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Differential Effects of the Putative GBF1 Inhibitors Golgicide A and AG1478 on Enterovirus Replication

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The genus Enterovirus, belonging to the family Picornaviridae, includes well-known pathogens, such as poliovirus, coxsackievirus, and rhinovirus. These small, nonenveloped viruses contain a single-stranded RNA genome of positive polarity. The genome is approximately 7.5 kb in length and encodes a single large polyprotein, which is processed into capsid proteins, encoded in the P1 region, and the nonstructural P2 and P3 region proteins that mediate viral RNA replication.

Brefeldin A (BFA), a fungal metabolite, is a well-known inhibitor of enteroviruses. BFA blocks transport of cargo from the endoplasmic reticulum (ER) to the Golgi vesicles by disrupting the Golgi vesicles and ER-Golgi intermediate compartment (ERGIC). BFA impedes replication of several enteroviruses through inhibition of Golgi-specific BFA resistance factor 1 (GBF1), a regulator of secretory pathway integrity and transport. GBF1 mediates the GTP exchange of Arf1, which in activated form recruits coatamer protein complex I (COP-I) to Golgi vesicles, a process important in transport between the endoplasmic reticulum and Golgi vesicles. Recently, the drugs AG1478 and Golgicide A (GCA) were put forward as new inhibitors of GBF1. The effects of AG1478, but not those of GCA, could be countered by overexpression of Arf1, indicating a difference in their molecular mechanism of action. Consistent with this idea, we observed that GCA drastically reduced replication of enteroviruses poliovirus and coxsackievirus B3 (CVB3) and other human enterovirus species, whereas AG1478 had no effect at all on enterovirus replication. Time-of-addition studies and analysis of RNA replication using a subgenomic replicon both showed that GCA suppresses RNA replication of CVB3, which could be countered by overexpression of GBF1. These results indicate that, in contrast to AG1478, GCA inhibits CVB3 RNA replication by targeting GBF1. AG1478 and GCA may be valuable tools to further dissect enterovirus replication.

The Enterovirus genus, belonging to the Picornaviridae family, includes many important human pathogens, such as poliovirus, human rhinovirus, echovirus, and coxsackievirus. These are small, nonenveloped viruses that contain a single-stranded RNA genome of positive polarity. The genome is approximately 7.5 kb in length and encodes a single large polyprotein, which is processed into capsid proteins, encoded in the P1 region, and the nonstructural P2 and P3 region proteins that mediate viral RNA replication.

Brefeldin A (BFA), a fungal metabolite, is a well-known inhibitor of enteroviruses. BFA blocks transport of cargo from the endoplasmic reticulum (ER) to the Golgi vesicles by disrupting the Golgi vesicles and ER-Golgi intermediate compartment (ERGIC). The inhibitory effect of BFA on enterovirus replication is attributed to the inhibition of GBF1 and does not seem to involve BIG1 or BIG2 (2, 11). Besides enteroviruses, other plus-strand RNA viruses, such as mouse hepatitis virus and hepatitis C virus, also seem to rely on GBF1 for efficient replication (2, 8, 11, 21). The viral protein 3A of the enteroviruses poliovirus and coxsackievirus B3 (CVB3) has been shown to interact directly with GBF1 (22, 22a, 23), but the exact function of this interaction remains to be established.

Recently, two compounds, AG1478 and Golgicide A (GCA), have been proposed to specifically inhibit GBF1. AG1478 was identified by screening a library of compounds for their ability to induce Golgi complex disassembly (13). AG1478, known as an inhibitor of the epidermal growth factor receptor (EGFR), had effects on the Golgi membranes highly similar to those of BFA through a mechanism not involving the inhibition of EGFR. Arf1-GTP pulldown assays showed that AG1478 inhibited Arf1 activation. Furthermore, overexpression of GBF1 was shown to counter the effect of AG1478 on COP-I localization. Based on these results, AG1478 was proposed to be a GBF1 inhibitor.

GCA was identified in a high-throughput screen for small molecules that protected Vero cells from the effects of Shiga toxin (15). Similar to AG1478 and BFA, GCA was reported to fragment the Golgi vesicles and to inhibit Arf1 activation. Furthermore, overexpression of either wild-type GBF1 or the...
BFA-resistant mutant GBF1-M832L relieved the effects of GCA. In addition, the authors constructed a structural model of the catalytic Sec7 domain of GBF1 in complex with GCA, showing that GCA binds GBF1 at the same site as BFA. Collectively, their results provided convincing lines of evidence that GCA specifically inhibits GBF1 in a manner similar to BFA and does not act on BIG1 and BIG2.

BFA has been instrumental in elucidating the membrane requirements for enterovirus replication. Therefore, we investigated the effects of AG1478 and GCA on enterovirus replication after first characterizing the effects of these drugs on BGM cells, the cell line that we routinely use in our studies on coxsackievirus B3 replication. Treatment with other AG1478 or GCA fragmented the Golgi vesicles and caused dissociation of Arf1 and COP-I from Golgi membranes, yet these drugs had different effects on GBF1 localization. Interestingly, the effects of AG1478, but not those of GCA, could be countered by overexpression of Arf1. Next, GCA was found to abrogate enterovirus replication, whereas surprisingly AG1478 did not affect replication at all. Together these results indicate that AG1478 on one hand and GCA and BFA on the other hand have different mechanisms of action, leading to a disparate effect on enterovirus replication.

**MATERIALS AND METHODS**

**Cells and reagents.** Buffalo green monkey (BGM) kidney cells, HeLa cells, and baby hamster kidney 21 (BHK-21) cells were grown at 37°C in minimal essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Brefeldin A (BFA) (Sigma-Aldrich) was dissolved in methanol, and dimethyl sulfoxide (DMSO) was used to dissolve AG1478 (Sigma-Aldrich) and Golgicide A (GCA) (15). Unless indicated otherwise, the concentrations of BFA, AG1478, and GCA used in experiments were 2 μM (7.1 μM), 25 μM, and 10 μM, respectively.

**Viruses.** Coxsackievirus B3 (CVB3) was obtained by transfecting in vitro-transcribed RNA derived from the p53CB3/T7 plasmid. This plasmid contains the cDNA of CVB3 strain Nancy driven by a T7 RNA polymerase promoter (24). The euchromomycoiditis virus (EMCV) strain used in this study is the mengovirus strain, which was obtained upon transfection of in vitro-transcribed RNA from cDNA clone pM161.6 (6). Enterovirus 71 (EV71) (BeCr) and coxsackievirus A21 (CVA21) (Coe) were obtained from the National Institute for Public Health and Environmental Research (RIVM, Netherlands).

**Plasmids.** The VSV-GFP plasmid, kindly provided by P. Keller and K. Simons (Max Planck Institute of Molecular Biology and Genetics, Dresden, Germany), encodes a green fluorescent protein (GFP) fusion of the temperature-sensitive ts045 vesicular stomatitis virus G protein (VSV-G protein) (19). The plasmid coding for Gaussia luciferase, pCMV-Glu (CMV stands for cytomegalovirus, and Gluc stands for Gaussia luciferase), and the control plasmid, pEGFP-C1 (EGFP stands for enhanced GFP), were purchased from New England Biolabs and Clontech, respectively. Plasmids pEYFP-GBF1 wt (wt) and pEYFP-Glu were digested with restriction enzymes, and DNA transfactions were performed with 200 ng plasmid DNA. A21 (CVA21) (Coe) were obtained from the National Institute for Public Health and Environmental Research (RIVM, Netherlands).

**Replicon assay.** Replicon assays were performed as described previously (20). The p53CB3-LUC subgenomic replicon used contains the CVB3 cDNA in which the Ic capsid coding region is replaced by the firefly luciferase gene (24). The plasmid was linearized, purified, and transcribed in vitro by T7 RNA polymerase, and transcript RNAs were transfected into BGM cells. After transfection, the cells were cultured in the presence or absence of drugs at 37°C. At 2, 4, 6, and 8 h posttransfection, the cells were lysed and analyzed for firefly luciferase activity.

**Analysis of viral polypeptide processing in vivo.** In vivo labeling studies were performed as described previously (4). Briefly, BGM cells in a 24-well plate were infected with CVB3 at a multiplicity of infection (MOI) of 50. At 5 h.p.i., the cells were starved by incubating them in methionine-free medium for 30 min. Subsequently, 35S-labeled methionine was added for 30 min. At 6 h.p.i., the cells were lysed, and translation products were analyzed on a 10% polyacrylamide gel containing SDS, fixed, and then exposed to Kodak XAR film.

**RESULTS AND DISCUSSION**

AG1478 and GCA inhibit cargo transport through the secretory pathway. We determined the effects of the putative Golgi-specific BFA resistance factor 1 (GBF1) inhibitors AG1478 and Golgicide A (GCA) on the function of the secretory pathway in BGM cells, the cell line that we routinely use in our studies on enterovirus replication. First, we investigated the transport of the membrane-associated VSV-G protein by use of a temperature-sensitive mutant that is retained in the ER at a temperature of 40°C. When the temperature is switched to 32°C, the VSV-G protein is allowed to progress further along the secretory pathway to the plasma membrane (Fig. 1A). In BFA-treated cells, the VSV-G protein was absent from the plasma membrane after the temperature switch. Instead, it displayed a reticular and punctate staining which was previously identified to be the ER and ER exit sites (23). Treatment with either AG1478 or GCA had a similar effect, indicating that all drugs tested inhibit transport of membrane-bound proteins.

Second, we tested the effects of the drugs on secretion of soluble proteins by use of Gaussia luciferase (Gaussia-luc), a novel form of luciferase that is secreted by the cells in the supernatant (1, 16). At 16 h after transfection of BGM cells with pCMV-Glu, the medium was replaced, and drugs were added to the cells. After 2.5 h, the supernatant was analyzed for Gaussia-luc activity. The supernatant of untreated cells exhibited high luciferase activity, whereas a severe decrease in luciferase activity was detected upon treatment with BFA, AG1478, or GCA (Fig. 1B).

Together, these results show that, similar to BFA, AG1478 and GCA inhibit transport of both membrane-anchored proteins and secreted soluble factors.
AG1478 and GCA cause Golgi dispersal and inhibit COP-I recruitment. Next, we determined the effects of AG1478 and GCA on the Golgi integrity and localization of GBF1, Arf1, and COP-I in BGM cells. All drugs caused Golgi dispersal as shown by the fragmented localization of cis-Golgi matrix protein GM130 after a 1-h treatment (Fig. 1C). In untreated cells, GBF1, the putative target of the drugs, is localized to the Golgi apparatus (Fig. 1C). Upon addition of BFA or GCA, GBF1...
redistributed to a more diffuse localization. In contrast, GBF1 displayed a punctate pattern in AG1478-treated cells. Although AG1478 and GCA had a different effect on GBF1 localization, they had similar effects on Arf1 and COP-I localization. Arf1 and COP-I localized to the Golgi apparatus in untreated BGM cells, but drug-treated cells displayed a diffuse staining of Arf1 and COP-I with no apparent difference between the drugs (Fig. 1C).

The effects of GCA can be countered only by overexpression of GBF1, while the effects of AG1478 can also be countered by overexpression of Arf1. Previously it was shown that the effect of BFA on COP-I can be countered by overexpressing GBF1 (3, 10, 12) and that the BFA-resistant GBF1-M832L mutant was even more effective (12). This mutant was also reported to confer resistance to GCA (15). To test the ability of GBF1 to counter drug-induced COP-I dissociation, cells overexpressing GBF1 or Arf1 were treated for 1 h with BFA, AG1478, or GCA. Subsequently, the cells were fixed and stained with an antibody against COP-I. GBF1-wt, wild-type GBF1.

![Figure 2A](https://example.com/figure2a.png)

**Fig. 2.** Overexpression of GBF1 counters the effects of BFA, GCA, and AG1478, while overexpression of Arf1 counters only the effects of AG1478. (A) Plasmids encoding GBF1 or Arf1 were transfected into BGM cells. At 24 h posttransfection, the cells were treated for 1 h with BFA, AG1478, or GCA. Subsequently, the cells were fixed and stained with an antibody against COP-I. GBF1-wt, wild-type GBF1. (B) A Gaussia-luc construct was cotransfected with GBF1-M832L or Arf1 constructs into cells. The next day, the medium was replaced with (drug-containing) medium, and after 2.5 h, the supernatant was analyzed for luciferase activity. Gaussia-luc activity is expressed as a percentage of luciferase activity in the supernatant of untreated cells. The experiment was performed in triplicate, and the bars represent means ± SEMs (error bars).

![Figure 2B](https://example.com/figure2b.png)

In addition, we tested whether overexpression of Arf1, the effector of GBF1, was able to counter the effects of the drugs on COP-I localization. Figure 2A shows that in cells treated with AG1478 or GCA, GBF1 localized to the Golgi apparatus.

![Figure 2C](https://example.com/figure2c.png)
with either BFA or GCA, both COP-I and the overexpressed Arf1-GFP have a diffuse distribution, indicating that the effects of BFA or GCA could not be countered by overexpression of wild-type Arf1, which is consistent with previous observations (17). The constitutively active mutant Arf1-Q71L was also not able to counteract the effects of BFA and GCA (data not shown). Surprisingly, overexpression of Arf1 did counter the effects of AG1478 on COP-I localization, since COP-I localized to Arf1-GFP-containing membranes in transfected cells.

Next, we examined whether overexpression of GBF1-M832L or Arf1 could relieve the effects of the drugs on a functional level by measuring the secretion of Gaussia-luc. Gaussia-luc secretion was inhibited by all three drugs, but in all cases this effect could be countered by overexpression of GBF1-M832L (Fig. 2B). The inhibitory effect of AG1478, but not that of BFA or GCA, could be relieved by overexpressing Arf1 or, to a stronger extent, by overexpressing the Arf1-Q71L mutant (Fig. 2B). Collectively, these results imply that AG1478 has a different mechanism of action than BFA and GCA.

**GCA, but not AG1478, inhibits enterovirus replication.** GBF1 is a crucial factor for the replication of enteroviruses (2, 11). Therefore, we tested whether AG1478 and GCA were able to inhibit replication of coxsackievirus B3 (CVB3), a human enterovirus B species (HEV-B species) member. A representative growth curve of CVB3 is shown in Fig. 3A, which shows that virus production reaches a plateau after about 8 h. Therefore, we evaluated the effects of AG1478 and GCA on CVB3 replication by determining the virus yield at 8 h.p.i. Interestingly, AG1478 did not inhibit replication of CVB3, not even in concentrations higher than those required for fragmentation of the Golgi apparatus (Fig. 3B). GCA, however, impaired CVB3 replication as shown in Fig. 3C. Concentrations of 1 or 3 μM GCA had no effect on the virus yield, but with 10 μM GCA, the concentration used by Saez et al. (15) which also in our hands had a very strong effect on BGM cells, virus replication was strongly inhibited. Virus replication was completely abolished in the presence of 30 μM GCA with virus yields being similar to input levels. As GCA has been reported to specifically inhibit GBF1 and not the other guanine nucleotide exchange factors (GEFs), such as BIG1 and BIG2 (15), these results provide further evidence that GBF1 is the only GEF that is required for enterovirus replication.
The spectrum of GCA activity on replication of picornaviruses was examined by testing its effect on two other enterovirus species: enterovirus 71 (EV71), a member of the HEV-A species, and coxsackievirus A21 (CVA21), a member of the HEV-C species. In addition, we tested whether mengovirus, a member of the cardiovirus genus known to be resistant to BFA (7, 9), is sensitive to treatment with GCA. BGM cells (CVB3/EV71), HeLa cells (CVA21), and BHK-21 cells (mengovirus) were infected with the different viruses and incubated in the presence or absence of GCA. We determined the virus yield after 8 h, since at later time points (determined at 16 h), we observed partial recovery of the Golgi structure and the GBF1 localization (data not shown), which might be caused by metabolism of GCA or alternatively by a cellular compensatory mechanism. Consistent with this observation, the inhibition of CVB3 replication by GCA was also somewhat decreased after 16 h as measured by virus growth curves (data not shown), providing further evidence for a correlation between enterovirus replication and GBF1 activity.

Figure 3D shows that besides CVB3, the two other enteroviruses tested (EV71 and CVA21) also showed sensitivity to GCA. Mengovirus, on the other hand, was resistant to GCA and replicated to titers in the presence of GCA that were similar to the titers in the absence of drugs. Virus yields were determined at 8 h.p.i. The experiment was performed in triplicate, and bars represent means ± SEMs.

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Figure 3D shows that besides CVB3, the two other enteroviruses tested (EV71 and CVA21) also showed sensitivity to GCA. Mengovirus, on the other hand, was resistant to GCA and replicated to titers in the presence of GCA that were similar to the titers in the absence of this drug in both BHK-21 cells (Fig. 3D) and HeLa cells (data not shown). Taken together, these results show that the antiviral activity of GCA is strongly reminiscent of that of BFA, which supports the idea that GCA targets GBF1 and thereby inhibits virus replication in a manner similar to BFA.

**GCA impairs enterovirus RNA replication by targeting GBF1.** To investigate which stage of the virus life cycle is inhibited by GCA, we first performed time-of-addition studies,
In which we added GCA to the cells after infection with CVB3 at 1-h intervals and determined the virus yield at 8 h.p.i. The life cycle of picornaviruses is rapid, with virus entry, uncoating, and translation occurring within 2 h.p.i., while viral RNA replication is initiated at 2.5 to 3 h.p.i. Virus production starts at 5 to 6 h.p.i (Fig. 3D) and reaches a plateau after approximately 8 h.p.i (25). Figure 4A shows that GCA lost its efficacy when added early (0 to 2 h.p.i) or late (5 to 7 h.p.i), respectively.

In vivo labeling studies were performed to examine the possibility that GCA impairs translation or processing of the viral proteins. To this end, BGM cells were infected with CVB3 in the absence of drugs. At 5.5 h.p.i., a time point when there is entry or assembly, it would have exerted its effect when added drug exerts its effect in the stage of viral protein translation and viral RNA replication. If the drug was targeting either virus entry or assembly, it would have exerted its effect when added early (0 to 2 h.p.i) or late (5 to 7 h.p.i), respectively.

Concluding remarks. In this study, we showed that AG1478, GCA, and BFA all inhibited the function of the secretory pathway as shown by defective transport of VSV-G protein and the Golgi apparatus, and dispersal of Arf1 and COP-I. This observation strongly suggests that the inhibitory effect of BFA and GCA on GBF1 function and/or its localization is crucial to suppress CVB3 RNA replication. In sum, AG1478 and GCA disturb the secretory route in a different manner. Therefore, these drugs may be useful new tools to study enterovirus replication.

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