Flavopiridol inhibits P-TEFb and blocks HIV-1 replication

Received for publication, July 10, 2000
Published, JBC Papers in Press, July 21, 2000, DOI 10.1074/jbc.C000446200

Sheng-Hao Chao‡, Koh Fujinaga‡, Jon E. Marion‡, Ran Taube‡, Edward A. Saussville‡, Adrian M. Senderowicz‡,‡‡, B. Matija Peterlin¶, and David H. Price‡‡

From the §Molecular Biology Program and the ‡Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242, the ¶Departments of Medicine, Microbiology and Immunology, Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, California 94113, and the **Developmental Therapeutics Program (DTP), DTP Clinical Trials Unit, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health, Bethesda, Maryland 20892

Flavopiridol (L86-8275, HMR1275) is a cyclin-dependent kinase (Cdk) inhibitor that is in clinical trials as a cancer treatment because of its antiproliferative properties. We found that the flavonoid potently inhibited transcription by RNA polymerase II in vitro by blocking the transition into productive elongation, a step controlled by P-TEFb. The ability of P-TEFb to phosphorylate the carboxyl-terminal domain of the large subunit of RNA polymerase II was inhibited by flavopiridol with a Ki of 3 nM. Interestingly, the drug was not competitive with ATP. P-TEFb composed of Cdk9 and cyclin T1 is a required cellular cofactor for the human immunodeficiency virus (HIV-1) transactivator, Tat. Consistent with its ability to inhibit P-TEFb, flavopiridol blocked Tat transactivation of the viral promoter in vitro. Furthermore, flavopiridol blocked HIV-1 replication in both single-round and viral spread assays with an IC50 of less than 10 nM.

Flavopiridol is a potential anti-cancer therapeutic agent currently being tested in phase I and II clinical trials (1). It has been proposed to target Cdk's controlling the cell cycle (2, 3) and induce apoptosis in various types of cancerous cells (1). Biochemical evidence indicated that flavopiridol most strongly inhibited Cdk1, Cdk2, and Cdk4 and was less potent on other Cdk's tested including Cdk7 (1). A structure of Cdk2 complexed with flavopiridol revealed that the drug docks in the ATP binding site (4), which helps to explain why inhibition of Cdk2 is competitive with ATP. Two previous studies demonstrated an effect of flavopiridol on transcription. Mammalian cells treated with the deacetylase showed a decreased transcription of the gene encoding cyclin D1 (5), and high levels of flavopiridol affected levels of 63 different mRNAs in Saccharomyces cerevisiae (6). The transcriptional inhibition observed in these studies could have been direct or a consequence of altered progression through the cell cycle.

The present study focuses on P-TEFb, a protein kinase composed of Cdk9 and a cyclin subunit derived from one of three different genes (7, 8). P-TEFb controls the elongation phase of transcription by RNA polymerase II (9) and is a cellular cofactor required for activation of transcription of the HIV-1 genome by the viral transactivator Tat (10, 11). Tat forms a triple complex with P-TEFb containing Cdk9 and cyclin T1 and the nascent transcript from the HIV-1 promoter (TAR) (12–14). This recruitment of P-TEFb activates transcription by causing an increase in the number of RNA polymerase II molecules that synthesize full-length mRNAs (10, 15). Our results demonstrated that flavopiridol inhibited P-TEFb causing inhibition of transcription, especially from the HIV-1 promoter in the presence of Tat. The properties of the inhibitor toward P-TEFb and on Tat transactivation suggest that the drug should be examined as a potential HIV-1 therapy.

EXPERIMENTAL PROCEDURES

Transcription and Kinase Assays—Pulse-chase transcription assays were carried out as described previously (16, 17) except for modifications indicated in the text. Kinase assays were as described in Marshall et al. (18) using Drosophila RNA polymerase II as the substrate. A 10 mM stock of flavopiridol (Aventis Inc.) in Me2SO was stored at −80 °C. The stock was diluted to 0.1 mM in Me2SO, and a set of serial dilutions in 4% Me2SO was used to give the indicated concentration of flavopiridol. The final concentration of Me2SO in transcription or kinase assays was less than 1%.

Single-round HIV-1 Infection Assays—50 μl of high titer viral stock generated from transfecting 293T cells with the HIV-1Lai provirus was added to 105 Sx221 cells that contain one copy of the HIV-1 long terminal repeat linked to the β-galactosidase reporter gene (19). Cells were incubated for 5 h to allow entry and integration of HIV-1, washed, and grown for an additional 36 h before being fixed and stained for β-galactosidase (19). Flavopiridol was added to the culture 12 h prior to the infection, and cells were grown in its presence for the duration of the assay.

Multiple-round Viral Spread Assay—In the presence of 10 μg of Polybrene, HIV-1NL4–3 viral particles (containing 2 ng of p24m) were added to 5 × 106 Jurkat cells. After 5 h virus was removed by extensive washing. Flavopiridol was added at indicated concentrations from 1.5 to 25 nM. On days 2, 5, 8, 11, and 15, supernatants were collected and cells were incubated with fresh medium and flavopiridol. Reverse transcriptase activity was measured on 10 μl of the supernatant. With the high multiplicity of infection used most Jurkat cells were killed after 10 days of infection, and viral titers returned to base-line levels.

RESULTS

To investigate the effects of flavopiridol on transcription an in vitro assay with a template containing the cytomegalovirus (CMV) promoter and HeLa nuclear extract was used. The inclusion of increasing concentrations of flavopiridol in the reaction resulted in a dramatic inhibition of the appearance of the 660-nucleotide run-off transcript (Fig. 1A). The radioactivity in run-off transcripts was quantitated, and the IC50 for the inhibitory effect was determined to be 34 nM. To determine if flavopiridol inhibited initiation or elongation a pulse-chase assay was used to separate the two processes. Flavopiridol was added

This paper is available on line at http://www.jbc.org

Vol. 275, No. 37, Issue of September 15, pp. 28345–28348, 2000
Printed in U.S.A.
Flavopiridol Inhibits P-TEFb

To determine if flavopiridol directly inhibited RNA polymerase II, early elongation complexes were isolated under stringent washing conditions that eliminate all known factors able to affect the elongation properties of the polymerase (17). The polymerases in these complexes are able to efficiently, albeit slowly, elongate their nascent transcripts as indicated by the 1-, 2-, 3-, 4-, and 5-min chase time points (Fig. 1D). Flavopiridol had no effect on the elongation properties of RNA polymerase II because transcripts produced in a 5-min chase with increasing amounts of flavopiridol were all the same length (Fig. 1D). Flavopiridol did not have any affect on transcription in vitro by RNA polymerase I or III (data not shown). The data in Fig. 1 strongly suggest that flavopiridol affects the action of P-TEFb.

We next examined the effect of flavopiridol on the ability of P-TEFb to phosphorylate the carboxyl-terminal domain (CTD) of RNA polymerase II. The kinase assay (18) utilized recombinant P-TEFb, composed of human Cdk9 and cyclin T1 (8), and purified RNA polymerase II. P-TEFb was dramatically inhibited by flavopiridol in a standard assay using 10 μM ATP (Fig. 2A). The IC50 was calculated to be 6 nM. Assays were also carried out using 30, 100, and 300 μM ATP. We were surprised to find that the IC50 did not vary significantly (6–10 nM) at the different concentrations of ATP (Fig. 2, A–D). The data from Fig. 2 were fit to equations derived for competitive, noncompetitive, and uncompetitive inhibition (21). The data fit uncompetitive inhibition best and gave an apparent Ks of 3 nM. This is 4 orders of magnitude below the Km for ATP of 36 μM. Even though the kinetic analysis suggested that the drug was not competitive with ATP, a competitive mechanism is possible if very tight binding of the inhibitor essentially inactivates the enzyme. Regardless of the mechanism, these results indicate that flavopiridol is the most effective P-TEFb inhibitor described so far.

Because P-TEFb comprised of Cdk9 and cyclin T1 is required into the reactions during the formation of preinitiation complexes (preincubation), initiation (pulse), or elongation (chase) (Fig. 1B). Initiation was not inhibited by the drug as indicated by the uniform production of short transcripts during the pulse (Fig. 1B, lanes marked P). However, the addition of flavopiridol at any step resulted in decreased levels of run-off transcripts during the subsequent chase (Fig. 1B, lanes marked C). Under the influence of flavopiridol the elongation complexes produced shorter, incomplete transcripts indicating that flavopiridol affected the elongation stage of transcription. Almost identical results were obtained when 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was used instead of flavopiridol (Fig. 1C). DRB inhibits P-TEFb, a cyclin-dependent kinase that controls the number of polymerases that enter into productive elongation (20). This similarity suggests that flavopiridol might inhibit P-TEFb function, but an alternative explanation, that the drug might slow the rate of elongation by RNA polymerase II, was also possible.

Flavopiridol affects the action of P-TEFb. Kinase assays were performed and quantitated as described (18) at the indicated ATP concentrations: 0 μM (A), 10 μM (B), 30 μM (C), 100 μM (D). Insets show autoradiographs from SDS-protein gels in which the phosphorylated polymerase was resolved. The hypophosphorylated (Iα) and hyperphosphorylated (Iβ) forms of the large subunit of RNA polymerase II are indicated. The lanes (left to right) are from reactions containing 0, 0.1, 0.3, 1, 3, 10, 30, 100, and 300 nM flavopiridol. The extent of phosphorylation was quantitated from dried gels using an InstantImager (Packard). The IC50 values were calculated by fitting the data to a logistic dose-response curve using the program TableCurve (Jandel Scientific). Each inhibition curve was performed more than twice on different days with the same results.

Fig. 1. Effect of flavopiridol on transcription in vitro. Pulse-chase transcription using the CMV promoter is shown. A, run-off transcripts generated in the presence of indicated amounts of flavopiridol were analyzed in a 6% gel (inset) and quantitated with an InstantImager (Packard). B, flavopiridol (1 μM) was added at different stages of a pulse-chase reaction as indicated: none, no drug added; pre-inc., preincubation. Reactions were stopped after the pulse (P) or after the chase (C). C, identical experiment with DRB (50 μM) instead of flavopiridol. D, early elongation complexes generated during a pulse labeling and isolated with a protocol (17) incorporating a 1 M KCl, 1% Sarkosyl wash were analyzed in a 6% gel (inset) and quantitated with an InstantImager (Packard).
for activation of the HIV-1 promoter by Tat, we examined the effect of flavopiridol on the ability of Tat to activate transcription from the HIV-1 promoter in vitro. Tat stimulated the appearance of a 694-nucleotide run-off transcript from the HIV-1 promoter about 5-fold (Fig. 3A). As has been found before, the P-TEFb inhibitor DRB blocked the formation of long run-off transcripts (Fig. 3A). When increasing concentrations of flavopiridol were included in Tat transactivation reactions the amount of run-off transcription was reduced to background levels. Quantitation of the results indicated that the IC₅₀ was 7 nM (Fig. 3B). These results are consistent with the ability of flavopiridol to inhibit the kinase activity of P-TEFb.

Flores et al. (22) demonstrated that reducing the activity of P-TEFb via the expression of a dominant negative Cdk9 protein or treatment with P-TEFb inhibitors reduced HIV-1 replication in cells. Therefore, effects of flavopiridol on the single round of infection by HIV-1 in Sx22-1 indicator cells and viral replication of HIV-1NL4–3 in Jurkat cells were examined (Fig. 4). Sx22-1 cells are HeLa cells that contain one copy of the HIV-1 promoter linked to the β-galactosidase reporter gene and can be efficiently infected by the HIV-1NL4–3 strain. Following the infection by HIV-1, the production of Tat leads to the expression of β-galactosidase, which is detected by the blue staining of Sx22-1 cells with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (19). The addition of flavopiridol reduced the number of blue cells to background levels and exhibited an IC₅₀ of 8 nM (Fig. 4A). Of note, the Sx22-1 cells remained viable even at the highest concentrations of flavopiridol (100 nM) as determined by trypan blue exclusion (data not shown). These findings were extended with a viral spread assay using Jurkat cells infected with the HIV-1NL4–3 virus (Fig. 4B). Using high multiplicities of infection, these cells produce maximal viral titers 5 days after the initial infection after which the cells begin to die. Reverse transcriptase assays again demonstrated that flavopiridol reduced the production of virus in a dose-dependent fashion. As was found in the single-round assay, a dramatic block of HIV-1 replication occurred at a concentration of flavopiridol between 6 and 12.5 nM (Fig. 4B). Cells that did not replicate HIV-1 remained viable at the highest concentra-

**FIG. 3. Effect of flavopiridol on Tat transactivation in vitro.** Continuous labeling transcription assays using the HIV-1 promoter were performed as described earlier (10). A, autoradiograph of run-off transcripts analyzed by denaturing PAGE. Tat (20 ng/reaction), DRB (50 μM), and flavopiridol were added to the indicated reactions. B, run-off transcripts were quantitated, and an IC₅₀ was determined as described in Fig. 1. The assay shown is representative of more than 5 assays performed with different preparations of Tat.

**FIG. 4. Effect of flavopiridol on HIV-1 infection.** A, effect of flavopiridol on the single round of infection in Sx22-1 indicator cells. Single-round infection assays were carried out as described under “Experimental Procedures.” The plot shows the number of blue cells in one well of a 96-well culture plate versus the concentrations of flavopiridol. Experiments are representative of two infections performed in triplicate. B, effect of flavopiridol on viral spread and replication in Jurkat cells. Viral spread assays were carried out as described under “Experimental Procedures.” Experiments are representative of two infections performed in duplicate where the standard errors of the mean were less than 20%.

**DISCUSSION**

The results presented here demonstrate that flavopiridol inhibits P-TEFb more potently than the previously suspected targets Cdk1 and Cdk4. Our data indicate that the IC₅₀ for flavopiridol against P-TEFb (3 nM) is significantly lower than the IC₅₀ for either Cdk1 (41 nM) (2) or Cdk4 (65 nM) (3). Because flavopiridol is not competitive with ATP on P-TEFb but is competitive with ATP on Cdk1 and Cdk4, there should be an even greater difference in IC₅₀ between P-TEFbs compared with Cdk1 or Cdk4 at the high ATP levels found in vivo. Because flavopiridol inhibits P-TEFb more potently than other Cdk, it is possible that the antiproliferative effects of the drug might be due to inhibition of P-TEFb and the subsequent negative effect on transcription.

The lack of competition with ATP we found for P-TEFb inhibition by flavopiridol suggests that the drug binds relatively tightly to P-TEFb. Because a crystal structure indicates that the drug docks in the ATP binding site of Cdk2 (4), it is likely that the ATP binding site of P-TEFb is also targeted. It is also possible that flavopiridol binds to another site and this is supported by the kinetic data that fit an uncompetitive model best. A structure of P-TEFb complexed with flavopiridol would be useful in resolving this issue.
The involvement of P-TEFb in the regulation of transcription from the HIV-1 promoter is well documented, but it is not clear why replication of the virus is more sensitive to reduction of P-TEFb activity than other viral or cellular promoters. One study used both an in vitro and in vivo assay to screen a library of over 100,000 compounds for molecules that blocked Tat transactivation of the HIV-1 promoter but not expression from the CMV promoter. Every compound identified was found to inhibit P-TEFb (23). Three of these inhibitors were shown to block HIV-1, but not human T-cell lymphotropic virus, type 1 replication at concentrations that were on average about 14 times lower than concentrations that caused cytotoxic effects (22). Significantly, expression of a dominant negative Cdk9 transgene at levels equal to the wild-type Cdk9 dramatically reduced the ability of the cells to support HIV-1 replication (22). HIV-1 replication in T cells requires activation of the cells, which is coupled to up-regulation of cyclin T1 levels (24). Initiation from the HIV-1 promoter in T cells before activation is not accompanied by efficient elongation to make viral mRNAs. After T cell activation, when the levels of P-TEFb containing cyclin T1 have increased, viral mRNAs are produced. This tight control of expression in vivo suggests that the HIV-1 promoter is strongly controlled by the action of negative factors that increase the dependence on P-TEFb. Two factors, negative elongation factor and DRB sensitivity-inducing factor, play a general role in elongation control (25–27), and DRB sensitivity-inducing factor has been shown to play a role in Tat transactivation (28). It is also possible that a CTD phosphatase might be especially active at the HIV-1 promoter. Presumably, the phosphatase would have a negative effect on elongation by reversing the P-TEFb-dependent phosphorylation of the CTD. Support for this idea comes from the finding that the phosphatase is inhibited by Tat (29, 30). Studies aimed at elucidating the mechanism of enhanced sensitivity of transcription from the HIV-1 promoter compared with other viral and cellular promoters are needed. These future studies should emphasize in vitro assays with virus or stably integrated viral promoters (22), because less than a 5-fold difference was seen between the HIV-1 and CMV promoter in vivo (Figs. 1 and 3) and in transient transfection assays (not shown).

Because P-TEFb is a key factor in HIV-1 infection and flavopiridol blocks HIV-1 propagation in cultured cells, we suggest that flavopiridol should be evaluated as a potential AIDS therapy. Currently the drug must be administered parenterally, and at its maximal tolerated dose in cancer patients (200–400 nM), it caused diarrhea and a proinflammatory syndrome (31). However, selection of lower dose levels that achieve the nanomolar drug levels predicted from these experiments to be potentially useful (10–20 nM) might alleviate these problems. The concept of using a P-TEFb inhibitor, however, may be broadly applicable because P-TEFb is also required for HIV-2, equine infectious anemia virus, simian immunodeficiency virus, and bovine immunodeficiency virus (11). Moreover, treatment with current drugs results in the selection and propagation of resistant viral strains. Because P-TEFb is a cellular factor it is unlikely that resistant strains will arise.

Acknowledgment—We thank Bryce Plapp (University of Iowa) for help with the kinetic analysis of flavopiridol inhibition of P-TEFb.

REFERENCES
1. Senderowicz, A. M., and Sausville, E. A. (2000) J. Natl. Cancer Inst. 92, 376–387
2. Lossewicz, M. D., Carlson, B. A., Kaur, G., Sausville, E. A., and Worland, P. J. (1994) Biochem. Biophys. Res. Commun. 201, 589–595
3. Carlson, B. A., Dubay, M. M., Sausville, E. A., Bizuela, L., and Worland, P. J. (1996) Cancer Res. 56, 2973–2978
4. De, A. W. J., Mueller-Dieckmann, H. J., Schulze-Gahmen, U., Worland, P. J., Sausville, E. A., and Kim, S. H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2735–2740
5. Carlson, B., Lahusen, T., Singh, S., Loiaiza-Perez, A., Worland, P. J., Pestell, R., Albanese, C., Sausville, E. A., and Senderowicz, A. M. (1999) Cancer Res. 59, 4634–4641
6. Gray, N. S., Wodicka, L., Thunnissen, A. M., Norman, T. C., Kwon, S., Espinoza, F. H., Morgan, D. O., Barnes, G., Le Clerc, S., Meijer, L., Kim, S. H., Lockhart, D. J., and Schultz, P. G. (1998) Science 281, 533–538
7. Fu, T.-J., Peng, J., Price, D. H., and Flores, O. (1999) J. Biol. Chem. 274, 34527–34530
8. Peng, J., Zhu, Y., Milton, J. T., and Price, D. H. (1998) Genes Dev. 12, 755–762
9. Price, D. H. (2000) Mol. Cell. Biol. 20, 3628–3634
10. Zhu, Y., Pevry, T., Peng, J., Ramanathan, Y., Marshall, N. F., Marshall, T., Amendt, B., Mathews, M. B., and Price, D. H. (1997) Genes Dev. 11, 2622–2632
11. Taupe, R., Fujinaga, K., Wimmer, J., Barboric, M., and Peterlin, B. M. (1999) Virology 264, 245–253
12. Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. A. (1998) Cell 92, 451–462
13. Garber, M. E., Wei, P., KewalRamani, V. N., Mayall, T. P., Herrmann, C. H., Rice, A. P., Littman, D. R., and Jones, K. A. (1998) Genes Dev. 12, 3512–3527
14. Bieniasz, P. D., Greda, T. A., Bogerd, H. P., and Cullen, B. R. (1999) Mol. Cell. Biol. 19, 4592–4599
15. Mareniak, R. A., and Sharp, P. A. (1991) EMBO J. 10, 4189–4196
16. Marshall, N. F., and Price, D. H. (1992) Mol. Cell. Biol. 12, 2078–2090
17. Peng, J., Liu, M., Marion, J., Zhu, Y., and Price, D. H. (1998) Cold Spring Harbor Symp. Quant. Biol. 63, 365–370
18. Marshall, N. F., Peng, J., Xie, Z., and Price, D. H. (1996) J. Biol. Chem. 271, 27176–27183
19. Fackler, O. T., Kienzle, N., Kremmer, E., Boese, A., Schramm, B., Klimkait, T., Kucherer, C., and Mueller-Lantzsch, N. (1997) Eur. J. Biochem. 247, 843–851
20. Marshall, N. F., and Price, D. H. (1995) J. Biol. Chem. 270, 12335–12338
21. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
22. Flores, O., Lee, G., Kessler, J., Miller, M., Schlief, W., Tomassini, J., and Hazuda, D. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7208–7213
23. Manezoe, H. S., Lee, G., Flygare, J., Tomassini, J., Liu, P., Zhu, Y., Peng, J., Blau, C., Hazuda, D., Price, D. H., and Flores, O. (1997) Genes Dev. 11, 2633–2644
24. Garriga, J., Peng, J., Parreno, M., Price, D. H., Henderson, E. E., and Grana, X. (1998) Oncogene 17, 3093–3102
25. Wada, T., Takagi, T., Yamaguchi, Y., Watanabe, D., and Honda, H. (1998) EMBO J. 17, 7393–7403
26. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Honda, H. (1999) Cell 97, 41–51
27. Garber, M. E., and Jones, K. A. (1999) Curr. Opin. Immunol. 11, 460–465
28. Wu-Baer, F., Lane, W. S., and Gaynor, R. B. (1998) J. Mol. Biol. 277, 179–197
29. Marshall, N. F., Dahmus, G. K., and Dahmus, M. E. (1998) J. Biol. Chem. 273, 31726–31730
30. Cho, H., Kim, T. K., Manezo, H., Lane, W. S., Flores, O., and Reinberg, D. (1999) Genes Dev. 13, 1540–1552
31. Senderowicz, A. M., Headlee, D., Stinson, S. F., Lush, R. M., Kalil, N., Villalba, L., Hill, K., Steinberg, S. M., Figg, W. D., Tompkins, A., Arboth, S. G., and Sausville, E. A. (1998) J. Clin. Oncol. 16, 2986–2999
Flavopiridol Inhibits P-TEFb and Blocks HIV-1 Replication
Sheng-Hao Chao, Koh Fujinaga, Jon E. Marion, Ran Taube, Edward A. Sausville, Adrian M. Senderowicz, B. Matija Peterlin and David H. Price

J. Biol. Chem. 2000, 275:28345-28348.
doi: 10.1074/jbc.C000446200 originally published online July 21, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000446200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 31 references, 20 of which can be accessed free at
http://www.jbc.org/content/275/37/28345.full.html#ref-list-1