11. G. Michaelsson, *Br. J. Derm.* **103**, 351 (1980).
12. C. L. Malmsten, J. Palmblad, A. Uden, O. Radmark, L. Engstedt and B. Samuelsson, *Acta physiol. scand.* **110**, 449 (1980).
13. M. A. Bray, A. W. Ford-Hutchinson and M. J. H. Smith, *Prostaglandins* **22**, 213 (1981).
14. S. C. Carr, G. A. Higgs, J. A. Salmon and J. A. Spayne, *Br. J. Pharmac.* **73**, 253P (1981).
15. M. A. Bray, F. M. Cunningham, A. W. Ford-Hutchinson and M. J. H. Smith, *Br. J. Pharmac.* **72**, 483 (1981).
16. A. L. Tappel, W. O. Lundberg and P. D. Boyer, *Archs Biochem. Biophys.* **42**, 293 (1953).
17. W. L. Smith and W. E. M. Lands, *J. biol. Chem.* **247**, 1038 (1972).
18. W. P. Kingston, *Br. J. Pharmac.* **74**, 919P (1981).
19. K. R. Palmer, J. R. Goepel and C. D. Holdsworth, *Br. Med. J.* **282**, 1571 (1981).
20. M. Campieri, G. A. Lanfranchi, G. Bazzocchi, C. Brignola, F. Sarti, G. Franzin, A. Battocchia, G. Labo and P. R. Dal Monte, *Lancet* **II**, 270 (1981).
21. C. P. Willoughby, J. Piris and S. C. Truelove, *Scand. J. Gastroent.* **15**, 715 (1980).
22. B. R. Baker, *Design of Active-Site-Directed Irreversible Enzyme Inhibitors*, pp. 202–4. John Wiley, New York (1967).
23. M. Binazzi, G. De Panfilis and G. Landi, *Drugs exptl clin. res.* **VII** (1), 57 (1981).
24. M. A. Peppercorn and P. Goldman, *Gastroenterology* **64**, 240 (1973).
25. W. F. Stenson and E. Lobos, *J. clin. Invest.* **69**, 494 (1982).

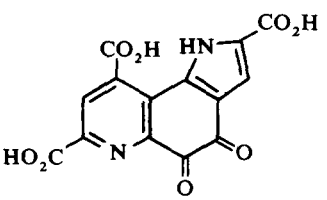
Biochemical Pharmacology, Vol. 32, No. 1, pp. 172–174, 1983.
Printed in Great Britain.

0006-2952/83/010172-03 $03.00/0

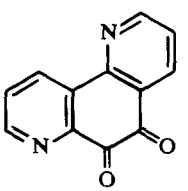
Effects of 1,7- and 1,10-phenanthroline dione on tissue culture cells*

(*Received* 20 *April* 1982; *accepted* 2 *August* 1982)

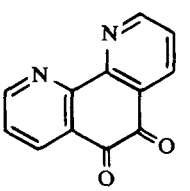
Methoxatin is a recently discovered coenzyme which functions in various bacterial NAD(P)-independent alcohol, glucose, aldehyde and, perhaps, methylamine dehydrogenases [1–4]. Because of the scarcity of this coenzyme, 1,7-phenanthroline dione (1,7-PAD) and 1,10-phenanthroline dione (1,10-PAD) have been studied as chemical models of methoxatin [5]. Both analogs were found to participate in diverse reactions such as 1-electron transfer, transamination, redox reactions, and condensation with nucleophiles. 1,10-PAD also has obvious structural analogy with *ortho*-phenanthroline which impairs cell proliferation [6, 7], and both have a quinone moiety found in a number of potentially useful anti-neoplastic agents [8–10]. Indeed, phenanthrenequinone and a number of related analogs have been shown to be cytotoxic towards cells in culture and to have activity in some experimental animal tumor models [9]. With these considerations, we undertook a study of the effects of 1,7- and 1,10-PAD on cells grown in tissue culture. A preliminary report of our findings is described here.



Methoxatin



1,7-PAD



1,10-PAD

Materials and methods

1,10-PAD was obtained from Alfa-Ventron and 1,7-PAD was prepared as previously described [5]. [CH_3-^{3}H]dThd (50 Ci/mmole) and [5-^{3}H]Urd (20 Ci/mmole) were purchased from Moravek Biochemicals, and [4,5-^{3}H]Ileu (100 Ci/mmole) was obtained from New England Nuclear. All other materials were of the purest grade commercially available. S49 mouse lymphoma cells were grown as suspensions in Dulbecco's Modified Eagle (DME) H16 media supplemented with 10% heat-inactivated horse serum. S180 mouse cells were grown as suspensions in DME H21 supplemented with 10% heat-inactivated fetal calf serum. Cell number was determined daily using a Coulter counter ZBI. The EC_{50} values refer to the concentration of drug which inhibited the growth rate of cells by 50% as compared to controls not containing drug.

Cloning assays of S49 cells were performed by a modfication of a reported procedure [11]. Primary mouse epithelial fibroblasts were grown as monolayers in DME H21 media supplemented with 10% heat-inactivated fetal calf serum. One day prior to cloning, confluent cultures were trypsinized, resuspended in media, and replated in 2.4 × 1.7 cm tissue culture wells at *ca.* 50–70% confluency as determined microscopically. The cloning medium was freshly prepared by adding 100 ml DME H16 medium (at 44°) containing 10% horse serum, 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer (pH 7.4), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin to an autoclaved solution of 0.44 g agarose in 10 ml water. The medium was kept at 44°. At least 1 hr before use, media from primary mouse epithelial cell cultures were aspirated, and cells were overlayed with 2.0 ml of the cloning media. Plates were briefly cooled and kept at 37° until use. S49 cells, treated as specified, were diluted

* This research was supported by USPHS Grants CA 14266 from the National Cancer Institute and BC-383 from the American Cancer Society.

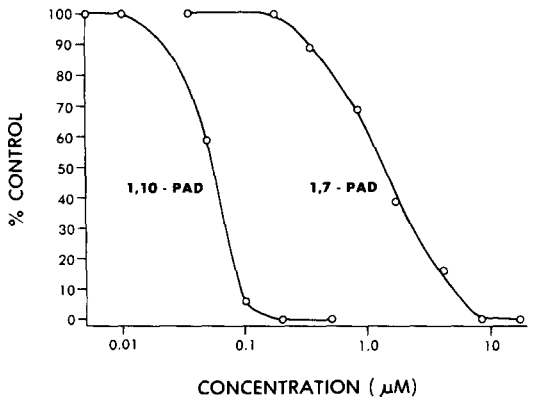


Fig. 1. Inhibition of growth rate of S-49 cells by 1,7- and 1,10-PAD. Cells were seeded at *ca.* 5×10^4 cells/ml and treated with indicated concentrations of the drugs. Aliquots of duplicate cultures were counted at 24, 48 and 72 hr. The percentage of control growth is 100 times the slope of the log of the number of cells versus time, divided by the slope of the untreated control culture [14].

to *ca.* 100–10,000 cells/ml in growth medium, and 0.5 ml of the suspension was diluted with 3 vol. of cloning medium. Aliquots (0.5 ml) were plated above wells containing cloning medium over feeder layers and incubated at 37°. After 14 days, clones were counted under a dissecting microscope. With unperturbed, exponentially growing S49 cells, cloning efficiency exceeded 90%.

dThd, Urd and Ileu incorporation into macromolecules was determined as follows. Cells at a density of *ca.* 10^6/ml were incubated at 37° with specified concentrations of the drug. At specified intervals, aliquots were transferred to sterile tubes containing the appropriate radiolabeled precursor, and incubation was continued. At 10-min intervals, 0.2-ml aliquots (*ca.* 2×10^5 cells) were removed and added to an equal volume of ice-cold 20% trichloroacetic acid (TCA) in a 1.5-ml Eppendorf microfuge tube and mixed with a vortex. After 10 min at 0°, with several agitations, tubes were centrifuged at 15,000 *g* for 15 sec. The supernatant solution was removed and the pellet was washed three times with ice-cold 5% TCA. The pellet was resuspended in 0.2 ml water, transferred to a scintillation vial, and solubilized by incubation with 1.5 ml Protosol at 50° for 45 min. Radioactivity was determined after addition of 0.1 ml acetic acid and 12 ml Aquasol.

Acid-soluble cell extracts were prepared as previously described [12]. Ribonucleotide pools were determined by high performance liquid chromatography (HPLC) on a Partisil-Sax column and eluted with an ionic strength-pH gradient [12]. Deoxyribonucleotide triphosphates were determined by HPLC after periodate-methylamine treatment [13].

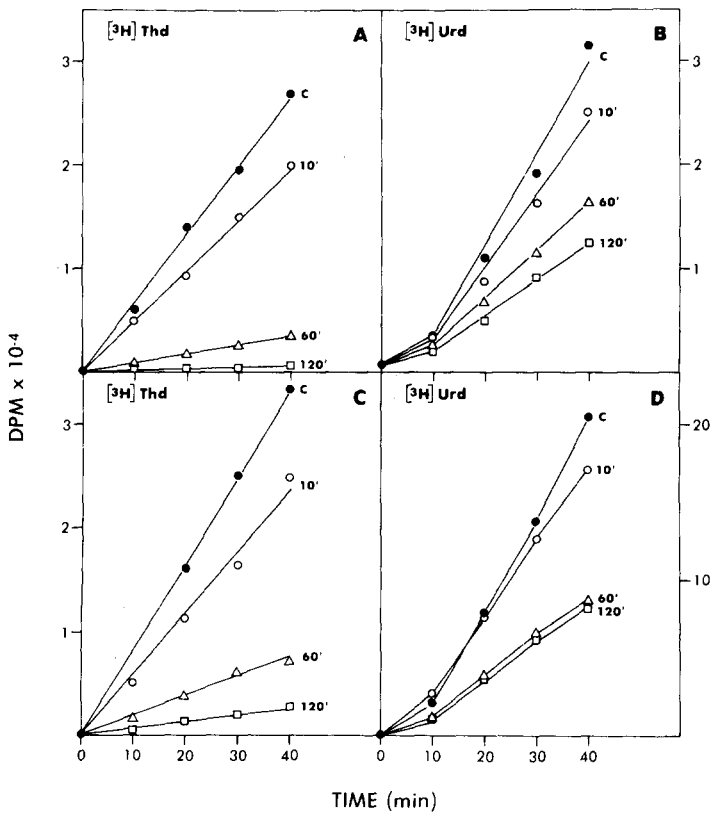


Fig. 2. Effects of 1,10-PAd and 1,7-PAD on incorporation of dThd and Urd into nucleic acids of S49 cells. The line labeled C is a control not exposed to the drug. The time of drug exposure (in minutes) prior to the addition of the radioactive precursor is indicated next to the other lines. At specified times, 2 ml containing 0.5 to 1×10^6 cells/ml were treated with [^{3}H]dThd (2.0 Ci/mmole) or [5-^{3}H]Urd (20 Ci/mmole). Aliquots (0.2 ml) were analyzed for acid-insoluble radioactivity at 10-min intervals as described in Materials and Methods. The panels show the effect of 1,10-PAD on incorporation of (A) 0.5 μCi [^{3}H]dThd or (B) 1.0 μCi of [5-^{3}H]Urd, and of 1,7-PAD on incorporation of (C) 0.5 μCi of [^{3}H]dThd or (D) 10 μCi of [5-^{3}H]Urd.

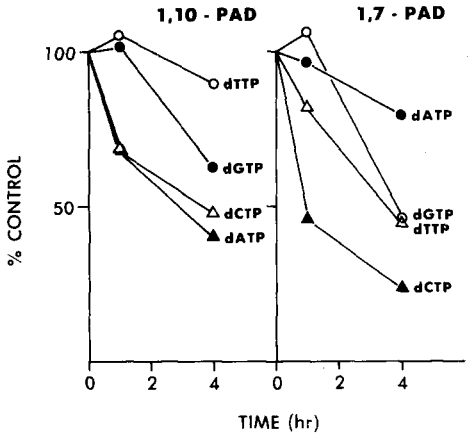


Fig. 3. Deoxynucleoside-5′-triphosphate pool size changes during exposure to 1,10-PAD and 1,7-PAD. Control values (pmoles/10^6 cells) were dATP, 65; dCTP, 70; dGTP, 24; and dTTP, 80.

Results and discussion

Both 1,7- and 1,10-PAD were potent growth inhibitors of S49 (Fig. 1) and S180 (data not shown) cells in culture. 1,7-PAD showed EC_{50} values of 1.4 and 1.3 μM with S49 and S180 cells respectively. 1,10-PAD was even more potent, with EC_{50} values of 0.056 and 0.042 μM for S49 and S180 cells. Similar EC_{50} values have been observed for 1,7- and 1,10-PAD against HKSV28, Balb 3T3 and Swiss 3T3 cell lines (Dr. S. J. Benkovic, personal communication).

When S49 cells were exposed to 11 μM 1,7-PAD or 0.56 μM 1,10-PAD for 6 or more hr, there was over a 2-log cell-kill as determined by cloning assays. Under conditions where controls showed 110 ± 7.7 (S.D.) clones/well, no clones grew from cells exposed to the drugs (N = 6). With 0.56 μM 1,10-PAD, cell-kill was also shown to be time dependent with estimated cell-kills of 80, 99 and over 99.7% after 5, 10 and 23 hr of drug exposure respectively. While additional experiments are required to accurately quantitate the extent of cell-kill, it is clear that the growth inhibitory properties of these compounds are due to cytocidal effects and not arrest of growth.

As shown in Fig. 2A, exposure of S49 cells to 1,10-PAD resulted in a dramatic decrease in the rate of [^{3}H]dThd incorporation into DNA. DNA synthesis was decreased as a function of the duration of exposure to 1,10-PAD with 87% reduction after 1 hr and essentially complete inhibition after 2 hr of exposure. Incorporation of [5-^{3}H]Urd into RNA was also inhibited, albeit to a lesser extent (Fig. 2B). A 50% reduction in the rate of RNA synthesis was observed after 2–3 hr of drug exposure. There appeared to be a negligible effect on the rate of protein synthesis as measured by the incorporation of [^{3}H]Ileu (10 μCi/ml, 100 Ci/mmole) after 3 hr of drug exposure (not shown). Essentially, the same effects on DNA, RNA and protein syntheses were observed with 1,7-PAD, although, as with cytotoxicity studies, a higher concentration of drug was necessary (Fig. 2C and D).

After a 4-hr exposure of S49 cells to 11 μM 1,7-PAD or 0.56 μM 1,10-PAD, HPLC analysis indicated that ribonucleotide pool sizes were unchanged. In contrast, these compounds caused interesting effects on the deoxyribonucleotide pools (Fig. 3). With 1,7-PAD there was a slight decrease in dATP with significant depletion of dGTP, dTTP and dCTP pools. In contrast, 1,10-PAD showed no significant change in dTTP levels, a moderate decrease in dGTP, and significant decreases in dCTP and dATP levels.

In summary, we have found that 1,7- and 1,10-diones are extremely potent cytotoxic agents towards cells in tissue culture. From the data available, it is reasonable to point to the inhibition of DNA synthesis and RNA synthesis as major components of the cytotoxic effects of 1,7- and 1,10-PAD. Although the diverse chemical reactions in which these compounds may participate raise the suspicion that they might act as general cell poisons, this is unlikely because of the very low concentrations at which they are cytotoxic, their lack of effect on protein synthesis and ribonucleotide pools, and their specific effects on deoxyribonucleotide pools. Nevertheless, it is difficult to suggest a single target which accounts for all of the effects of these compounds, and we presume at this time that they have more than one mode of action. Furthermore, since the effects of the two drugs on dXTP levels are qualitatively different, 1,7- and 1,10-PAD probably differ in at least one of their target sites. In this regard, we note that 1,10-PAD chelates metal ions, whereas 1,7-PAD is incapable of chelation [5]. The potent cytotoxic effects of 1,7- and 1,10-PAD, in particular the latter, as well as the likelihood that they represent a new mechanistic and structural class of agents, warrant testing of their efficacy as anti-neoplastic agents. Further elucidation of the mechanism of these drugs will await the results of such studies.

Departments of Pharmaceutical Chemistry and Biochemistry and Biophysics, and the Cancer Research Institute
Department of Medicine
University of California
San Francisco, CA 94143
U.S.A.

DAVID IGDALOFF
DANIEL V. SANTI*

Department of Chemistry
University of California
Santa Barbara, CA 93106,
U.S.A.

TIMOTHY S. ECKERT
THOMAS C. BRUICE

* Author to whom correspondence should be addressed.

REFERENCES

1. S. A. Salisbury, H. S. Forrest, W. B. T. Cruse and O. Kennard, *Nature, Lond.* **280**, 843 (1979).
2. J. A. Duine, J. Frank and J. K. VanZeeland, *Fedn Eur. Biochem. Soc. Lett.* **108**, 443 (1979).
3. M. Ameyama, K. Matsushita, Y. Ohno, E. Shinagawa and O. Adachi, *Fedn Eur. Biochem. Soc. Lett.* **130**, 179 (1981).
4. R. DeBeer, J. A. Duine, J. Frank and P. J. Large, *Biochim. biophys. Acta* **622**, 370 (1980).
5. T. S. Eckert, T. C. Bruice, J. A. Gainor and S. M. Weinreb, *Proc. natn. Acad. Sci. U.S.A.* **79**, 2533 (1982).
6. K. H. Falchuk and A. Krishan, *Cancer Res.* **37**, 2050 (1977).
7. C. Krishnamurti, L. A. Saryan and D. H. Petering, *Cancer Res.* **40**, 4092 (1980).
8. N. R. Bachur, S. L. Gordon and M. V. Gee, *Cancer Res.* **38**, 1745 (1978).
9. J. S. Driscoll, G. F. Hazard, Jr., H. B. Wood, Jr. and A. Goldin, *Cancer Chemother. Rep.* (Part 2) **4** (2), 1 (1974).
10. L. L. Bennett, Jr., D. Smithers, L. M. Rose, D. J. Adamson and H. J. Thomas, *Cancer Res.* **39**, 4868 (1979).
11. V. D. Courtenay, *Br. J. Cancer* **34**, 39 (1976).
12. A. L. Pogolotti, Jr. and D. V. Santi, *Analyt. Biochem.*, in press.
13. C. Garrett and D. V. Santi, *Analyt. Biochem.* **99**, 268 (1979).
14. M. Umeda and C. Heidelberger, *Cancer Res.* **28**, 2529 (1968).