

Possible Mechanisms of Diarrheal Side Effects Associated with the Use of a Novel Chemotherapeutic Agent, Flavopiridol¹

Melissa E. S. Kahn, Adrian Senderowicz,
Edward A. Sausville, and Kim E. Barrett²

Division of Gastroenterology, Department of Medicine, University of California, San Diego, School of Medicine, San Diego, California 92103 [M. E. S. K., K. E. B.], and Developmental Therapeutics Program Clinical Trials Unit, Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892 [A. S., E. A. S.]

ABSTRACT

The novel cyclin-dependent kinase inhibitor flavopiridol has recently completed Phase I trials for the treatment of refractory neoplasms. The dose-limiting toxicity observed with this agent was severe diarrhea. Because the compound otherwise showed promise, the present study sought to determine possible mechanisms underlying the diarrheal side effects. Flavopiridol was tested for its ability to modify chloride secretory responses of the human colonic epithelial cell line, T₈₄. Studies were conducted *in vitro* in modified Ussing chambers. High concentrations of flavopiridol (10⁻⁴ M), above those likely to be clinically relevant, had a direct stimulatory effect on chloride secretion, probably ascribable to an increase in cyclic AMP. Lower, clinically relevant concentrations of flavopiridol (10⁻⁶ M) had no effect on chloride secretion by themselves but potentiated responses to the calcium-dependent secretagogue, carbachol. The drug also potentiated responses to thapsigargin and taurodeoxycholate and reversed the inhibitory effects of carbachol and epidermal growth factor on calcium-dependent chloride secretion. Pretreatment with the cyclic AMP-dependent secretagogue, forskolin, potentiated responses to flavopiridol, but not *vice versa*. Thus, diarrheal side effects induced by flavopiridol are likely multifactorial in origin and may involve interactions with endogenous secretagogues such as acetylcholine and bile acids. A better understanding of the diarrhea induced by flavopiridol should allow optimization of therapy with this otherwise promising drug and/or the development of related agents with improved toxicity profiles.

INTRODUCTION

The external environment manipulates cellular proliferation and differentiation by stimulating or inhibiting certain signal transduction pathways that impinge on the cell cycle (1–3). Each component of the cell cycle machinery, as a final executor in cell division, has the potential to elicit or to contribute to a neoplastic phenotype (4). When normal cells sense external stimuli, such as contact inhibition, they stop proliferating. However, in transformed cells, some of the controls exerted on progression through the cell cycle are lost. Checkpoints at the G₁-S and G₂-M transitions are surveillance mechanisms that monitor the completion of critical cell cycle transitions (5). In transformed cells, these checkpoints are less stringent or even absent (6). Because transformed cells have handicapped checkpoints, cancer has been described as a cell cycle disease (6).

Cell division involves the recruitment of a group of serine/threonine kinases, called CDKs.³ These are activated and inactivated in an orderly manner at specific time points throughout the cell cycle (6). The kinase activity of CDKs is controlled by their association with cyclins, proteins that are present at particular times in the cell cycle, as well as by posttranslational modifications (4). To date, nine CDKs have been discovered, each correlating with specific cyclins during different stages of the cell cycle. Most of the cyclin-CDKs complexes remain well-regulated in transformed cells, in terms of their coordination with DNA replication and mitosis. What is lacking is the ability to modulate the complexes in response to external factors and to DNA damage, most obviously at the G₁-S and G₂-M checkpoints. Approximately 90% of human tumors are associated with aberrations in cell cycle signaling pathways such as deletion of the retinoblastoma gene product (a tumor suppressor that normally blocks cell cycle progression), deletion of CDK inhibitors (such as p15 or p16), overexpression of cyclin D, or amplification of cdk4 or cdk6 with consequent activation of the G1 kinase and retinoblastoma inactivation (1, 2). Thus, CDKs are attractive chemotherapeutic targets, and drugs that could act as CDK inhibitors might be expected to be effective cytostatic drugs (7).

Flavopiridol is a recently discovered agent that, at doses attained in clinical settings, acts as a specific inhibitor of several CDKs including cdk1, cdk2, cdk4, and cdk7 (8). As such, the drug inhibits cell cycle progression in G₁ and G₂ (8). Recent studies have also revealed that flavopiridol also inhibits expression of cyclin D1, an effect which would also contribute to the ability of the drug to cause cell cycle arrest (9). At low concentrations, flavopiridol is not toxic to resting or confluent cells *in vitro*. However, at higher concentrations (~1 μM) toxicity can occur in resting as well as cycling cells (10). Initial clinical trials

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² To whom requests for reprints should be addressed, at University of California San Diego Medical Center 8414, 200 West Arbor Drive, San Diego, CA 92103. Phone: (619) 543-3726; Fax: (619) 543-6969; E-mail: kbarrett@ucsd.edu.

³ The abbreviations used are: CDK, cyclin-dependent kinase; EGF, epidermal growth factor; cAMP, cyclic AMP.

have suggested antineoplastic effects of this agent against refractory cancers, in particular prostate, renal, and colon cancers, as well as lymphoma (11, 12). The major dose-limiting toxicity of the drug was severe diarrhea, which necessitated the cessation of treatment in some cases (11, 12). However, unlike diarrhea associated with the use of more classical chemotherapeutic agents, the diarrheal side effects of flavopiridol were associated with little or no apparent damage to the intestinal epithelium and instead had "secretory" electrolyte features (11). We therefore hypothesized that flavopiridol might have direct effects on the chloride secretory properties of epithelial cells, and that these effects might result in diarrhea *in vivo*. The present study represents an *in vitro* approach, using the human colonic epithelial cell line T₈₄, to test this hypothesis.

MATERIALS AND METHODS

Materials. Carbachol and forskolin (Sigma Chemical Co., St. Louis, MO), EGF (Genzyme, Cambridge, MA), genistein, and thapsigargin (LC Laboratories, Woburn, MA) were purchased from the sources indicated. Taurodeoxycholic acid was the generous gift of Dr. Alan Hofmann (University of California, San Diego, CA). Flavopiridol was supplied by Dr. Jennifer Dumont (Hoechst Marion Roussel, Bridgewater, NJ). All other reagents used were of at least analytical grade and were obtained commercially.

Cells. The human colonic epithelial cell line T₈₄ was used for all experiments in this study. Cells were maintained in DME/F12 media with 5% newborn calf serum and in a humidified atmosphere of 95% air, 5% CO₂. For chloride secretion experiments and cAMP assays, T₈₄ cells were seeded onto 12-mm Millicell-HA culture plate inserts (permeable supports) and grown for 8–12 days. At the time of study, cell monolayers had stable values of transepithelial resistance in excess of 1000 Ω.cm².

Measurement of Chloride Secretion. Chloride secretory responses of T₈₄ cell monolayers grown on permeable supports were measured by mounting the cells in modified Ussing chambers, as described previously (13), and quantitated as changes in short circuit current (I_{sc}). Previous studies have shown that I_{sc} is wholly reflective of changes in transepithelial chloride secretion in response to a variety of agonists in this model (14, 15). Studies were conducted under short-circuited conditions except during brief intervals (3–5 s at each time point) when the open circuit potential difference across the monolayer was assessed. All studies were conducted in Ringers' solution (140 mM Na⁺, 5.2 mM K⁺, 1.2 mM Ca²⁺, 0.8 mM Mg²⁺, 119.8 mM Cl⁻, 25 mM HCO₃⁻, 2.4 mM H₂PO₄⁻, 0.4 mM HPO₄⁻, and 10 mM glucose).

cAMP Assay. cAMP was measured in lysates of stimulated or control cell monolayers using a commercially available cAMP enzyme immunoassay kit (Amersham, Arlington Heights, IL). Cells grown on Millipore inserts were rinsed thoroughly with Ringers' solution and allowed to equilibrate in this solution at 37°C for 15 min. Cells were then stimulated with flavopiridol (10⁻⁴ M) on the apical side or forskolin (10⁻⁵ M) on the apical and basolateral sides as a positive control for various times as indicated by the experimental design. Ice-cold ethanol/Ringers' (2:1, v/v) solution was added to stop the reaction, and

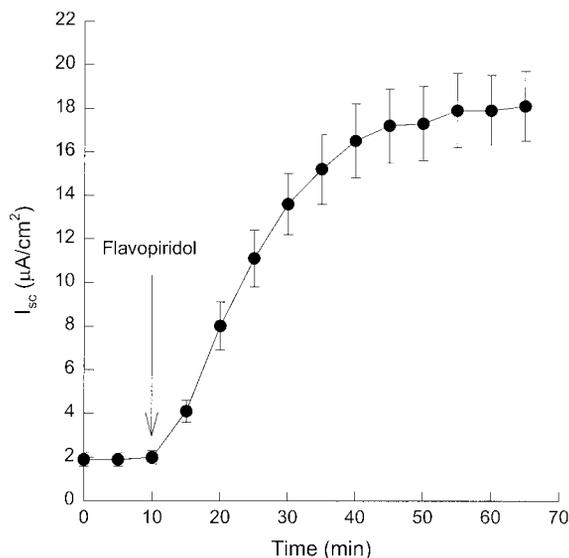


Fig. 1 Effect of flavopiridol (10⁻⁴ M) on chloride secretion across monolayers of T₈₄ cells. Flavopiridol was added apically at 10 min (arrow), and changes in chloride secretion were monitored as short circuit current (I_{sc}). Values are means for 27 experiments; bars, SE.

the lysates were kept at -70°C for no longer than 24 h before completing the assay.

Statistical Analysis. All data are expressed as means ± SE for a series of experiments. Statistically significant differences between mean values were analyzed by Student's *t* test or ANOVA with Bonferroni post-hoc tests, as appropriate.

RESULTS

Effect of Flavopiridol on Chloride Secretion. We first examined whether flavopiridol alone could stimulate chloride secretion across T₈₄ cell monolayers. Various doses of the drug were applied to either the apical or basolateral side and any change in I_{sc} noted. Flavopiridol stimulated a slow but sustained chloride secretory response when applied apically to T₈₄ monolayers (Fig. 1). The maximal I_{sc} response of 17.9 ± 1.6 µA/cm² (n = 27) was recorded at a dose of 10⁻⁴ M. Flavopiridol stimulated dose-dependent increases in I_{sc} when added to either the apical or basolateral side of T₈₄ cells, but the apical response was more pronounced (data not shown). To determine the mechanism of this direct effect on chloride secretion, cAMP levels were measured. As shown in Fig. 2, flavopiridol (10⁻⁴ M) caused a significant increase in intracellular cAMP levels that was similar to the increase in cAMP induced by forskolin, used as the positive control. However, this dose of flavopiridol has a smaller effect on I_{sc} than does forskolin, underscoring the complexity inherent in the regulation of chloride secretion, and the fact that simple correlations between second messenger levels and transport responses are not possible. Moreover, the concentration of flavopiridol (10⁻⁴ M) that caused increases in I_{sc} and cAMP is considerably in excess of blood levels of the drug measured in clinical settings (11, 12); therefore, the significance of these findings is somewhat unclear. At lower doses of flavopiridol (≤10⁻⁶ M) that might be seen in patients treated with

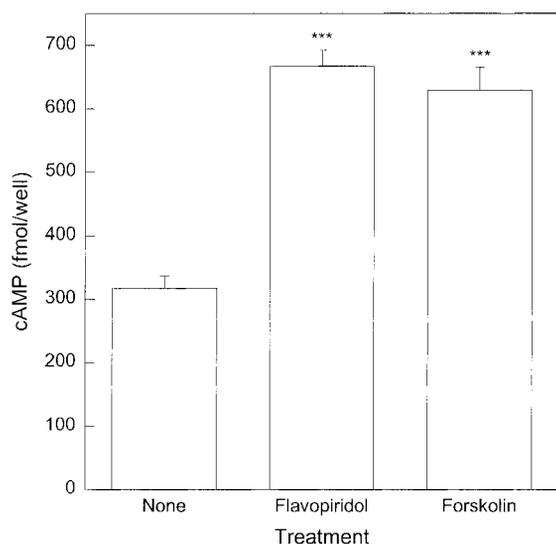


Fig. 2 Effect of flavopiridol (10^{-4} M) on cAMP levels in T_{84} cell monolayers. Cells were either left untreated or treated with flavopiridol or forskolin (10^{-5} M) as a positive control for 15 min. cAMP levels in cell lysates were measured by ELISA, and are means for six experiments; bars, SE. ***, significant elevations compared with baseline values in untreated control cells, $P < 0.001$ by ANOVA with Bonferroni post-hoc test.

the drug (11, 12), flavopiridol had no direct effect on chloride secretion ($I_{sc} < 1 \mu A/cm^2$) and no measurable effect on cAMP levels (data not shown).

Effect of Flavopiridol on Chloride Secretion Induced by Calcium-dependent Agonists. Because flavopiridol had little independent effect on chloride secretion at clinically achievable doses, it was unlikely that its direct effect on secretion could account for its diarrheal side effects. We therefore tested whether flavopiridol might instead potentiate responses to other known chloride secretagogues. Initial studies focused on calcium-dependent agonists, such as the muscarinic agonist carbachol (14). Pretreatment with flavopiridol led to a significant potentiation of the secretory response to carbachol, in that the response was both increased in magnitude and prolonged. As shown in Fig. 3, potentiation was seen at 10^{-4} M flavopiridol, as well as at doses of the drug ($\leq 10^{-6}$ M) that do not, by themselves, cause significant chloride secretion. In fact, doses of flavopiridol $\geq 10^{-9}$ M caused significant potentiation of the response to carbachol (Fig. 3B).

To determine whether the ability of flavopiridol to potentiate calcium-dependent secretory responses was specific for carbachol, other secretagogues were also tested. Thus, cells were pretreated with flavopiridol then stimulated with thapsigargin, an agent that elevates intracellular calcium levels in a receptor-independent fashion (15, 16). As seen for carbachol, flavopiridol also potentiated the chloride secretory response to thapsigargin (Fig. 4). This effect was also seen using lower doses of flavopiridol ($\leq 10^{-9}$ M; data not shown). Furthermore, the synergistic interaction between flavopiridol and thapsigargin was still evident if the sequence of agonist addition was reversed. Thus, pretreatment with thapsigargin also potentiated the secretory response to flavopiridol (data not shown). We also

questioned whether flavopiridol might alter responses to bile acids, which are endogenous calcium-dependent chloride secretagogues (17, 18). As shown in Fig. 5, flavopiridol significantly potentiated the secretory response to one such bile acid, taurodeoxycholate, in T_{84} cells. Again, the effect could also be reproduced with lower doses of flavopiridol (data not shown).

Effect of Flavopiridol on Inhibition of Chloride Secretion Induced by Carbachol or EGF. Recent studies from our laboratory have shown that calcium-dependent chloride secretory responses are also subject to a number of inhibitory mechanisms that limit their duration and/or magnitude (19, 20). Carbachol initially stimulates a secretory response, as noted above, but then renders the cells refractory to further stimulation by calcium-dependent agonists (16, 21). In contrast, EGF inhibits calcium-dependent chloride secretion without itself acting as a stimulus of this process (22). At least in theory, a drug such as flavopiridol might cause diarrhea not only by stimulating or potentiating positive signals for chloride secretion but also by attenuating negative influences on this process.

We therefore tested whether flavopiridol had any effect on muscarinic receptor-mediated inhibition of calcium-dependent chloride secretion. As expected (16), carbachol pretreatment inhibited the subsequent chloride secretory response of T_{84} cells to a second calcium-dependent secretagogue, thapsigargin (Fig. 6). However, in the presence of flavopiridol, carbachol no longer inhibited thapsigargin-induced chloride secretion (Fig. 6). Indeed, the response to thapsigargin was increased to a comparable extent by flavopiridol pretreatment whether or not carbachol was present. These data suggest that flavopiridol is able to reverse completely the inhibitory effect of carbachol on subsequent thapsigargin-induced chloride secretion.

Similarly, when EGF was added to T_{84} cells prior to the addition of thapsigargin, there was no immediate effect of the growth factor alone on basal chloride secretion, but the response to thapsigargin was inhibited (Fig. 7). In the presence of flavopiridol, the response to thapsigargin in cells pretreated with EGF did not differ significantly from that obtained in cells treated with thapsigargin alone (Fig. 7). Thus, the ability of flavopiridol to reverse the inhibitory effect of EGF on chloride secretion could also contribute to excess chloride secretion *in vivo*.

Interaction of Flavopiridol with the cAMP-dependent Agonist, Forskolin. Finally, we tested the effect of flavopiridol on chloride secretory responses to the cAMP-dependent agonist, forskolin. In contrast to findings obtained with calcium-dependent stimuli of chloride secretion, pretreatment with flavopiridol did not potentiate the secretory response to forskolin; in this case, the response to forskolin was not statistically different from that obtained with forskolin alone (data not shown). Interestingly, however, pretreatment with forskolin significantly potentiated a subsequent response to flavopiridol (Fig. 8).

DISCUSSION

The experiments described in this report were designed to examine the possibility that diarrheal side effects experienced by patients taking flavopiridol could be related to alterations in chloride secretion. In keeping with this hypothesis, flavopiridol

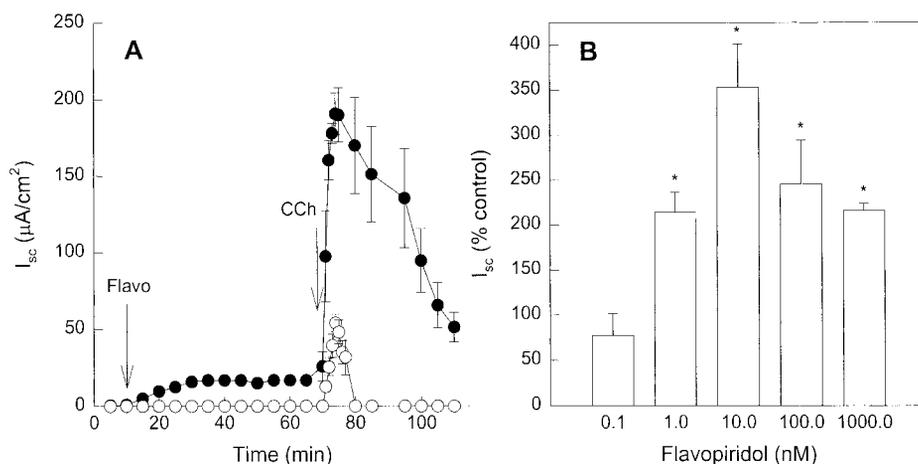


Fig. 3 Effect of flavopiridol on chloride secretion induced by carbachol (CCh, 10^{-4} M). A, time course of chloride secretory responses to carbachol in the absence (\circ) or presence (\bullet) of flavopiridol (Flavo, 10^{-4} M). Values are means for four experiments; bars, SE. B, effect of various nonsecretory doses of flavopiridol on chloride secretory responses to carbachol (10^{-4} M). Values are expressed as a percentage of the response obtained in the absence of flavopiridol pretreatment and are means for two to five experiments; bars, SE. *, values that represent significant potentiation of the response to carbachol, $P < 0.05$ by ANOVA with Bonferroni post-hoc test.

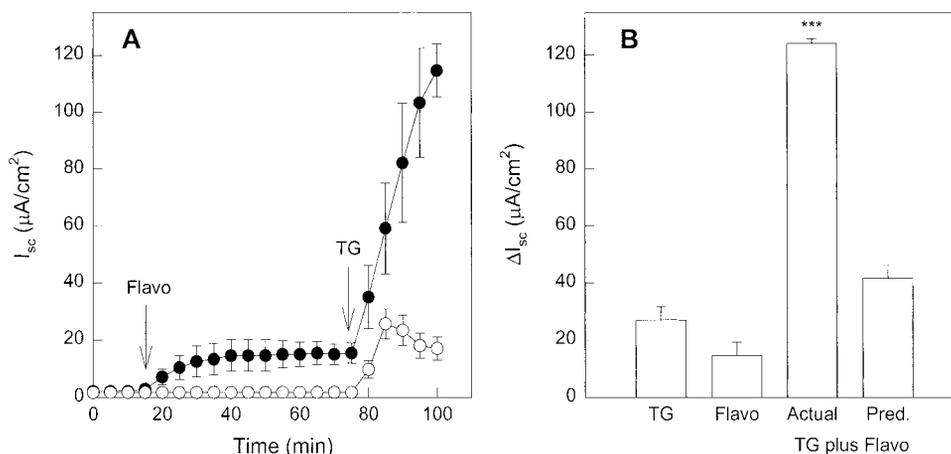


Fig. 4 Effect of flavopiridol on chloride secretion induced by thapsigargin (TG, 2×10^{-6} M). A, time course of chloride secretory responses to thapsigargin in the absence (\circ) or presence (\bullet) of flavopiridol pretreatment (Flavo, 10^{-4} M). Values are means for four experiments; bars, SE. B, increases in chloride secretion (ΔI_{sc}) induced by flavopiridol or thapsigargin alone or the combination of these agents (Actual). Pred., the predictive additive response calculated by addition of the individual responses to flavopiridol and thapsigargin. The response obtained with the combined agents significantly exceeded the predicted response, $P < 0.001$ by Student's t test. Values are means for four to five experiments; bars, SE.

was shown to modify various aspects of chloride secretion in an *in vitro* model, although some of the effects that were seen occurred at concentrations of the drug that are likely to be in excess of any that can be obtained clinically (11, 12).

Flavopiridol is a flavonoid, and other known flavonoids such as genistein are either direct chloride secretagogues at high doses or potentiate responses to other stimuli at low doses (23–25). In general, the ability of flavonoids to modify chloride secretion in T_{84} cells as well as in other systems has been ascribed to their ability to act as tyrosine kinase inhibitors. Some studies, however, also suggest a direct effect of the flavonoid genistein, at least, on CFTR chloride channels localized to the apical membrane of epithelial cells (23). Furthermore, we and others have determined that tyrosine kinase activity, including

that induced by binding of EGF to its receptor, serves to limit calcium-dependent chloride secretion (22, 26, 27). The mechanism of this effect is still the subject of investigation, although it appears likely to involve the tyrosine kinase-dependent generation of inhibitory intracellular messengers such as inositol 3,4,5,6-tetrakisphosphate and the products of phosphatidylinositol 3-kinase (20, 25, 27). In turn, these intracellular mediators directly or indirectly inhibit the activity of membrane chloride and potassium channels required for the overall process of chloride secretion (20, 28, 29). It is possible, therefore, that the ability of flavopiridol to potentiate responses to calcium-dependent secretagogues and to reverse inhibitory effects on chloride secretion might be related to some activity of the compound as a tyrosine kinase inhibitor. However, previous studies on this

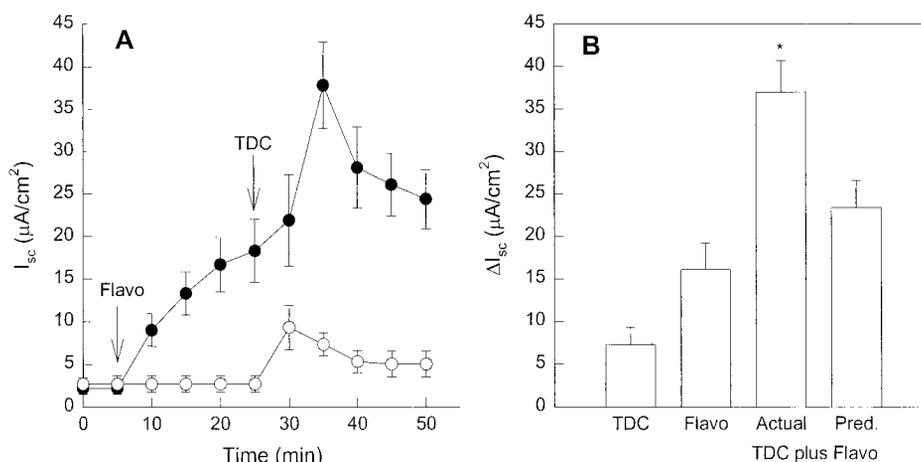


Fig. 5 Effect of flavopiridol on chloride secretion induced by taurodeoxycholate (TDC, 5×10^{-4} M). A, time course of chloride secretory response to taurodeoxycholate in the absence (\circ) or presence (\bullet) of flavopiridol pretreatment (Flavo, 10^{-4} M). Values are means for five to six experiments; bars, SE. B, increases in chloride secretion (ΔI_{sc}) induced by flavopiridol or taurodeoxycholate alone or the combination of these agents (Actual). Pred., the predictive additive response calculated by addition of the individual responses to flavopiridol and taurodeoxycholate. The response obtained with the combined agents significantly exceeded the predicted response, $P < 0.05$ by Student's t test. Values are means for five to six experiments; bars, SE.

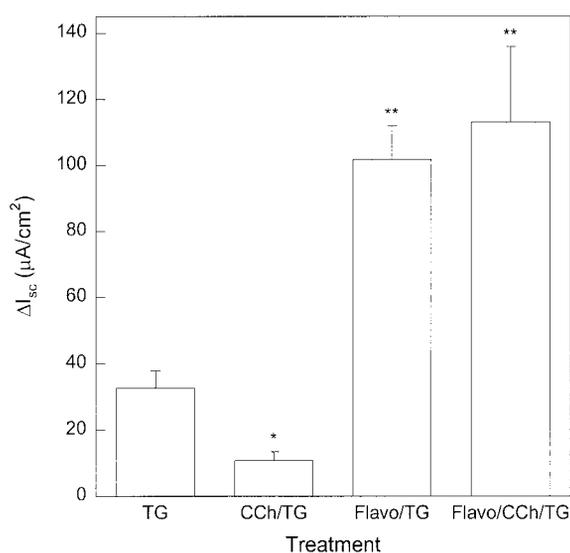


Fig. 6 Increases in chloride secretion (ΔI_{sc}) induced by thapsigargin (TG) alone or after pretreatment with carbachol (CCh, 10^{-4} M), flavopiridol (Flavo, 10^{-4} M), or the combination of carbachol plus flavopiridol. Values are means for 3–10 experiments; bars, SE. *, responses to TG that differed from the response obtained with TG alone ($*$, $P < 0.05$; $**$, $P < 0.01$ by ANOVA with Bonferroni post-hoc test). The response to TG in the presence of flavopiridol plus carbachol did not differ significantly from that obtained in the presence of flavopiridol alone ($P > 0.05$).

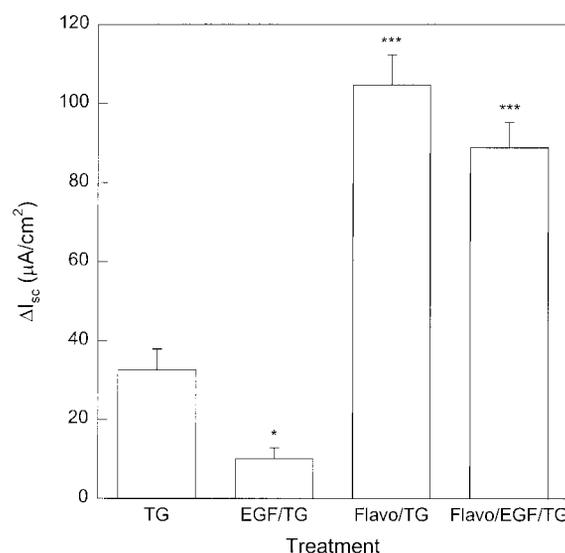


Fig. 7 Increases in chloride secretion (ΔI_{sc}) induced by thapsigargin (TG) alone or after pretreatment with EGF (100 ng/ml), flavopiridol (Flavo, 10^{-4} M), or the combination of EGF plus flavopiridol. Values are means for three to five experiments; bars, SE. *, responses to TG that differed from the response obtained with TG alone ($*$, $P < 0.05$; $***$, $P < 0.001$ by ANOVA with Bonferroni post-hoc test). The response to TG in the presence of flavopiridol plus EGF did not differ significantly from that obtained in the presence of flavopiridol alone ($P > 0.05$).

agent suggest that although flavopiridol can inhibit EGF receptor tyrosine kinase activity, this effect occurs at concentrations of the drug that do not correspond to the lower range of doses examined here (IC_{50} , 21 μM ; Ref. 8). Similarly, flavopiridol does not inhibit binding of EGF to its receptor, even at concentrations in excess of 2 mM (8). In contrast, effects of flavopiridol

on ErbB2 could conceivably be involved in its diarrheal effects, because we have shown that this receptor tyrosine kinase is pivotally involved in the inhibitory effects of EGF on epithelial chloride secretion (30, 31).

Alternative mechanisms of the effect of flavopiridol on chloride secretion can be considered. The drug is a weak inhib-

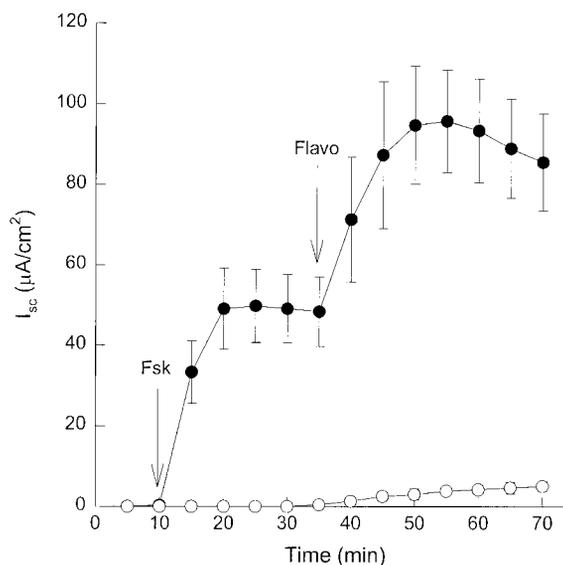


Fig. 8 Chloride secretion induced by flavopiridol (10^{-4} M) in the presence (○) or absence (●) of pretreatment with forskolin (Fsk, 10^{-5} M). Forskolin and flavopiridol were added at the times illustrated by the arrows. Values are means for four experiments; bars, SE. Where bars are not shown, SEs are contained within the symbols.

itor of protein kinase A (IC_{50} , 122 μM ; Ref. 8), although this activity would be expected to decrease chloride secretion rather than increase it (19). This activity might, however, account for the intriguing observation that pretreatment with forskolin potentiated responses to flavopiridol, but not *vice versa*. However, at present, we do not have a mechanistic understanding of this phenomenon. Alternatively, the effects of flavopiridol may relate to its ability to inhibit protein kinase C (IC_{50} , 6 μM ; Ref. 8) because we and others have shown that protein kinase C activation also appears to limit calcium-dependent chloride secretion via various mechanisms, including the generation of inositol 3,4,5,6-tetrakisphosphate and the inhibition of potassium channels (29, 32–34). However, an inhibitory effect of flavopiridol on protein kinase C activity is unlikely to account for the ability of the drug to reverse the inhibitory effect of carbachol on chloride secretion, because prior studies have shown that this effect is not reversed by the far more potent protein kinase C inhibitor, staurosporine (32).

It is also possible that the actions of flavopiridol on chloride secretion are somehow related to its relatively selective activity as an inhibitor of cyclin-dependent kinases (8). The drug inhibits cdk1, cdk2, cdk4, and cdk7 with IC_{50} s in the submicromolar range (8), certainly concentrations that are comparable with the lower doses of flavopiridol that were studied here. These kinases have been studied almost exclusively to date with respect to their roles in controlling the cell cycle, and limited information is available regarding other substrates for these kinases that might participate in the control of alternate cellular functions. However, cdk5, at least, has been shown to phosphorylate components of the actin cytoskeleton (35). If this activity is shared by other CDKs, it conceivably could alter chloride secretion in turn by regulating the insertion of pumps, channels, and cotransporters into the membrane (36–39).

The ability of flavopiridol to potentiate the chloride secretory response to taurodeoxycholate has an interesting clinical correlate. In preliminary clinical studies with flavopiridol, it was discovered that the administration of cholestyramine reduced the intensity of patients' diarrhea and in some cases, permitted escalation of the dose of flavopiridol that could be given (11, 12). In fact, this therapeutic strategy was prompted, in part, by the *in vitro* studies reported here, attesting to the validity of this approach to understand the adverse effects encountered (12). The clinical efficacy of cholestyramine might reflect an ability of the resin to bind flavopiridol and its known biliary metabolite, which would decrease the exposure of intestinal epithelial cells to flavopiridol (11). However, it is also tempting to speculate that at least part of the efficacy of cholestyramine in this setting relates to removal of endogenous secretagogues (*i.e.*, secretory bile acids) that might otherwise synergize with, and thereby uncover, a prosecretory action of flavopiridol. Similarly, anecdotal evidence suggests that flavopiridol-induced diarrhea could be diminished in some patients by administration of anticholinergic drugs (11).

In summary, flavopiridol, a cyclin-dependent kinase inhibitor, has demonstrated promising antineoplastic effects toward certain malignancies (11, 12). However, patients administered flavopiridol experience severe diarrheal side effects (11, 12). The current studies have shown that high doses of flavopiridol directly stimulate chloride secretion across monolayers of human colonocytes in modified Ussing chambers. This may be a result, at least in part, of the ability of flavopiridol to raise intracellular cAMP levels. Additionally, flavopiridol potentiates chloride secretory responses to calcium-dependent agonists and reverses cellular inhibitory mechanisms that normally limit the extent of calcium-dependent chloride secretion. Because these latter actions of flavopiridol can occur at doses that are clinically relevant (11, 12), they may well contribute to the side effects seen in patients treated with the drug. Whatever the intracellular/biochemical mechanism(s) of these effects, they could certainly provide an explanation as to why this medication causes severe diarrhea. A better understanding of the diarrhea induced by flavopiridol should allow optimization of therapy with this promising drug and/or the development of related agents with improved toxicity profiles (40).

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