptotic Cell Death—**After a 24-h incubation with AMF-26, the sub-G1 population increased in a dose-dependent manner over that of the DMSO-treated controls, i.e. 8.4 (AMF-26, 100 nM) versus 1.9% (Fig. 7A). These differences in the sub-G1 population were even greater after 48 h, i.e. AMF-26-treated cells 13.1 (AMF-26, 100 nM) versus 2.1% (Fig. 7A). Furthermore, cleavage products of PARP were assessed by Western blot analysis of cell lysates (Fig. 7B). After incubation for 24 h, a band corresponding to cleaved PARP (89-kDa) was significantly stronger in cell extracts of AMF-26-treated cells compared with DMSO-treated cells. After 48 h, the larger accumulation of cleaved PARP in AMF-26-treated cells was obvious, suggesting apoptosis of AMF-26-treated cells.

**Analysis of the Antitumor Efficacy of Orally Administered AMF-26 Using Xenografts of Human Breast Cancer BSY-1—**Finally, we evaluated the antitumor activity of AMF-26 against human breast cancer BSY-1 xenografts. After formation of the tumors (100–300 mm3), mice were orally administered 0 (control vehicle), 83, or 100 mg/kg of AMF-26 for 5 consecutive days. Administration of AMF-26 at 83 or 100 mg/kg induced almost complete tumor regression on day 21 (Fig. 7C, *upper panel*). To assess toxicity, we measured the body weight of the tumor-bearing mice. The weight of the tumor-bearing mice was slightly reduced by administration of AMF-26. After termination of the administration, the weight was rapidly regained but was not fully recovered during the observation (Fig. 7C, *lower panel*). These data suggested that AMF-26 treatment did not cause serious irreversible side effects.

**DISCUSSION**

In this study, we identified a novel Golgi disruptor, AMF-26 using COMPARE analysis followed by computer modeling/MD simulation and biological validations. AMF-26 is thought to induce Golgi disruption via the inhibition of Arf1 activation. The Golgi disrupting activity of AMF-26 was significantly correlated with its growth-inhibiting activity. Finally, our results showed that oral administration of AMF-26 induced the regression of human breast cancer BSY-1 xenografts.

The Arf family of small GTPases plays a major role in maintaining Golgi structure and driving Golgi membrane traffic (5, 7). It is known that the BFA-sensitive large ArfGEFs, such as GBF1, BIG1, and BIG2, are predominantly localized to the cis-Golgi and TGN. After activation by GBF1, Arf1 mediates COPI...
A previous study described the antitumor activity of BFA in early stage mouse subcutaneous models (13). In the course of...
these studies, BFA was injected intraperitoneally twice a day. Frequent administration was necessary because the clearance of BFA is rapid (less than 20 min) (13), consistent with the observation that this compound is a substrate of glutathione S-transferase (63). Here, we demonstrated that oral administration of AMF-26 once a day induced almost complete regression of human breast cancer BSY-1 xenografts in nude mice. To the best of our knowledge, this is the first report describing the oral administration of an inhibitor targeting Arf1 activation that elicits strong antitumor activity without severe body weight loss. Further study on the pharmacokinetic profile of AMF-26 remains to be performed.

Very recently, AMF-26 was identified as an angiogenesis inhibitor by Watari et al. (64), who revealed that inhibition of VEGF receptor phosphorylation and NF-κB signaling was involved in antiangiogenic activity in human umbilical vein endothelial cells. Therefore, the in vivo antitumor activity of AMF-26 shown in the present study may have partly resulted from its antiangiogenic activity in addition to its direct tumor growth inhibition.

The COMPARE analysis is a powerful tool that can be used to identify small-molecule enzyme inhibitors, for example, telomerase inhibitor (FJ-5002) (45), a topoisomerase I and II inhibitor (MS-247) (19), and PI3K inhibitor (ZSTK474) (22, 46, 47). In this study, we exploited the COMPARE analysis to screen BFA-like small molecule inhibitors. Using this approach, we identified AMF-26 as a novel inhibitor of Arf1 activation, despite the fact that its chemical structure does not resemble that of BFA. From these results, the screening of chemicals guided by COMPARE analysis appears to be useful for the discovery of novel drug candidates targeting various biological functions including the Golgi system.

In conclusion, we identified a novel Golgi disruptor targeting Arf1 activation, AMF-26, and showed that it induced complete regression of human breast cancer BSY-1 xenografts. These data suggest that AMF-26 is a novel drug candidate for cancer treatment targeting Arf1 activation.

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