ABSTRACT

In most eukaryotic cells, entry into mitosis is tightly controlled and requires completely replicated and undamaged DNA. We show that the antitumor drug, fostriecin, interferes with this control; it induces cycling cells to enter mitosis prematurely, and it can overcome the mitotic entry checkpoint, forcing into mitosis cells that were arrested in the division cycle by treatment with the DNA replication inhibitor aphidicolin or with the DNA-damaging agents camptothecin and teniposide. This effect was observed in all rodent, simian, and human cell lines tested. Fostriecin also prevents progression through the later stages of mitosis as determined by the absence of normal half-spindles, anaphase figures, and telophase figures. The only previously known target for fostriecin is topoisomerase II, which is inhibited in vitro with a 50% inhibitory concentration of 40 nM (T. J. Boritzki, T. S. Wolfard, J. A. Besserer, R. C. Jackson, and D. W. Fry. Inhibition of type II topoisomerase by fostriecin. Biochem. Pharmacol., 37: 4063–4068, 1988). We show that fostriecin is a more potent inhibitor of protein phosphatase 1, with a 50% inhibitory concentration of 4 μM and protein phosphatase 2A, with a 50% inhibitory concentration of 40 nM. Inhibition of the mitotic entry checkpoint and inhibition of protein phosphatases are novel properties for antitumor drugs with potential or proven therapeutic value.

INTRODUCTION

Entry into mitosis is a tightly regulated process that is dependent upon the completion of previous cell cycle events. Cells treated with inhibitors of DNA synthesis or with agents that damage DNA usually delay entry into mitosis, a state that is termed G2 arrest. It is generally believed that the arrest is necessary for the cells to complete DNA synthesis or repair before a commitment to mitosis is made (1). G2 arrest is caused by the activation of a negative feedback mechanism called the mitotic entry checkpoint (2). Mutation, deletion, or over-expression of certain genes disrupts the checkpoint and allows cells with unreplicated or damaged DNA to enter mitosis (reviewed in Refs. 3–5). The proteins encoded by these genes are thought to participate in a regulatory mechanism that can sense incompletely replicated or damaged DNA and convey the message to protein kinases and phosphatases that regulate the activity of Cdc2 kinase, the major mitotic inducer (5).

Many antitumor drugs are DNA-damaging agents and cause G2 arrest (1, 6). The cytotoxic activity of these drugs correlates with their ability to induce G2 arrest (7). The major mitotic inducer, Cdc2 kinase, is negatively regulated in such G2-arrested cells (1, 8–13), suggesting that a functional mitotic entry checkpoint is required for the drugs to exert their cytotoxic action.

INTRODUCTION

There is also mounting evidence that checkpoint control is not entirely normal in cancer cells. When treated with X-irradiation or fluorescent light during late S or G2, human cancer cells and cells transformed in culture show more broken chromosomes than normal cells at the next metaphase (14, 15). This suggests that cancer cells have a slightly defective mitotic entry checkpoint (16). A human lymphoma cell line with unusual sensitivity to the DNA-damaging agent nitrogen mustard has also recently been shown to have a defective checkpoint (13). Thus, a better understanding of the biochemical mechanism of checkpoint control is relevant not only to our understanding of the normal cell cycle but also to an understanding of the differences between normal and cancer cells and the mechanism of action of antitumor drugs.

It has recently been suggested that inhibitors of checkpoints may be valuable antitumor agents (3). They may further weaken the checkpoints of cancer cells while having little effect on the checkpoints of normal cells, leading to more selective killing of tumor cells (3). Thus far only a limited number of checkpoint inhibitors have been found, as indicated by their ability to induce entry into mitosis in cells arrested with unreplicated or damaged DNA. These are caffeine (17–19), the protein kinase inhibitors 2-amino purine and 6-dimethylaminopurine (20, 21), and the protein phosphatase inhibitors okadaic acid and calyculin A (22–25). None of these agents is used in cancer therapy.

Fostriecin is an antitumor drug active against lung, breast, colon cancer, and leukemia cells and is now in phase I clinical trials (26). We show that fostriecin is an inhibitor of the mitotic entry checkpoint and that it is a potent inhibitor of PPI1 and PP2A.

MATERIALS AND METHODS

Cell Culture and Drug Treatment

BHK-21 cells were grown as monolayers in Dulbecco’s minimal essential medium supplemented with 7.5% FBS and antibiotics at 37°C in humidified 10% CO2. Human cell lines were grown in RPMI containing 10% FBS and antibiotics at 37°C in humidified 10% CO2. Fostriecin (NSC 339638, 94528) was obtained as vials containing 25 mg fostriecin, 39 mg ascorbic acid (as an antioxidant), and NaOH to neutralize to pH 7. It was added from a fresh stock solution in phosphate-buffered saline. Controls were treated with ascorbic acid neutralized to pH 7. Colcemid (Sigma Chemical Co.) was from a 0.5 mg/ml stock in ethanol at −20°C, aphidicolin (Sigma) was from a 1 mg/ml stock in DMSO, VM-26 (Bristol-Myers) was from a 5 mM stock in DMSO, camptothecin (Sigma) was from a 0.5 mM stock in DMSO, and okadaic acid (GIBCO) was from a 0.5 mM stock in 10% DMSO.

Aphidicolin Block. Asynchronously growing BHK cells were first subjected to serum deprivation (0.25% FBS in culture medium) for 16 h, or they were incubated in isoleucine-free medium containing 10% dialyzed FBS for 12 h, followed by a 12 h treatment with 2.5 μg/ml aphidicolin. Asynchronously
growing human cells were treated with isoleucine-free medium containing 10% dialyzed FBS followed by a 12 h treatment with 2.5 μg/ml aphidicolin in complete medium.

**Camptothecin Block.** Asynchronously growing BHK cells were treated with 0.5 μm camptothecin or the equivalent volume of DMSO. After 1 h, the medium was removed, and the cultures were washed twice in fresh medium.

**VM-26 Block.** Asynchronously growing cells were treated with 25 μM VM-26 as described in Roberge et al. (10).

**Fluorescence Microscopy**

After drug treatment, floating cells were collected and pooled with attached cells harvested by trypsinization. The cells were stained with the fluorescent DNA dye bisbenzimide for chromosome visualization following fixation with 3.7% formaldehyde (10) or following hypotonic swelling and treatment with Carnoy’s fixative (27). The latter treatment gave superior structural preservation but could not be used in conjunction with antibody treatment. At least 10 random fields containing more than 20 cells/field were selected for microscopic examination using a Zeiss standard microscope equipped for epifluorescence. Nuclear lamins were detected by indirect immunofluorescence as described in Roberge et al. (10) except that fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was used as the secondary antibody. Microtubules were detected by indirect immunofluorescence with monoclonal antibody E7 to β-tubulin (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as a secondary antibody.

**Cdc2 Kinase Purification**

Cells were harvested and washed twice with 100 μM EDTA in phosphate-buffered saline and twice with 10 mM Tris-buffered saline. The cells were then lysed by adding 50 μl/10^6 cells of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 1% Triton X-100, 250 mM NaCl, 15 mM MgCl_2, 1 mM dithiothreitol, 50 mM NaF, 10 μg/ml nucodazole, 80 mM 2-glycerophosphate, 40 mM p-nitrophenyl phosphate, 0.25 mM sodium o-vanadate, 0.1 μM okadaic acid, 10 μg/ml each of aprotinin, leupeptin, and pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride. They were kept on ice for 10 min and then centrifuged at 12,000 x g for 15 min at 4°C. A sample of 100 μg of protein was mixed with 40 μl of 1 mg/ml p13^wys^ beads and KII buffer containing 12.5 mM 3-(N-morpholino)propanesulfonic acid, 12.5 mM 2-glycerophosphate, 5 mM ethyleneglycol bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 75 mM MgCl_2, and 0.5 mM NaF (pH 7.2) to a final volume of 400 μl. The mixture was incubated with agitation for 30 min at 4°C and then centrifuged at 500 x g for 30 s. The beads were washed three times with KII buffer and suspended in 20 μl of KII buffer and SDS sample buffer. Samples were boiled for 3 min before loading on 10% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose by electroblocting, reacted with 2 μg/ml antibodies to the COOH terminus of p34^cdcd2^, and visualized using a chemiluminescent method (Amersham ECL). Histone H1 kinase activity was determined (Fig. 1). Unexpectedly, fostriecin did not bring about G2 arrest. Rather, it stimulated entry into mitosis. More than 30% of the cells had condensed chromosomes after 2 h of treatment with 375 μM fostriecin and Colcemid compared with about 7% after treatment with Colcemid alone. This effect was dose dependent and was half-maximal at about 125 μM fostriecin. Fostriecin induced entry into mitosis in all cell lines tested, including simian COS cells, human leukemia Jurkat, and human myeloid U937 (not shown).

To determine whether the premature mitotic state induced by fostriecin is normal, we next examined several morphological hallmarks of mitosis. Cells were treated with or without fostriecin in the absence of Colcemid. Within 1 h of treatment with fostriecin all cells had rounded up and could be detached from the culture dish by shaking (not shown). Chromosomes were fully condensed but scattered (Fig. 2, B, C, and G) compared with controls (Fig. 2, A and E). A small percentage of the mitotic cells displayed a punctate pattern of chromatin condensation (Fig. 2B, arrowhead) similar to the S-PCC characteristic of cells entering mitosis before completion of DNA replication (30–32). Immunolocalization of nuclear lamins showed that fostriecin caused nuclear lamin depolymerization in all cells with condensed chromosomes but not in cells with interphase chromatin (Fig. 2, C and D). Immunolocalization of microtubules with antibodies to β-tubulin in cells treated with fostriecin showed that all cells with condensed chromosomes had two separated spindle poles with astral microtubules but no typical half-spindles were seen (Fig. 2D) compared with controls (Fig. 2E). Thus, fostriecin induced the major morphological events characteristic of prometaphase: rounding up of cells; chromosome condensation; nuclear lamina depolymerization;...

**RESULTS**

**Fostriecin Induces Premature Entry into Mitosis in Cycling Cells.** Fostriecin inhibits topoisomerase II in vitro (29). Since many antitumor drugs are topoisomerase II inhibitors and induce G2 arrest, we first examined whether fostriecin also induced G2 arrest. Asynchronously growing BHK cells were treated with different concentrations of fostriecin and with the inhibitor of microtubule polymerization Colcemid to prevent exit from mitosis. After 1 or 2 h, the cells were harvested by trypsinization, fixed for microscopic examination, and stained with the fluorescent DNA dye bisbenzimid, and the percentage of cells with condensed mitotic chromosomes was determined (Fig. 1). Unexpectedly, fostriecin did not bring about G2 arrest. Rather, it stimulated entry into mitosis. More than 30% of the cells had condensed chromosomes after 2 h of treatment with 375 μM fostriecin and Colcemid compared with about 7% after treatment with Colcemid alone. This effect was dose dependent and was half-maximal at about 125 μM fostriecin. Fostriecin induced entry into mitosis in all cell lines tested, including simian COS cells, human leukemia Jurkat, and human myeloid U937 (not shown).

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Fostriecin Induces Entry into Mitosis in Cells with Incompletely Replicated DNA. We also examined the effects of fostriecin on cells arrested in the cycle as a result of incomplete replication. BHK cells were treated with aphidicolin, an inhibitor of DNA replication. This caused 80% of the cells to accumulate at the G1-S boundary (Table 1). The cells (59%) entered S phase synchronously upon release from the aphidicolin block and were still in S phase 3 h after release (Table 1). When cells were released from the aphidicolin block and treated immediately with Colcemid for up to 2 h, no significant entry into mitosis was detected (Fig. 4). By contrast, when cells were released from the aphidicolin block and treated with both Colcemid and fostriecin, about 60% showed S-PCC (Fig. 4). The S-PCC morphology is shown in Fig. 5. Similar experiments were performed with four human cell lines (Jurkat lymphoma cells, HL-60 promyelocytic leukemia cells, U937 histiocytic lymphoma cells, and CEM acute lymphoblastic leukemia cells). Fostriecin elicited S-PCC in all cell lines (Fig. 6) although the proportion was lower than it was for BHK cells.

To demonstrate unambiguously that the cells treated with fostriecin entered a mitotic state from S phase, BrdUrd was added to BHK cells upon release from the aphidicolin block to label cells undergoing DNA replication. After 20 min the BrdUrd was washed away, and the cells were treated with or without fostriecin for 2 h. Individual cells were monitored for BrdUrd incorporation in the nucleus by immunofluorescence and for S-PCC with the DNA dye bisbenzimide. Table 2 shows that 58% of the cells stained with BrdUrd showed S-PCC. In addition, all the cells in S-PCC had incorporated BrdUrd, demonstrating that they entered mitosis only from S phase and not from G1.

Table 1 shows that BHK cells have not completed S phase 2 h after release (Table 1). When cells were released from the aphidicolin block and were still in S phase 3 h upon release from the aphidicolin block to label cells undergoing DNA replication. After 20 min the BrdUrd was washed away, and the cells were treated with or without fostriecin for 2 h. Individual cells were monitored for BrdUrd incorporation in the nucleus by immunofluorescence and for S-PCC with the DNA dye bisbenzimide. Table 2 shows that 58% of the cells stained with BrdUrd showed S-PCC. In addition, all the cells in S-PCC had incorporated BrdUrd, demonstrating that they entered mitosis only from S phase and not from G1.

Table 1. Effect of aphidicolin treatment on the cell cycle distribution of BHK cells determined by flow cytometry.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 + M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>66</td>
<td>19</td>
<td>14</td>
</tr>
<tr>
<td>Blocked with aphidicolin</td>
<td>80</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Released from aphidicolin for 3 h</td>
<td>37</td>
<td>59</td>
<td>4</td>
</tr>
</tbody>
</table>

To examine in more detail the advancement of entry into mitosis, we pulse-labeled asynchronously growing cells with the thymidine analogue BrdUrd for 20 min and treated them with or without fostriecin for up to 2 h. The cells were then stained with fluorescently conjugated BrdUrd antibodies and bisbenzimide. The percentage of those cells that had incorporated BrdUrd and that had also displayed condensed chromosomes was scored. Fig. 3 shows that few of the cells that incorporated BrdUrd had entered mitosis after 1 or 1.5 h of treatment with Colcemid alone or Colcemid and ascorbic acid. By contrast, a significantly higher proportion of the BrdUrd-labeled cells treated with fostriecin had entered mitosis by 1 h, and close to 30% of the cells were in mitosis by 2 h. This shows that the cells entered mitosis prematurely, about 2 h ahead of untreated cells.

Fig. 3. Acceleration of the rate of entry into mitosis of BrdUrd-labeled BHK cells by fostriecin. Fostriecin was added for the indicated times to cells previously pulse-labeled with BrdUrd. The percentage of BrdUrd-labeled cells in mitosis was determined by first localizing BrdUrd-labeled cells by immunofluorescence and then determining the condensation state of their chromosomes with bisbenzimide. Fig. 3 shows that few of the cells that incorporated BrdUrd had entered mitosis after 1 or 1.5 h of treatment with Colcemid alone or Colcemid and ascorbic acid; •, Colcemid alone; ○, Colcemid and fostriecin. Bars, SD.
Fostriecin Overcomes G2 Arrest Caused by VM-26 and Camptothecin. Cell cycle progression can be arrested or delayed in late G2 by agents that induce DNA lesions. Tsao et al. (12) have shown that short incubation of S-phase cells with the topoisomerase I inhibitor camptothecin results in G2 arrest. Camptothecin stabilizes a cleavable complex between topoisomerase I and DNA in which DNA is nicked on one strand (33). The cell cycle arrest was confirmed in BHK cells by exposing cultures to 0.5 μM camptothecin followed by incubation in camptothecin-free medium, resulting in a complete block of entry into mitosis between 5 and 6.5 h after onset of treatment (Fig. 7, A). DMSO, the solvent for camptothecin, did not induce G2 arrest (Fig. 7, B). The addition of fostriecin to camptothecin-treated cells at h 5 caused a large proportion of the cells to enter mitosis 1.5 h later (Fig. 7, A). G2 arrest can also be induced by treating G2 cells with the topoisomerase II inhibitor VM-26 (10). Fig. 8 shows that exposure of BHK cells to 25 μM VM-26 resulted in a complete block of entry into mitosis. The addition of fostriecin to cells exposed to VM-26 overcame the block and induced cells to enter mitosis. Thus, fostriecin can overcome the cell cycle arrest induced by camptothecin and VM-26.

Fostriecin Induces Only a Minor Activation of Cdc2 Kinase in BHK Cells. Entry into mitosis is characterized by the activation of Cdc2 kinase (reviewed in Refs. 34 and 35). During the S and G2 phases of the cell cycle, p34CDC2 associates with cyclin B, a regulatory subunit, and the complex is maintained in a repressed state until mitosis. Activation of Cdc2 kinase at the onset of mitosis requires the dephosphorylation of critical amino acid residues in p34CDC2 (3, 36–38). Cells were synchronized in S phase by release from the aphidicolin block and were treated with fostriecin for 1.5 h. Cdc2 kinase was then isolated using p130cdc2 beads and analyzed by SDS gel electrophoresis followed by detection with antibodies specific to p34CDC2. Fig. 9 shows that treatment with fostriecin caused a downward shift in electrophoretic mobility characteristic of the dephosphorylated and activated form of Cdc2 kinase. Similarly, the addition of fostriecin to cells blocked in G2 with VM-26 also caused dephosphorylation of p34CDC2 (Fig. 9). In both cases, p34CDC2 dephosphorylation was accompanied by only a minor increase in Cdc2 kinase activity as measured by phosphorylation of histone H1 in vitro (Fig. 9). In a separate experiment, equal amounts of Cdc2 kinase isolated from cells treated with or without fostriecin were separated by SDS gel electrophoresis and probed with antibodies to phosphotyrosine. p34CDC2 was completely dephosphorylated at tyrosine residues in cells treated with fostriecin (not shown).

Fostriecin Is a Potent Inhibitor of PP1 and PP2A. To understand better how fostriecin causes the premature appearance of mitotic events we looked at changes in cellular protein phosphorylation caused by fostriecin. BHK cells were synchronized to S phase with aphidicolin and treated with fostriecin and 32P i for 2 h. Fostriecin caused a large increase in the phosphorylation of a Mr 55,000 protein (not shown). Okadaic acid is another agent that can overcome the block to entry into mitosis in cells with unreplicated DNA. Ma-hadevan et al. (39) have reported that okadaic acid stimulates the

Table 2 Induction of S-PCC by fostriecin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BrdUrd-labeled</th>
<th>BrdUrd-labeled</th>
<th>S-PCC cells that are</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells (%)</td>
<td>in S-PCC (%)</td>
<td>BrdUrd-labeled (%)</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>39</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Fostriecin</td>
<td>40</td>
<td>58</td>
<td>100</td>
</tr>
<tr>
<td>Ascorbic acid and aphidicolin</td>
<td>33</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Fostriecin and aphidicolin</td>
<td>41</td>
<td>68</td>
<td>100</td>
</tr>
</tbody>
</table>
inducing DNA breaks (29). Our original goal was to determine whether fostriecin also induces G₂ arrest. We found the opposite result; fostriecin stimulates entry into mitosis. Treatment of cycling cells with fostriecin caused the premature appearance of the morphological characteristics of early mitosis, rounding up of the cells, nuclear lamina depolymerization, chromosome condensation, spindle pole separation, and aster formation at least 2 h before schedule.

Cells that entered mitosis prematurely seemed unable to exit this mitotic state in the presence of fostriecin; later mitotic events such as metaphase alignment of the chromosomes at the equatorial plate, separation and segregation of sister chromatids, and cytokinesis were very rarely observed. Our immunofluorescence study of microtubules in cells treated with fostriecin showed that although spindle poles separated and astral microtubules were formed, morphologically normal half spindles were not formed, and chromosomes were scattered throughout the cell and apparently not attached to astral microtubules. The absence of a normal mitotic spindle may be the reason why cells fail to complete mitosis.

Fostriecin also forced entry into mitosis in cells blocked in S phase with the DNA polymerase inhibitor aphidicolin and in cells arrested in G₂ with the topoisomerase I inhibitor camptothecin or the topoisomerase II inhibitor VM-26. Thus, fostriecin overcomes the block imposed by the mitotic entry checkpoint. The observation that fostriecin induces entry into mitosis in cycling cells and in checkpoint-blocked cells suggests that its intracellular target is common to both normal cell cycle progression and the checkpoint pathway.

Other chemicals have been shown to induce premature mitosis or to overcome the mitotic entry checkpoint in mammalian cells. Millimolar concentrations of caffeine can overcome the checkpoint activated by DNA-damaging agents and potentiate their killing effects in many cell lines (17, 42). Caffeine can also overcome the checkpoint activated by unreplicated DNA but only in rodent cells (18, 19). Caffeine does not accelerate entry into mitosis in cycling cells (19) and its mechanism of action has not been elucidated. High concentrations of 2-aminopurine can also overcome the checkpoint activated by unreplicated or damaged DNA (20, 21). 2-Aminopurine is an inhibitor of protein phosphorylation of unknown specificity (43). Okadaic acid and calyculin A are potent inhibitors of PP1 and PP2A (41). Both can overcome the checkpoint activated by unreplicated DNA (24, 25, 44).

**DISCUSSION**

Antitumor drugs that target topoisomerases stabilize a complex in which topoisomerase is covalently linked to DNA via a single- or double-stranded DNA break (33). Cells treated with those drugs arrest in G₂, probably as a result of the induced DNA lesions. Fostriecin is an antitumor drug that inhibits topoisomerase II in vitro without phosphorylation of an uncharacterized Mr 55,000 protein in quiescent 3T3 cells. Since okadaic acid is a strong inhibitor of type 1 and 2A protein phosphatases (40, 41), we tested whether fostriecin also acts in this manner. Fostriecin was incubated in vitro with purified PP1, PP2A, or protein tyrosine phosphatase α. Fostriecin inhibited PP2A strongly, with an IC₅₀ of 40 nM, comparable to that of okadaic acid (Fig. 10). PP1 was inhibited to a lesser extent, with an IC₅₀ of about 4 μM. Fostriecin did not inhibit protein tyrosine phosphatase α, even at 100 μM.
Fostriecin does not bear obvious structural similarity to any of these chemicals. Identifying fostriecin targets is important as a step toward uncovering the elements involved in regulating normal entry into mitosis and checkpoint control. The only previously identified fostriecin target is topoisomerase II which is inhibited in vitro with an IC_{50} of 40 μM (29). We show that fostriecin inhibits PP2A with a 1000-fold lower IC_{50} of 40 nM and PP1 with a 10-fold lower IC_{50} of 4 μM. The lowest fostriecin concentration at which we observe stimulation of entry into mitosis is 37.5 μM, well above the IC_{50} for the phosphatases in vitro. The drug concentration required to inhibit an enzyme in the cell depends on several factors, including the intracellular concentration of the enzyme, the stability of the drug in the incubation medium, and the permeability of the plasma membrane to the drug (41). The intracellular concentration of PP1 and PP2A is usually in the 0.1–1 μM range (41). Inhibition would therefore be expected to require μM concentrations of fostriecin in the cell. Fostriecin is sensitive to oxidation, and we have determined that it loses 50% of its ability to promote entry into mitosis during the first 30 min of incubation in cell culture medium (not shown). We do not know if the plasma membrane constitutes a barrier to fostriecin penetration. Therefore, our observation that fostriecin stimulates entry into mitosis when initially present in the culture medium at 37.5 μM and above is not incompatible with protein phosphatases being the major intracellular targets.

Several more lines of evidence suggest that checkpoint inhibition is likely due to the inhibition of PP2A and/or PP1 rather than topoisomerase II: (a) although fostriecin inhibits purified topoisomerase II, it has no effect on the activity of this enzyme in cell extracts (45); (b) Chen and Beck (46) recently showed that fostriecin does not inhibit VM-26-mediated DNA-topoisomerase II complexes in living cells, strongly suggesting that it is not an in vivo inhibitor of topoisomerase II; (c) none of the other topoisomerase II inhibitors have been shown to induce premature mitosis while the PP1 and PP2A inhibitors okadaic acid and calyculin A do; and (d) topoisomerase II is not required for entry into mitosis (47) while PP2A negatively regulates Cdc2 kinase in Xenopus egg extracts (22, 48), and PP2A, is the major enzyme that dephosphorylates substrates for cyclin-dependent kinases (49). In addition, the M, 55,000 regulatory subunit of Drosophila PP2A is required for segregation of chromosomes at anaphase (50). Thus inhibition of PP2A by fostriecin could explain both why treated cells enter mitosis prematurely and why they fail to complete mitosis.

Identifying fostriecin targets is also important for understanding of how the drug exerts its antineoplastic activity. To our knowledge, inhibition of protein phosphatases is a novel property for antitumor drugs with potential or proven therapeutic values. If further research confirms our suggestion that fostriecin acts through inhibiting protein phosphatases, a search for other protein phosphatase inhibitors may yield valuable new drugs for cancer therapy. We are also not aware of other antitumor drugs that induce lethal entry into mitosis. If forced entry into mitosis and failure to exit mitosis proves to be a mechanism by which fostriecin kills cancer cells in tumors, then binary therapy with both a drug that induces cell cycle block (such as VM-26) and a drug like fostriecin that induces these cells to enter mitosis may provide more efficient treatment than either drug alone.

ACKNOWLEDGMENTS

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REFERENCES

INDUCTION OF MITOTIC EVENTS BY FOSTRIECIN


Antitumor Drug Fostriecein Inhibits the Mitotic Entry Checkpoint and Protein Phosphatases 1 and 2A

Michel Roberge, Christopher Tudan, Stewart M. F. Hung, et al.


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