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Discovery of inhibitors of membrane traffic from a panel of clinically effective anticancer drugs

Hiroko Kamata\textsuperscript{a,b}, Sotaro Sadahiro\textsuperscript{b} and Takao Yamori\textsuperscript{*a,†}

\textsuperscript{a}Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 3–8–31 Ariake, Koto-ku, Tokyo 135–8550, Tokyo 135-8550, Japan,

\textsuperscript{b}Tokai University, Graduate School of Medicine, 143 Shimosouya, Isehara-shi, Kanagawa 259-1193, Japan

\textsuperscript{†}Current affiliation: Pharmaceuticals and Medical Devices Agency, Shin-Kasumigaseki Building, 3-3-2 Kasumigaseki, Chiyoda-ku, Tokyo 100-0013, Japan

\textsuperscript{*}To whom correspondence should be addressed. e-mail: yamori-takao@pmda.go.jp
Summary

In addition to their major targets, clinically effective drugs may have unknown off-targets. By identifying such off-targets it may be possible to repurpose approved drugs for new indications. We are interested in the Golgi apparatus as a novel target for cancer therapy, but there is a paucity of candidate Golgi-disrupting drugs. Here, we aimed to identify Golgi-disrupting compounds from a panel of 34 approved anticancer drugs by using HBC-4 human breast cancer cells and immunofluorescence microscopy to visualize the Golgi apparatus. The screen identified five drugs having Golgi-disrupting activity. Four of them were vinca alkaloids (vinorelbine, vindesine, vincristine and vinblastine), and the fifth drug was eribulin. This is the first study to demonstrate that vinorelbine, vindesine and eribulin possess Golgi-disrupting activity. The 5 drugs are known to inhibit tubulin polymerization and to induce microtubule depolymerization. Interestingly, a microtubule-stabilizer paclitaxel did not induce Golgi-disruption, suggesting that the three-dimensionally preserved microtubules are partly responsible for maintaining the Golgi complex. Concerning eribulin, a noteworthy drug because of its high clinical efficacy against advanced breast cancer, we further confirmed its Golgi-disrupting activity in three different human breast cancer cell lines, BSY-1, MDA-MB-231 and MCF-7. Golgi-disruption may contribute to anticancer efficacy of eribulin. In conclusion, the present study revealed that four vinca alkaloids and eribulin possessed potential Golgi-disrupting activity among a panel of 34 approved anticancer drugs. Other drugs covering various molecular-targeted drugs and classical DNA-damaging drugs showed no Golgi-disrupting effect. These results suggest that tubulin polymerization-inhibitors might be promising candidate drugs with Golgi-disrupting activity.

Keywords: Anticancer Drug, Golgi-disrupting, Repurposing, Unknown target, Cancer Therapy, Drug Discovery,
**Introduction**

Cancer cells have heterogeneous malignant properties. Various cellular targets for therapeutic drugs have been identified to treat cancer. Such targets include signaling molecules \(^1\), the proteasome \(^2\), histone deacetylases \(^3\), heat-shock protein 90 \(^4\) and tumor angiogenesis-inducing factors \(^5\). Drugs that act on these targets (molecular targeted drugs) directly interfere with cancer cell-specific properties at the molecular level and therefore efficiently eliminate cancer cells. Accordingly, many of these anticancer drugs are highly effective and have been approved for clinical use. However, further development of novel molecular targets and identification of their inhibitors is required in order to eradicate cancers that are currently intractable.

We are interested in the Golgi apparatus as a novel target for anticancer drugs. The Golgi is a cellular organelle composed of a sac-like bilayer membrane, where proteins synthesized in the endoplasmic reticulum are stored and undergo glycosylation and processing. Membrane trafficking occurs from the endoplasmic reticulum to the Golgi and then to the cell membrane or other cellular compartments \(^6\) \(^7\). Colchicine, vinblastine and nocodazole, which are inhibitors of tubulin polymerization, reportedly induce Golgi disruption \(^8\). Brefeldin A (BFA) is a well-known Golgi-disruptor, which induces Golgi disruption by inhibiting the interaction between ADP-ribosylation factor 1 (Arf1) and guanine nucleotide exchange factor \(^9\) \(^10\). We recently identified a novel compound AMF-26 as a new Golgi-disruptor that has a mode of action similar to BFA \(^11\). Furthermore, we demonstrated that AMF-26 induced complete regression in the treatment of a xenograft of human breast cancer BSY-1 without severe loss of body weight \(^11\). Based on these results, the Golgi appears to be a promising target for cancer treatment. However, to date, only a few Golgi-disrupting compounds have been identified. Thus, we reasoned that it would be useful to screen for compounds with Golgi-disruptor activity.

In addition to their major targets, clinically effective drugs may also have unknown off-targets. Identification of such off-targets will enable approved drugs to be repurposed for new indications. In the present study, we aimed to discover potential Golgi-disrupting compounds from a panel of 34 approved anticancer drugs by using HBC-4 human breast cancer cells. As a result, five drugs were found to exhibit Golgi-disrupting activity. Four of them were vinorelbine, vindesine, vincristine and vinblastine, which belong to vinca alkaloids. The fifth one was eribulin, a recently approved drug with the indication of advanced breast cancer \(^12\) \(^13\) \(^14\). This is the first study to report that vinorelbine, vindesine and eribulin induces Golgi-disruption. Intriguingly, the primary target of the 5 drugs is tubulin polymerization.
Other drugs covering various molecular-targeted drugs and classical DNA-damaging drugs showed no Golgi-disrupting effect.

**Materials and Methods**

**Chemicals:** To screen Golgi-disruptors, we used 34 approved anticancer drugs. Vinorelbine, vindesine, vincristine, and vinblastine were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). Eribulin was manufactured by Eisai Co., Ltd. (Tokyo, Japan) as trade name Halaven. Other drugs used in this screen were obtained from SCADS inhibitor kit, which was kindly provided by Screening Committee of Anticancer Drugs supported by Grant-in-Aid for Scientific Research on Priority Area “Cancer” from the Ministry of Education, Culture, Sports, Science and Technology, Japan. All the drugs tested in this study are listed in Table 1. We used BFA and nocodazole (Sigma-Aldrich, Tokyo, Japan), which are known Golgi-disruptors, as positive controls for the screening process.

**Cell lines:** Human breast cancer cell lines HBC-4, BSY-1, MCF-7 and MDA-MB-231 cells, which are the members of a panel of 39 human cancer cell lines known as JFCR39, were used for the in vitro studies. All the cell lines were cultured in RPMI 1640 medium (Wako Pure Chemical Industries, Tokyo, Japan) 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.

**Evaluation of Golgi-disrupting activity by immunofluorescence microscopy:** Golgi-disrupting activity of test compounds was evaluated as described previously. Briefly, HBC-4 cells were plated in 24-well plates for 48 h and then treated with test compounds individually at a final concentration of 1 µM. Known Golgi disrupting compounds, BFA and AMF-26, reportedly induced Golgi disruption at 1 µM. To find a compound having comparable Golgi-disrupting activity to them, we chose a concentration of 1 µM for screening Golgi-disrupting compound. After 1 h incubation with a test compound, the 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 0.5% Triton X-100 (Sigma-Aldrich, Tokyo, Japan) 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 mouse anti-human Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor.
(GBF1, Golgi marker) antibody (clone 25; BD Biosciences, San Jose, CA, U.S.A.) diluted in blocking buffer. After washing in PBS, the cells were incubated for 1 h with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.). The cells were then washed in PBS, stained with DAPI (Molecular Probes, Eugene, OR, U.S.A.), and mounted with fluorescent mounting medium (Dako Japan, Tokyo, Japan). The immunostained cells were imaged using a fluorescence microscope IX81 (Olympus Corp., Tokyo, Japan) with a x40, NA 0.95 objective, and MetaMorph Software (Molecular Devices, Sunnyvale, CA, U.S.A.). The hit compounds were further examined for Golgi-disrupting activity against an additional panel of three human breast cancer cell lines, BSY-1, MCF-7 and MDA-MB-231, using the same immunostaining method.

**Results**

**Screening a panel of approved anticancer drugs for Golgi-disrupting activity**

We used anti-GBF1 antibody to visualize the Golgi apparatus. GBF1 is localized to the cis-Golgi apparatus and plays a role in vesicular trafficking by activating Arf1 \(^{(11)}\). In untreated cells GBF1 was observed in the perinuclear region, where it formed a ribbon-like (or a cap-like) structure (Figure 1). However, addition of a Golgi-disrupting compound caused a rapid release of GBF1 into the cytoplasm (Figure 1). Using this characteristic, we assessed the Golgi-disrupting effect of 34 approved anticancer agents in HBC-4 breast cancer cells. The Golgi-disrupting effect was evaluated by counting cells in a specified single field of view under light microscopy. A test specimen was considered positive for the Golgi-disrupting effect when disruption was observed in at least 80% of the counted cells. Nocodazole and BFA, an inhibitor of tubulin polymerization and Arf1 activation, respectively, were used as positive controls for Golgi-disruption \(^{(8)}\) \(^{(9)}\). We confirmed that both of these compounds induced Golgi-disruption in HBC-4 cells (Table 1).

Based on the results of these studies, five of the 34 drugs were identified as displaying a Golgi-disrupting effect (Table 1 and Figure 1). Four of these compounds were vinorelbine, vindesine, vincristine and vinblastine, which are vinca alkaloids and known inhibitors of tubulin polymerization \(^{(18)}\). The fifth compound identified in the screen showing a Golgi-disrupting effect was eribulin. Although its mode of action to tubulin is different from that of the 4 vinca alkaloids, eribulin is also a tubulin polymerization inhibitor \(^{(19)}\). Of note, paclitaxel, which is a microtubule-stabilizer, did not show a Golgi-disrupting effect. The other drugs screened in this study, including molecular-targeted drugs and classical DNA-damaging drugs, also displayed no Golgi-disrupting activity.
Dose-dependent Golgi-disrupting activity of the hit compounds

The dose-dependency of the hit compounds for Golgi-disruption was analyzed (Figure 2). The molar concentration of a compound required for 50% Golgi-disruption (EC50) in HBC4 cells was determined. All of the hit drugs showed Golgi-disrupting activity at sub-micro molar concentrations. The EC50s of nocodazole, vinorelbine, vindesine, vincristine, vinblastine and eribulin were 40.7 ± 4.4 nM, 19.4 ± 1.0 nM, 21.5 ± 4.9 nM, 19.4 ± 2.0 nM, 27.4 ± 3.5 nM and 120 ± 10.1 nM, respectively.

Visualization of the Golgi-disrupting effect of eribulin in four different human breast cancer cell lines

To examine whether eribulin exerts a Golgi-disrupting effect on breast cancer cells other than HBC4 cells, we repeated our analysis using three additional human breast cancer cell lines; BSY-1, MDA-MB-231 and MCF-7 (Figure 3). In all cases, GBF1 was localized in the vicinity of the nucleus of DMSO-treated cells (control). By contrast, all cell lines treated with 1 μM of eribulin showed GBF1 had dispersed into the cytoplasm. A similar observation was made when cells were treated with BFA. These results clearly demonstrated that eribulin had a Golgi-disrupting effect in all four human breast cancer cell lines tested in this study.

Discussion

An emerging strategy of drug development involves identifying off-targets of clinically effective drugs, thereby enabling approved drugs to be used for new indications. It is also becoming clear that a comprehensive understanding of the spectrum of effects exerted by an anticancer agent is fundamental for evaluating its efficacy and toxicity profile. We previously showed that Golgi is a promising novel target of anticancer drug. Therefore, in the present study, we aimed to identify Golgi-disrupting compounds from a panel of 34 approved anticancer drugs using HBC-4 breast cancer cells. Our screen gave 5 hits that displayed Golgi-disrupting activity. Four of them were vinorelbine, vindesine, vincristine and vinblastine, which are all vinca alkaloids. The fifth hit in our screen for Golgi-disrupting activity was eribulin, which is a synthetic analogue of the marine natural product halichondrin B. These 5 drugs are known to inhibit tubulin polymerization and to induce microtubule depolymerization. Interestingly, microtubules reportedly play an important role in the organization of the Golgi apparatus. Thus, it is perhaps unsurprising that these vinca alkaloids and eribulin induced Golgi-disruption. Indeed, Golgi-disruption by vinblastine and vincristine has been reported previously. However, this is the first study to experimentally demonstrate that vinorelbine, vindesine and eribulin...
possess Golgi-disrupting activity.

In contrast to the tubulin polymerization-inhibitors, a microtubule-stabilizer paclitaxel did not show a Golgi-disrupting effect. If the lack of Golgi-disrupting activity is confirmed in other microtubule-stabilizers such as docetaxel, it will be very interesting what makes such difference. The Golgi complex in mammalian cells is composed of cisternal stacks that function in processing and sorting of membrane and luminal proteins during transport from the site of synthesis in the endoplasmic reticulum to lysosomes, secretory vacuoles, and the cell surface. The Golgi stacks are usually arranged as an interconnected network in the region around the centrosome, which is the major organizing center for cytoplasmic microtubules. Intriguingly, Alvarez and Szul29) reported that BFA disrupted the organization of the microtubule and the actin cytoskeletons. A close relation thus exists between Golgi elements and microtubules 8) 26). Collectively, it might be considered that after disruption of microtubules by tubulin-polymerization inhibitors, the Golgi stacks are disconnected from each other, partly broken up, and dispersed in the cytoplasm. On the other hand, after treatment by microtubule-stabilizers, the three-dimensionally preserved microtubules might be partly responsible for the maintenance and function of the Golgi complex.

Among the 5 hit drugs, eribulin is most recently approved with the indication of metastatic breast cancer 12) 13) 14) and malignant soft tissue sarcoma 30). Eribulin is now attracting a lot of attention because of its high clinical efficacy against advanced breast cancer. In the mode of inhibiting tubulin polymerization, eribulin is unlike vinca alkaloids. Eribulin predominantly binds to a small number of high affinity sites at the plus end of existing microtubules 24) 25). The anticancer efficacy of eribulin is thought to involve a number of different mechanisms 31) 32), including reducing the migration and invasive capacity of tumor cells, reversing the tumor cell phenotype from epithelial–mesenchymal transition to mesenchymal–epithelial transition states, as well as reducing tumor microenvironment abnormality. In addition, we presently found that eribulin induces Golgi-disruption in HBC-4 cells, which may contribute to its anticancer action. As this was the first observation, we further confirmed this phenomenon in three additional breast cancer cell lines; BSY-1, MDA-MB-231 and MCF-7. Whether eribulin induces Golgi-disruption in cancers other than breast cancer is interesting problem to be examined in future.

In conclusion, the present study revealed that four vinca alkaloids and eribulin possessed potential Golgi-disrupting activity among a panel of 34 approved anticancer drugs. Other drugs covering various molecular-targeted drugs and classical DNA-damaging drugs
showed no Golgi-disrupting effect. These results suggest that tubulin polymerization-inhibitors might be promising candidate drugs with Golgi-disrupting activity.

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Conflict of interest

The authors declare no conflict of interest.
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Table 1 Screening for Golgi-disrupting compounds from a panel of approved anticancer drugs

| Drug name  | Golgi-Disruption | Known main mode of action |
|------------|------------------|---------------------------|
| Cisplatin  | N                | Crosslinking DNA          |
| Mitomycin C| N                | Crosslinking DNA          |
| Daunorubicin| N                | DNA Intercalation, Inhibition of topoisomerase II |
| Bleomycin  | N                | DNA Strand break          |
| Actinomycin D| N              | Inhibition of DNA-primed RNA synthesis |
| Camptothecin| N                | Inhibition of topoisomerase I |
| Doxorubicin| N                | DNA Intercalation, Inhibition of topoisomerase II |
| Aclarubicin| N                | DNA Intercalation, Inhibition of topoisomerase I/II |
| Etoposide (VP-16)| N          | Inhibition of topoisomerase II |
| Ubenimex  | N                | Inhibition of aminopeptidase B |
| Fluorouracil| N               | Inhibition of thymidylate synthase |
| Methotrexate| N               | Inhibition of dihydrofolate reductase |
| Paclitaxel | N                | Stabilization of microtubule |
| Vinorelbine| P                | Inhibition of tubulin polymerization |
| Vinodesine | P               | Inhibition of tubulin polymerization |
| Vinceristine| P               | Inhibition of tubulin polymerization |
| Vinblastine| P                | Inhibition of tubulin polymerization |
| Eribulin   | P                | Inhibition of tubulin polymerization |
| Flutamide  | N                | Antagonizing androgen receptor |
| Tamoxifen  | N                | Antagonizing estrogen receptor |
| Imatinib   | N                | Inhibition of BCR-ABL/KIT tyrosine kinase |
| Nilotinib  | N                | Inhibition of BCR-ABL/KIT tyrosine kinase |
| Dasatinib  | N                | Inhibition of BCR-ABL/SRC tyrosine kinase |
| Gefitinib  | N                | Inhibition of epidermal growth factor receptor tyrosine kinase |
| Erlotinib  | N                | Inhibition of epidermal growth factor receptor tyrosine kinase |
| Lapatinib  | N                | Inhibition of epidermal growth factor receptor /HER2 tyrosine kinase |
| Vorinostat | N                | Inhibition of histone deacetylases |
| Temsirolimus| N              | Inhibition of mammalian target of rapamycin |
| Everolimus | N                | Inhibition of mammalian target of rapamycin |
| Sorafenib  | N                | Inhibition of multi-kinases |
| Sunitinib  | N                | Inhibition of multi-kinases |
| Pazopanib  | N                | Inhibition of multi-kinases |
| Bortezomib | N                | Inhibition of proteasome |
| Nocodazole | P                | Inhibition of tubulin polymerization |
| Brefeldin A| P                | Disruption of Golgi |
| Cytochalasin D| N          | Inhibition of actin polymerization |
The Golgi-disrupting activities of 34 approved anticancer drugs were tested. HBC4 human breast cancer cells were seeded and cultured for 48 h and exposed to individual drugs at a final concentration of 1 μM for 1 h at 37 °C. The cells were then fixed and stained with an antibody to the Golgi marker, GBF1 as described in Materials and Methods. The photographs of Golgi-disruption induced in HBC-4 cells by tested drugs are shown in Figure 1. The Golgi-disrupting effect was measured by visually counting the cells in which Golgi was dispersed in a specified single field of view under light microscopy. A test specimen was considered positive (P) for the Golgi-disrupting effect when the disruption was observed in at least 80% of the counted cells. Samples not reaching this threshold were considered negative (N).
HBC4 human breast cancer cells were exposed to individual drugs at a final concentration of 1 μM for 1 h at 37 °C. The cells were then fixed and stained with an antibody to the Golgi marker, GBF1 as described in the Materials and Methods. The nucleus appeared blue by DAPI-staining, and the Golgi appeared green by immunostaining with anti-GBF1 antibodies. When Golgi was intact, it clearly localized in the vicinity of the nucleus as indicated by white arrowheads in the panel of DMSO. The Golgi was dispersed into the cytoplasm when the cells were treated by vinblastine, vincristine, vindesine, vinorelbine or eribulin, all of which are tubulin polymerization inhibitors. In contrast, paclitaxel, a stabilizer of microtubules, showed no apparent effect on Golgi structure as indicated by white arrowheads.
To investigate the dose-dependency of Golgi-disrupting activity of the five hit compounds, HBC4 cells were treated with each hit compound or a known Golgi-disruptor (nocodazole) at the indicated concentrations for 1 h as described in the Materials and Methods. The number of cells with disrupted Golgi and the total cell number were counted in eight separate fields of view. The ratio of the number of cells with disrupted Golgi was then plotted. Symbols used are indicated in the figure.
Figure 3 Golgi-disrupting activity of eribulin in four different human breast cancer cell lines

Four human breast cancer cell lines (HBC-4, BSY-1, MDA-MB-231, and MCF-7) were exposed to 1 μM eribulin at 37°C for 1 h, and subjected to Golgi staining with an anti-GBF1 antibody and nuclear staining with DAPI as described in the Materials. In DMSO-treated cells, the Golgi apparatus was observed to have converged in the vicinity of the nucleus as indicated by white arrowheads. In cells treated with BFA, which is a known Golgi inhibitor, the Golgi apparatus was dispersed into the cytoplasm. All the four cell lines treated with eribulin showed dispersed Golgi.