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

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Received 22 December 2009/Accepted 15 May 2010

The genus Enterovirus, belonging to the family Picornaviridae, includes well-known pathogens, such as poliovirus, coxsackievirus, and rhinovirus. Brefeldin A (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 coat 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. In this study, we investigated the effects of these putative GBF1 inhibitors on secretory pathway function and enterovirus replication. We show that both drugs induced fragmentation of the Golgi vesicles and caused dissociation of Arf1 and COP-I from Golgi membranes, yet they differed in their effect on GBF1 localization. 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 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.
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 BigG1 and BigG2.

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 GCF1 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 drugs. Unless indicated otherwise, the concentrations of BFA, AG1478, and GCA used in experiments were 2 μg/ml (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 echovirum (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). Enterovirus 71 (EV71) (BeCr) and coxsackievirus A21 (CVA21) (Coc) were obtained from the National Institute for Public Health and Environmental Protection (RIVM, Netherlands).

Plasmids. The VSVG-GFP plasmid, kindly provided by P. Kellen 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-Gluc (CMV stands for cyto-megalovirus, 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-GFB1 wt (EYFP stands for enhanced yellow fluorescent protein, and wt stands for wild type), pEYFP-GFB1-M832L (12), pArf1-EGFP wt (5), and pArf1-Q71L-EGFP (11) were described previously. DNA transfections were performed with 200 ng plasmid DNA and the transfection reagent Fugene (Roche) according to the manufacturer's instructions 1 day prior to the assays.

VSV-G trafficking. BGM cells grown on coverslips in 24-well plates were transfected with pVSVG(ts045)-GFP and incubated at 40°C. At 16 h posttransfection, BFA, AG1478, or GCA was added to the cells. Following a 30-min incubation, the temperature was switched to 32°C. After 2 h, the cells were fixed, stained with anti-GFP antibody (diluted 1:100; Molecular Probes) and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G (Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular Probes) and analyzed with a Leica BMR microscope.

Virus infections. Replication of CVB3 and EV71 was studied on subconfluent layers of BGM cells, while CVA21 and mengovirus were studied on HeLa and BHK-21 cells, respectively. After 30 min of virus adsorption, the cells were washed, and fresh (drug-containing) medium was added to the cells. For the time-of-addition studies, GCA was added to the cells at 1-h intervals. At 8 h postinfection (h.p.i.), the cells were freeze-thawed three times to release intracellular virus particles, and virus titers were determined by endpoint titration by the method of Reed and Muench and expressed as 50% cell culture infective doses (CCID50) per ml (14).

Replicon assay. Replicon assays were performed as described previously (20). The p53CB3-LUC subgenomic replicon used contains the CVB3 cDNA in which the P1 capsid coding region is replaced by the firefly luciferase gene (29). The replicon 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 polyprotein 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-Gluc, 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 the different drugs, after which COP-I was stained. Figure 2A shows that COP-I was present on membranes in drug-treated cells that overexpress yellow fluorescent protein (YFP)-tagged GBF1 (GBF1-YFP), while COP-I appeared cytosolic in untransfected, drug-treated cells. Also, the Golgi apparatus morphology seemed to be restored in the drug-treated, transfected cells with GBF1-YFP localizing to the Golgi apparatus. Thus, expression of GBF1 was indeed able to prevent the dissociation of COP-I from membranes induced by the different drugs. Overexpression of GBF1-M832L showed similar results (data not shown).

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 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. 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 \( \mu \)M GCA had no effect on the virus yield, but with 10 \( \mu \)M 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 \( \mu \)M 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.

**FIG. 3.** GCA, not AG1478, inhibits replication of enteroviruses. (A) BGM cells were infected with CVB3 at an MOI of 5. Total virus titers were determined at the indicated time points. The virus titer was measured as log 50% cell culture infective doses per ml (log CCID50/ml). (B) Various concentrations of AG1478 were added to the cells immediately after infection with CVB3. Virus titers were determined after 8 h. (C) The cells were infected with CVB3 and treated with various concentrations of GCA. Concentrations of 10 \( \mu \)M GCA or higher significantly inhibited CVB3 replication \( (P < 0.001, \text{one-way analysis of variance [ANOVA]}) \). (D) BGM, HeLa, or BHK-21 cells were infected with CVB3, EV71, CVA21, or mengovirus and treated with GCA or BFA. Virus titers were determined after 8 h. Experiments were performed in triplicate, and the bars represent means ± SEMs (error bars).
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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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 later than 4 h after infection, which suggests that the 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.

**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 efficient virus replication, BGM cells were pulse-labeled for 30 min with [35S]methionine. During these 30 min, drugs were also added to the cells to test possible effects on viral protein synthesis and processing in a situation where only viral proteins are translated due to host shutdown. Neither GCA nor BFA affected translation or processing of viral proteins (Fig. 4C).

Subsequently, the effect of GCA on RNA replication was studied directly by use of a subgenomic replicon pCB3/T7-Luc, in which the P1 region has been replaced with the firefly luciferase gene. The level of luciferase activity in cells is a measure of viral RNA replication. Figure 4B shows that, similar to BFA, GCA strongly inhibits firefly luciferase accumulation, demonstrating that GCA hinders viral RNA replication.

Since the dissociation of COPI-I from Golgi membranes induced by GCA could be countered by overexpression of GBF1 or GBF1-M832L, we tested whether GBF1-M832L overexpression was also able to counter virus replication in the presence of GCA. To this end, BGM cells overexpressing GBF1-M832L or EGFP as a negative control were infected with CVB3, and virus titters were determined at 8 h.p.i. Overexpression of GBF1-M832L had no effect on CVB3 replication in untreated cells. However, in the presence of 10 μM GCA or 2 μg/ml BFA, virus titters were significantly higher in cells overexpressing GBF1-M832L than in EGFP-expressing cells (Fig. 4D). Similar results were obtained with 30 μM GCA, a concentration that completely blocked CVB3 replication, indicating that GBF1-M832L was able to relieve the effect of the drugs on CVB3 replication.

**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 VSVG protein and Gaussia-luc. The drugs fragmented the Golgi membranes and dispersed Arf1 and COPI-I in a similar manner, but they differed in their effects on the localization of GBF1. Furthermore, overexpression of Arf1 could counter the effect of AG1478, but not of GCA or BFA, on COPI-I localization and function of the secretory pathway. Finally, unlike GCA, AG1478 did not inhibit replication of enteroviruses, which is known to be GBF1 dependent (2, 11). Together, these results question whether AG1478 is a genuine inhibitor of GBF1.

Remarkably, AG1478 did not inhibit enterovirus replication, despite the fact that it has many effects in common with BFA and GCA, such as inhibition of the secretory pathway, disassembly of the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus, and dispersal of Arf1 and COPI-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.

**ACKNOWLEDGMENTS**

We thank D. Haslam for kindly providing Goldgicide A.

This work was supported by grants from The Netherlands Organization for Scientific Research (NWO-VID1-917.46.306 and NWO-ECHO-700.57.001 to F.J.M.V.K.) and a research grant to F.J.M.V.K. and J.N. in the framework of the “Conventant K.U. Leuven-Radboud University Nijmegen.”

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