Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 133–138

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

IN VIVO ANTI-HIV ACTIVITY OF (+)-CALANOLIDE A IN THE HOLLOW FIBER MOUSE MODEL

Ze-Qi Xu,* Melinda G. Hollingshead,[a] Suzanne Borgel,[b] Cindy Elder,[b] Albert Khilevich, and Michael T. Flavin

MediChem Research, Inc. and Sarawak MediChem Pharmaceuticals, Inc., 12305 South New Avenue, Lemont, IL 60439, U.S.A; [a]Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, and [b]SAIC-Frederick, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD 21701, U.S.A

Received 22 October 1998; accepted 20 November 1998

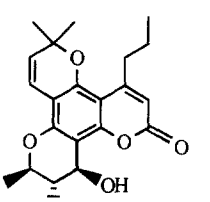
Abstract: In vivo anti-HIV efficacy of (+)-calanolide A has been evaluated in a hollow fiber mouse model. It was demonstrated that the compound was capable of suppressing virus replication in two distinct and separate physiologic compartments (ip and sc) following oral or parenteral administration on a once- or twice-daily treatment schedule. A synergistic effect was observed for the combination of (+)-calanolide A and AZT.

Introduction

(+)-Calanolide A, a natural product isolated from several tropical plants of the genus *Calophyllum*, has been demonstrated to be active against HIV-1.[1] Evaluation of its activity against HIV-1 RT and NNRTI-resistant viruses,[2-5] as well as detailed enzyme kinetics for RT inhibition,[6] has suggested that (+)-calanolide A represents a novel class of HIV-1 specific RT inhibitor. The antiviral features of (+)-calanolide A are highlighted below.

(1) **Activity against a wide range of HIV-1 strains**:[1-5] (+)-Calanolide A provides cytoprotection to both established and fresh human cells, including fresh peripheral blood leukocytes and macrophages, against all laboratory and clinical isolates of HIV-1, including both syncytium-inducing and nonsyncytium-inducing clinical isolates.

(2) **Unique sensitivity profile to drug-resistant virus isolates**:[1,2,6] While being fully active against AZT- and 3TC-resistant mutations, (+)-calanolide A exhibits enhanced anti-HIV activity against virus isolates with the Y181C mutation and against virus isolates with both Y181C and the AZT-resistance engendering mutations. Even though the compound possesses reduced activity against K103N mutant virus (four to ten fold), it remains fully active against virus isolates that express both the Y181C and K103N mutations. These two mutations are the most commonly observed mutations in both laboratory and clinical virus isolates and have proven highly resistant to nearly all other NNRTIs, including nevirapine (Viramune®), delavirdine (Rescriptor®), and efavirenz (Sustiva™).



(+)-Calanolide A

0960-894X/99/$ - see front matter
PII: S0960-894X(98)00713-6

(3) **Unique mutation resistant to (+)-calanolide A**:[7-10] In the in vitro resistance studies, (+)-calanolide A predominantly selects for a drug-resistant virus isolate having a previously unknown mutation at amino acid residue 139 (T139I), which is, however, susceptible to almost all other anti-HIV agents including NNRTIs.

(4) **Unique mechanism and synergistic effect in combination with other antiretroviral agents**:[6,7] Two binding sites/modes on HIV-1 RT have been proposed for (+)-calanolide A binding, indicating that it may interact with the HIV-1 RT in a mechanistically unique fashion. In the in vitro cell culture assays, (+)-calanolide A exhibits synergistic inhibition of HIV when used in combination with nucleoside RT inhibitors, protease inhibitors, or certain other NNRTIs.

(5) **Penetration to viral reservoirs**:[11] (+)-Calanolide A, due to its lipophilic nature, has been demonstrated to readily distribute into brain as well as lymph after oral and intravenous administration in rats.

Currently, (+)-calanolide A is in clinical trials to evaluate its safety and pharmacokinetics as single and multiple doses in both normal healthy and HIV-infected volunteers. After oral administration, the drug was generally well tolerated and no patterns indicative of a safety concern were observed.[12] Plasma drug concentrations in humans were higher than anticipated from animal data; AUC and C_{max} values increased with increasing dose. It appeared that therapeutic levels can be achieved in humans, based on in vitro EC_{90} values. However, the in vivo anti-HIV efficacy of (+)-calanolide A has never been demonstrated. Herein, we wish to report the in vivo results obtained in the hollow fiber mouse model, an animal model which has been demonstrated to be amenable to evaluation of efficacy of anticancer and antiviral agents.[13-15]

Materials and Methods

The hollow fiber-based anti-HIV efficacy assay that measures the ability of a compound to inhibit acute replication of HIV-1 in CEM-SS cells in an *in vivo* environment has been described previously.[13-15] Briefly, CEM-SS cells were acutely infected with the III_B strain of HIV-1.[16,17] These infected cells were immediately loaded into conditioned polyvinylidene fluoride hollow fibers (Spectrum Medical Corporation, Houston, TX) with a molecular mass exclusion of >500,000 Dalton and an internal diameter of 1 mm. The fiber samples were heat sealed at 2 cm intervals with hot needle holders (270-300 °F) and separated at the center of the seal and kept in medium at 4 °C until implanted into mice. Each 2-cm sample contained approximately 20 μL of cell inoculum. Three fiber cultures were implanted subcutaneously (sc) and three fiber cultures were implanted intraperitoneally (ip) into each SCID mouse (NCI Animal Production Facility, NCI-FCRDC, Frederick, MD), thus providing six experimental data points per mouse.

For each experiment, groups of mice received treatment with the vehicle, the test compound, or the positive control compound, ddC. The treatment route and schedule were varied among several experiments. The vehicles were sesame oil for oral gavage and DMSO for sc administration. The ddC control (40 mg/kg q8h) was solubilized in physiologic saline for administration by the ip route. Treatments were administered on Day 0 (day of fiber implant) through Day 6 with sample collection on Day 7. The samples collected included the fibers for cell viability determination and RT quantitation as well as serum and peritoneal washes for p24 antigen quantitation. The p24 antigen concentrations were determined with a commercially available enzyme-linked

immunosorbent assay (ELISA) kit (Coulter Diagnostics, Hialeah, FL). Cell viability was determined with a stable end-point MTT dye conversion assay.[18] (+)-Calanolide A was synthesized as previously described.[19]

Results and Discussion

A dose range-finding study was initially conducted with 3 mice per dose group. (+)-Calanolide A was administered to mice by oral gavage at various doses every 8 h (q8h), every 12 h (q12h), or once daily (q24h). The results indicated that (+)-calanolide A, given twice daily by the oral route at 150 mg/kg/dose (q12h) or once daily at 200 mg/kg/dose (q24h), produced significant anti-HIV activity in both subcutaneous and intraperitoneal compartments, as demonstrated by an increase in viable cell mass and a decrease in RT activity in the hollow fiber cultures, as well as a decrease in serum p24 concentrations (data not shown). Since the hollow fiber samples from the treated mice contained adequate viable cells, the decreases in both RT activity and p24 antigen production cannot be attributed to a lack of host cell replication. Furthermore, these doses were not lethally toxic to the host mice, while doses above 200 mg/kg q12h and 300 mg/kg q24h were found to be toxic. With a q8h dosing schedule, a dose of 100 mg/kg/dose only produced a decrease in RT activity, without protection against decreased cell viability; a dose of 150 mg/kg/dose (q8h) prevented increases in RT activity and p24 antigen production with an accompanying increase in cell viability, but it was toxic (data not shown).

Two groups of mice bearing only three sc or three ip fibers were treated with (+)-calanolide A at 100 mg/kg q12h by ip injection. The viral replication in the sc fibers was suppressed to the same level as that in the ip fibers, indicating that the compound was able to diffuse out of the peritoneal cavity and be delivered to the sc site.

These results encouraged further studies. Reproducible evidence of in vivo anti-HIV activity of (+)-calanolide A was obtained with once- or twice-daily dosing by the oral route (six mice per dosing group). Controls included saline-treated, DMSO-treated, and vehicle-treated mice (five mice per group). Again, the p24 antigen concentration in serum and peritoneal wash (Figure 1A), as well as RT activity in the hollow fiber culture (Figure 1B), indicated a significant reduction in viral replication following treatment. The dose-dependent reduction was not the result of cytotoxicity to the CEM-SS cells, since the viable cell mass in the hollow fiber samples exceeded the starting cell mass for all compound-treated groups (Figure 1C). Of equal importance was the fact that neither organ nor cellular toxicity was observed at antiviral doses.

To evaluate the correlation of the anti-HIV activity of (+)-calanolide A with its plasma concentrations, blood samples were collected from mice on Day 7 at the 24-h time period after the last dose, prior to the fiber collection. Plasma samples obtained were analyzed for the unchanged drug using a validated HPLC method.[20] Even though the plasma concentrations were found to be highly variable (the concentrations from the samples of 100 and 200 mg/kg/dose q24h groups were below the limit of quantification), they generally increased with the increasing of dosages and correlated with the anti-HIV activity observed (i.e., reduction in p24 antigen and RT activity). As illustrated in Figure 1D, the higher plasma concentrations led to a greater reduction in serum p24 antigen levels, with the exception of the 175 mg/kg q12h dose, which may be due to possible incomplete drug administration.

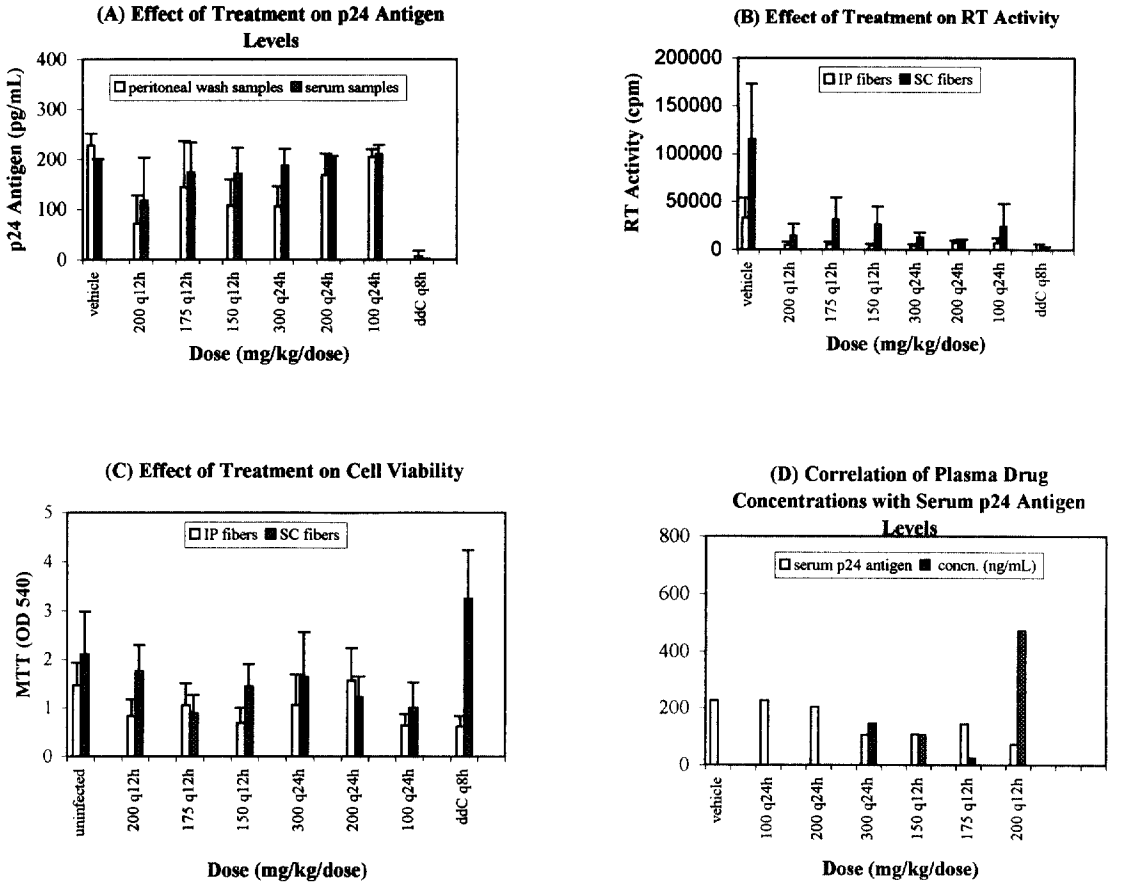
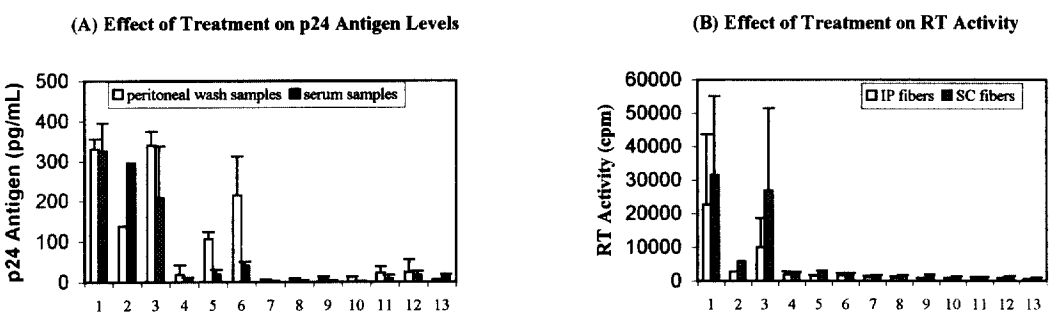


Figure 1. Anti-HIV Activity of (+)-Calanolide A in the Hollow Fiber Model

It was reported that the combination of (+)-calanolide A with AZT in the in vitro cell culture assay yielded a synergistic effect.[7] This synergy has been confirmed in this in vivo model. Two daily doses of (+)-calanolide A (50 and 100 mg/kg/dose, q12h) were orally administered into mice with both ip and sc implants for 6 days, along with three daily intraperitoneal doses of AZT (37.5, 70, and 150 mg/kg/dose, q8h), respectively. A profound reduction in p24 antigen and RT activity was observed even at a dose as low as 50 mg/kg of (+)-calanolide A when combined with 37.5 mg/kg of AZT (Figure 2). The combination reduced the p24 antigen in both serum and peritoneal wash to levels far below those seen with the single agents at the same dose levels.



1) vehicle q12h po, 2) (+)-calanolide A 100 mg/kg/dose q12h po, 3) (+)-calanolide A 50 mg/kg/dose q12h po, 4) AZT 150 mg/kg/dose q8h ip, 5) AZT 75 mg/kg/dose q8h ip, 6) AZT 37.5 mg/kg/dose q8h ip, 7) (+)-calanolide A 100 mg/kg/dose q12h po + AZT 150 mg/kg/dose q8h ip, 8) (+)-calanolide A 100 mg/kg/dose q12h po + AZT 75 mg/kg/dose q8h ip, 9) (+)-calanolide A 100 mg/kg/dose q12h po + AZT 37.5 mg/kg/dose q8h ip, 10) (+)-calanolide A 50 mg/kg/dose q12h po + AZT 150 mg/kg/dose q8h ip, 11) (+)-calanolide A 50 mg/kg/dose q12h po + AZT 75 mg/kg/dose q8h ip, 12) (+)-calanolide A 50 mg/kg/dose q12h po + AZT 37.5 mg/kg/dose q8h ip, 13) ddC 40 mg/kg/dose q8h ip

Figure 2. Anti-HIV Activity of (+)-Calanolide A in Combination with AZT

Conclusion

Evaluation of (+)-calanolide A in a hollow fiber culture-based in vivo assay of antiviral efficacy showed significant anti-HIV activity following oral or parenteral administration on a once- or twice-daily treatment schedule. The compound was capable of suppressing virus replication in two distinct and separate physiological compartments (subcutaneous site and peritoneal cavity), provided adequate dose levels were administered. This supports the conclusion that the compound is well distributed in the host following oral absorption. Furthermore, a synergistic effect was observed for the combination of (+)-calanolide A and AZT. Therefore, the in vivo efficacy demonstrated here, along with results of a variety of in vitro studies, suggests that (+)-calanolide A has characteristics favorable for its development as a clinical candidate against AIDS.

Acknowledgment: The authors would like to thank Dr. Joseph M. Covey at the Pharmacology and Toxicology Branch, DTP, DCTD, National Cancer Institute, Bethesda, Maryland for informative discussions. We are also indebted to Dr. Robert A. Newman and his group at University of Texas M. D. Anderson Cancer Center, Houston, Texas for analyzing the plasma samples. The work was supported in part by NCI contract NO1-CO-56000. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

References

1. Kashman, Y.; Gustafson, K. R.; Fuller, R. W.; Cardellina, J. H., II; McMahon, J. B.; Currens, M. J.; Buckheit, R. W.; Hughes, S. H.; Cragg, G. M.; Boyd, M. R. *J. Med. Chem.* **1992**, *35*, 2735.
2. Flavin, M. T.; Rizzo, J. D.; Khilevich, A.; Kucherenko, A.; Sheinkman, A. K.; Vilaychack, V.; Lin, L.; Chen, W.; Mata, E.; Pengsuparp, T.; Pezzuto, J. M.; Hughes, S. H.; Flavin, T. M.; Cibulski, M.; Boulanger, W. A.; Shone, R. L.; Xu, Z.-Q. *J. Med. Chem.* **1996**, *39*, 1303.
3. Currens, M. J.; Gulakowski, R. J.; Mariner, J. M.; Moran, R. A.; Buckheit, R. W., Jr.; Gustafson, K. R.; McMahon, J. B.; Boyd, M. R. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 645.
4. Boyer, P. L.; Currens, M. J.; McMahon, J. B.; Boyd, M. R.; Hughes, S. H. *J. Virol.* **1993**, *67*, 2412.
5. Hizi, A.; Tal, R.; Shaharabany, M.; Currens, M. J.; Boyd, M. R.; Hughes, S. H.; McMahon, J. B. *Antimicrob. Agents Chemother.* **1993**, *37*, 1037.
6. Currens, M. J.; Mariner, J. M.; McMahon, J. B.; Boyd, M. R. *J. Pharmacol. Exp. Ther.* **1996**, *279*, 652.
7. Buckheit, R. W., Jr.; Fliakas-Boltz, V.; Decker, W. D.; Roberson, J. L.; Stup, T. L.; Pyle, C. A.; White, E. L.; McMahon, J. B.; Currens, M. J.; Boyd, M. R.; Bader, J. P. *Antiviral Res.* **1995**, *26*, 117.
8. Buckheit, R. W., Jr.; Fliakas-Boltz, V.; Decker, W. D.; Roberson, J. L.; Pyle, C. A.; White, E. L.; Bowdon, B. J.; McMahon, J. B.; Boyd, M. R.; Bader, J. P.; Nickell, D. G.; Barth, H.; Antonucci, T.K. *Antiviral Res.* **1994**, *25*, 43.
9. Buckheit, R. W., Jr.; Fliakas-Boltz, V.; Yeagy-Bargo, S.; Weislow, O.; Mayers, D. L.; Boyer, P. L.; Hughes, S. H.; Pan, B.-C.; Chu, S.-H.; Bader, J. P. *Virology* **1995**, *210*, 186.
10. Buckheit, R. W., Jr.; Kilnjerski, T. L.; Fliakas-Boltz, V.; Russell, J. D.; Stup, T. L.; Palansch, L. A.; Brouwer, W. G.; Dao, D. C.; Harrison, W. A.; Schultz, R. J.; Bader, J. P.; Yang, S. S. *Antimicrob. Agents Chemother.* **1995**, *39*, 2718.
11. Frank, P.; Flavin, M. T.; Roca-Actin, J.; Xu, Z.-Q. Safety Assessment of (+)-Calanolide A, A Naturally Occuring Anti-HIV Agent. *4th Conference on Retroviruses and Opportunistic Infections*, Washington, D.C., January 22-26, 1997, Abstract 225.
12. Creagh, T.; Xu, Z.-Q.; Ray, L.; Giltner, J.; Nayer, T.; Ruckle, J. *5th Conference on Retroviruses and Opportunistic Infections*, Chicago, February 1-5, 1998, Abstract 652.
13. Hollingshead, M. G.; Roberson, J.; Decker, W.; Buckheit, R. W., Jr.; Elder, C.; Malspeis, L.; Mayo, J.; Grever, M. *Antiviral Res.* **1995**, *28*, 265.
14. Buckheit, R. W., Jr.; Hollingshead, M. G.; Stinson, S.; Fliakas-Boltz, V.; Palansch, L. A.; Roberson, J.; Decker, W.; Elder, C.; Borgel, S.; Bonomi, C.; Shores, R.; Siford, T.; Malspeis, L.; Bader, J. P. *AIDS Res. Human Retrovirsuses* **1997**, *13*, 789.
15. Hollingshead, M. G.; Alley, M. C.; Camalier, R. F.; Abbott, B.. J.; Mayo, J. G.; Malspeis, L.; Grever, M. R. *Life Sci.* **1995**, *57*, 131.
16. Buckheit, R. W., Jr.; Swanstrom, R. *AIDS Res. Human Retrovirsuses* **1991**, *7*, 295.
17. Redd, L. J.; Muench, H. *Am. J. Hyg.*, **1938**, *27*, 493.
18. Alley, M. C.; Pacula-Cox, C. M.; Hursey, M. L.; Rubinstein, L. R.; Boyd, M. R. *Cancer Res.* **1991**, *51*, 1247.
19. Khilevich, A.; Mar, A.; Flavin, M. T.; Rizzo, J. D.; Dzekhtser, S.; Brankovic, D.; Lin, L.; Zhang, H.; Chen, W.; Liao, S.; Zembower, D. E.; Xu, Z.-Q. *Tetrahedron: Asymmetry* **1996**, *7*, 3315.
20. Newman, R. A.; Chen, W.; Madden, T. *J. Liq. Chrom. Rel. Technol.* **1997**, *20*, 651.