Biochemical Pharmacology, Vol. 16, pp. 1933–1940. Pergamon Press Ltd. 1967. Printed in Great Britain

EQUIVALENCE OF OLIGOMYCIN AND 2-CHLORO-4′,4″-DI(2-IMIDAZOLIN-2-YL)TEREPHTHALANILIDE IN THEIR EFFECTS ON MITOCHONDRIAL SWELLING*

MARY LOU MELONI and WILLIAM I. ROGERS

Life Sciences Division, Arthur D. Little, Inc., Cambridge, Mass., U.S.A.

(*Received* 29 *November* 1966; *accepted* 17 *March* 1967)

Abstract—2-Chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide (NSC 60339) has been compared with oligomycin and 2,4-dinitrophenol in its effects on a rat liver mitochondrial swelling system. The substituted phthalanilide and oligomycin are equivalent in their effects in the following swelling experiments. Neither alters spontaneous or calcium- or phosphate-induced swelling; both inhibit succinate-induced swelling; both prevent ATP inhibition of calcium- and phosphate-induced swelling; and both inhibit ATP reversal of calcium-induced swelling in fresh or aged mitochondria.

The known binding of phthalanilides to specific phospholipids and the inhibitory effects described suggest that NSC 60339 may be useful in studies on the nature and role of lipids in mitochondrial swelling and oxidative phosphorylation.

WODINSKY *et al.*, Burchenal *et al.* and Skipper *et al.* and others have shown that several of the substituted phthalanilides are active against experimental lymphocytic, mast cell, and granulocytic leukemias in mice and rats and in many cases are 'curative'.[1–9] Several substituted phthalanilides have been shown to inhibit a wide variety of biochemical reactions, among which are DNA, RNA, protein and lipid synthesis [10–13] and mitochondrial function.[10, 14, 15] The subject has been reviewed by Burchenal,[8] by Bennett,[9] and by Kensler *et al.*[16]

The observations that 2-chloro-4′,4″-di(2-imidazolin-2-yl)terepthalanilide (NSC 60339) is found in the nuclei and mitochondria of kidney, liver, and tumor cells[10, 16–19] and is bound to a new class of phospholipids in P388 lymphocytic leukemia cells[17, 20] prompted the speculation that alteration of membrane integrity might share a common mechanism by which this drug exerts its chemotherapeutic and toxic effects. In the accompanying paper[19] it is shown that the substituted phthalanilide, NSC 60339, is found primarily in the mitochondrial and nuclear fractions of P388 murine lymphocytic leukemia and rat liver cells that have been exposed to the drug.

It is also shown that the phthalanilide completely inhibits phosphorylation but inhibits succinate oxidation only 50 per cent even at very high drug concentrations. This incomplete inhibition of succinate oxidation accompanied by complete inhibition of phosphorylation is similar to the results of Lardy *et al.*[21] for oligomycin and of Hollunger[22] for guanidine. They reported that respiration not tightly coupled to phosphorylation is not as greatly inhibited as respiration using NAD-linked substrates. This similarity to oligomycin in effect on respiration made it of interest to compare the

* Supported by the Cancer Chemotherapy National Service Center Contract no. PH-43-65-61, National Cancer Institute, National Institutes of Health.

phthalanilide to oligomycin, which is thought to inhibit the formation of a high-energy intermediate at a site not on the direct oxidative–phosphorylation pathway, and to 2,4-dinitrophenol, which uncouples oxidative phosphorylation. A preliminary report of this work has been made.[23]

MATERIALS AND METHODS

Materials

The 2-chloro-4′,4″-di(2-imidazolin-2-yl)terephthalanilide was synthesized at Dr. Wander, S. A., Berne, Switzerland and supplied to us by the Cancer Chemotherapy National Service Centre of the National Cancer Institute, U.S. Public Health Service. The oligomycin (A 65–70 per cent B 20–25 per cent C 5–10 per cent) was obtained from Mann Research Laboratories. Adenosine 5′-triphosphate was obtained from Sigma Chemical Co. Other reagents were the purest grades that were commercially available.

Preparation of mitochondria

Mitochondria were isolated by the method of Hogeboom, Schneider and Pallade[24] from the livers of healthy Sprague–Dawley rats weighing 100–150 g. The concentration of the stock mitochondrial particulate was equivalent to 0·75 g rat liver per ml of 0·25 M sucrose. The mitochondria were used immediately after preparation to minimize any aging effect, which reportedly produces ATPase.[25] For experiments in which aged mitochondria were used, the mitochondria were aged for 2 hr at 0° in 0·25 M sucrose.

Measurement of swelling

Swelling experiments were carried out in 1 × 10-cm tubes and O.D. was followed with a Bausch & Lomb Spectronic 20 spectrophotometer. The incubation medium consisted of 133 mM KCl 10 mM Tris-HCl at 7·4. The experiments were initiated by adding 0·10 ml of the stock mitochondrial suspension in 0·25 M sucrose and mixing the contents by inversion of the tubes. This concentration of mitochondria particulate gave a zero-time O.D. of approximately 0·950 at 500 mμ. Tubes containing a total volume of 3·00 ml were read every 5 min for a total of 30 min.

RESULTS

Effect on spontaneous and orthophosphate-induced mitochondrial swelling

Neither the phthalanilide nor oligomycin affected the shallow, spontaneous swelling of intact mitochondria over the 20-min experimental period (Fig. 1). Mitochondrial swelling was induced with 3 mM orthophosphate. Neither the phthalanilide nor oligomycin added at zero time inhibited the phosphate-induced swelling (Fig. 1).

The effect of 2,4-dinitrophenol and ATP is shown in Fig. 2. ATP or 2,4-dinitrophenol added at zero time caused complete inhibition of the phosphate-induced swelling. This inhibition of swelling by ATP was considerably diminished when either the phthalanilide or oligomycin was added to the system with the ATP. However, the inhibition of phosphate-induced swelling by 2,4-dinitrophenol was not affected by the addition of either phthalanilide or oligomycin.

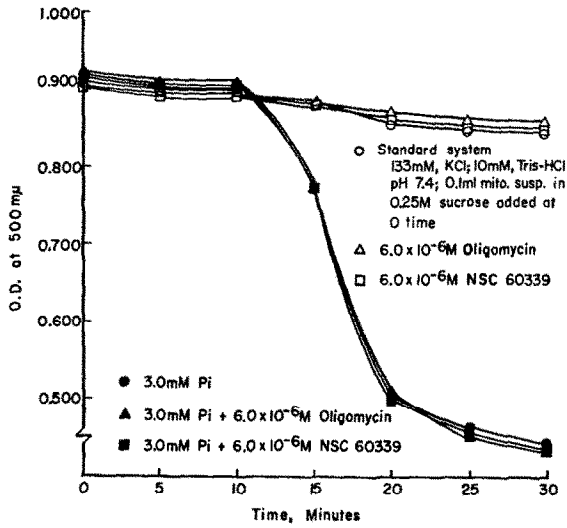

FIG. 1. Effect of phthalanilide and oligomycin on spontaneous and orthophosphate-induced mitochondrial swelling.

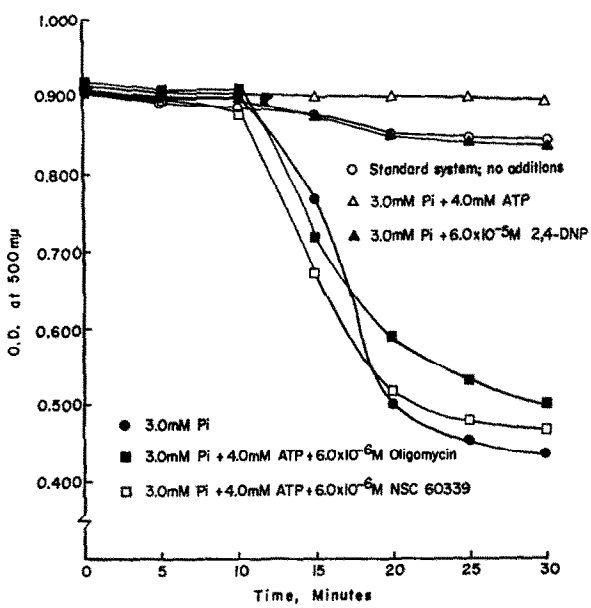

FIG. 2. Effect of phthalanilide and oligomycin on the ATP or 2,4-dinitrophenol inhibition of orthophosphate-induced mitochondrial swelling.

Effect on calcium-induced swelling

Mitochondrial swelling was rapidly induced by 3×10^{-5} M calcium chloride without a lag period (Fig. 3). Neither the time of onset of swelling, the rate of swelling, nor the extent of swelling was affected by the phthalanilide or oligomycin.

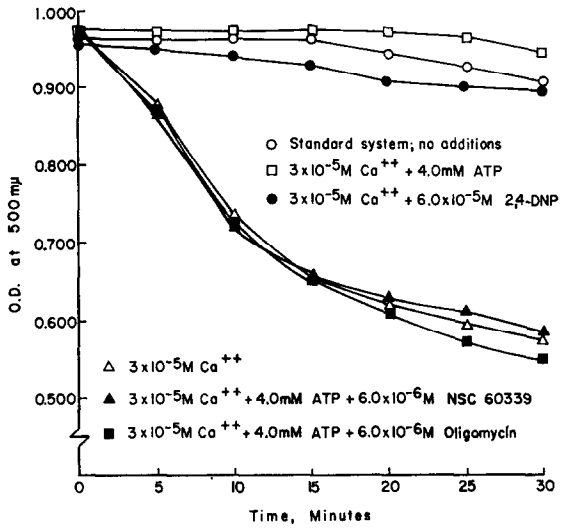

FIG. 3. Effect of phthalanilide and oligomycin on the ATP or 2,4-dinitrophenol inhibitions of Ca^{2+}-induced mitochondrial swelling.

Calcium-induced swelling was inhibited by either ATP or 2,4-dinitrophenol. As in the case of phosphate-induced swelling, the inhibitory effect of ATP on swelling was abolished by the phthalanilide or oligomycin at 6×10^{-6} M (Fig. 3). Thus, the mitochondria swelled in the presence of the drug as if no ATP were present. The effect of adding ATP 10 min after the onset of calcium-induced swelling is shown in Fig. 4. The mitochondria stopped swelling immediately upon the addition of ATP and apparently contracted slightly. However, as seen in the lower two curves, the addition of ATP at 10 min did not affect the rate or extent of swelling when either the phthalanilide or oligomycin was present at the time the ATP was added.

Unless oligomycin or the phthalanilide was used in amounts that were sufficient to prevent ATP utilization, ATP could overcome the inhibition by these agents. It is seen in Fig. 5 that the ATP inhibition of calcium-induced swelling could be titrated with either the phthalanilide or oligomycin. The inhibition by 4 mM ATP was not affected by 6×10^{-7} M phthalanilide or oligomycin, but was completely abolished by either agent at 6×10^{-6} M. When either of the drugs was present at 1×10^{-6} M, the 4 mM ATP was sufficient to decrease the extent of the calcium-induced swelling. The data suggest that a slight contraction occurred. It is interesting to note that the initial rate of swelling was not affected by this intermediate concentration of phthalanilide or oligomycin. This suggests that a titratable, time-dependent process was necessary before the inhibition by ATP was observed.

Calcium chloride was also used to induce swelling in mitochondria, which were aged for 2 hr at 0° in 0·25 M sucrose. As with the freshly prepared mitochondria,

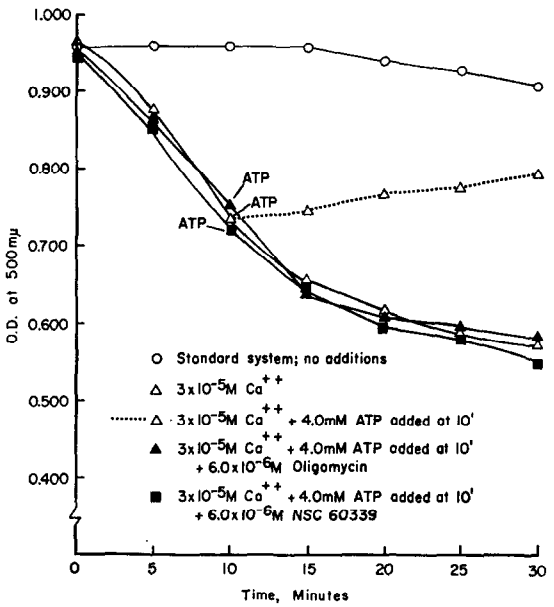

FIG. 4. Effect of phthalanilide and oligomycin on the ATP inhibition of Ca^{2+}-induced mitochondrial swelling.

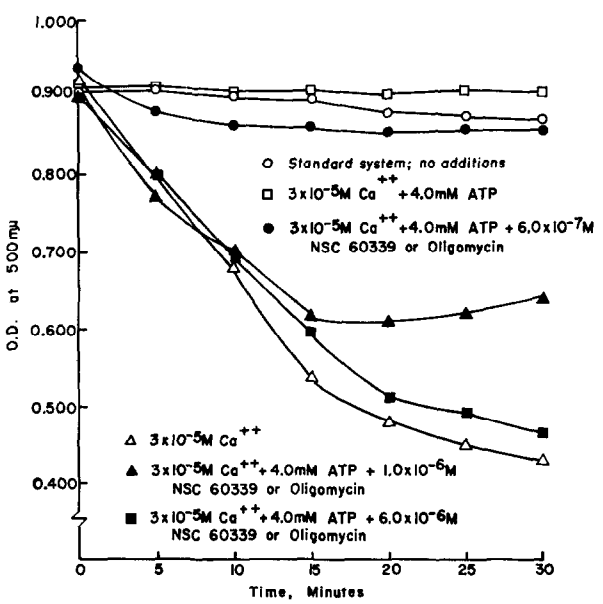

FIG. 5. Titration of the effect of phthalanilide and oligomycin on the ATP inhibition of Ca^{2+}-induced mitochondrial swelling.

swelling was completely inhibited by 2,4-dinitrophenol or ATP, but not by the phthalanilide or oligomycin.

Although 4 mM ATP added at zero time was sufficient to inhibit calcium-induced swelling in fresh mitochondria, no inhibition took place in aged mitochondria without an additional 4 mM ATP. When either oligomycin or the phthalanilide was present under these same conditions with aged mitochondria, no reversal of swelling took place with the additional amount of ATP.

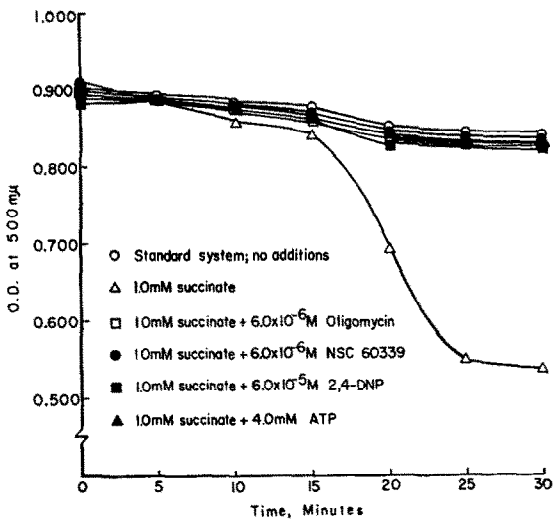

FIG. 6. Effect of phthalanilide, oligomycin, 2,4-dinitrophenol and ATP on succinate-induced mitochondrial swelling.

Effect on succinate-induced swelling

In the presence of 1 mM succinate, the time of onset of mitochondrial swelling was 15 min (Fig. 6). The rate of swelling was about the same as that induced by orthophosphate, but the extent of swelling was slightly less. The succinate-induced swelling was completely inhibited by ATP, 2,4-dinitrophenol, oligomycin or the phthalanilide.

DISCUSSION

Although the effects of many drugs on mitochondrial swelling have been studied,[26–29] the precise biochemical mechanisms of swelling have not been fully defined. The regulation of swelling and contraction is related to electron flow from substrates, the association of electron flow with phosphorylation, and the nature of the suspending media.[30] Orthophosphate- and calcium-induced mitochondrial swelling are inhibited by respiration inhibitors such as antimycin A, cyanide, azide, and some uncouplers such as 2,4-dinitrophenol and dicoumarol. However, the substituted phthalanilide (NSC 60339) neither induces swelling nor inhibits orthophosphate- or calcium-induced swelling. Therefore, it does not appear to act at the electron-transfer sites on the respiratory chain or at the terminal phosphorylating site of mitochondria from mammalian cells.

Oligomycin and NSC 60339 have been shown to be equivalent in their capacity to inhibit several mitochondrial swelling systems. The substituted phthalanilide is also similar to oligomycin in its incomplete inhibition of succinate oxidation but complete inhibition of phosphorylation in the coupled system.[19] Thus, the similarity of the phthalanilide's effects on various liver mitochondrial swelling systems to those of oligomycin suggests that a possible mode of phthalanilide action might be in the prevention of ATP utilization. It has been suggested by Racker that oligomycin acts in this manner by inhibition of ATPase activity[31] and by Lardy and Connelly that the mechanism is by inhibition of ATP—$^{32}P_i$ and P_i–$H_2{}^{18}O$ exchange reactions.[26]

Although the phthalanilide's effects may be directly on enzyme systems, they may be acting by forming complexes with specific lipids. It has been argued that the phospholipid components of the mitochondrial membrane are important in the control of swelling.[32] It has been shown that the substituted phthalanilide can be isolated in complexes with phospholipids of liver and leukemic cells in general,[20, 33] and of mitochondria from kidney, liver, and leukemia cells in particular.[10, 17] This suggests that the drug may be binding to lipid sites which are involved in critical enzyme systems or to lipid sites which may be essential for the control of uptake of water by mitochondria, as postulated for spermine.[34]

Both spermine and the phthalanilide have a repeating sequence of hydrophobic areas between nitrogen atoms. Both inhibit rat liver mitochondrial swelling. Spermine has a high affinity for phospholipids and nucleic acids;[34] the phthalanilide can be isolated as phospholipid complexes[17] and binds extensively to DNA *in vitro*.[35]

Since some phthalanilides, including NSC 60339, can be isoated in a complex with a new class of phospholipids from brain,[36] leukemia cells,[20] and probably from tissues in general,[17, 37] and since NSC 60339 is similar to oligomycin in its effects on mitochondrial swelling systems, it may be useful in studies on the chemistry and action of phospholipids in mitochondrial swelling and oxidative phosphorylation.

Acknowledgements—The authors wish to thank Dr. C. J. Kensler for his continued encouragement and support throughout this study. We are grateful to Mr. Isidore Wodinsky for his generosity in supplying animals.

REFERENCES

1. R. Hirt and R. Berchtold, *Experientia* **17**, 418 (1961).
2. S. A. Schepartz, I. Wodinsky and J. Leiter, *Cancer Chemother. Rep.* **19**, 1 (1962).
3. J. H. Burchenal, M. S. Lyman, J. R. Purple, V. Coley, S. Smith and E. Bucholz, *Cancer Chemother. Rep.* **19**, 19 (1962).
4. L. W. Law, *Cancer Chemother. Rep.* **19**, 13 (1962).
5. J. M. Venditti, A. Goldin and I. Kline, *Cancer Chemother. Rep.* **19**, 5 (1962).
6. R. F. Pittillo, L. L. Bennett, Jr., W. A. Short, A. J. Tomisek, G. J. Dixon, J. R. Thomson, W. R. Laster, Jr., M. Trader, L. Mattil, P. Allan, B. Bowdon, F. M. Schabel, Jr. and H. E. Skipper, *Cancer Chemother. Rep.* **19**, 41 (1962).
7. R. Hirt, in *Chemotherapy of Cancer* (Ed. P. A. Plattner), p. 228. Elsevier, Amsterdam (1964).
8. J. H. Burchenal, in *Chemotherapy of Cancer* (Ed. P. A. Plattner), p. 233. Elsevier, Amsterdam (1964).
9. L. L. Bennett, Jr., in *Progress in Experimental Tumour Research* (Ed. F. Homburger), vol. 7, p. 259. Hafner, New York (1965).
10. W. I. Rogers, D. W. Yesair, I. Wodinsky, A. Mahoney, A. Sivak and C. J. Kensler, *Proc. Am. Ass. Cancer Res.* **5**, 54 (1964).
11. D. W. Yesair, W. I. Rogers, P. E. Baronowsky, I. Wodinsky, P. S. Thayer and C. J. Kensler, *Cancer Res.* **27**, 314 (1967).

12. M. OCHOA, JR., A. GELLHORN and W. B. BENJAMIN, *Cancer Res.* **24**, 480 (1964).
13. A. GELLHORN, M. WAGNER, M. RECHLER, Z. KOREN and W. BENJAMIN, *Cancer Res.* **24**, 400 (1964).
14. M. J. PINE and J. A. DIPAOLO, *Cancer Res.* **26**, 18 (1966).
15. W. I. ROGERS, S. A. SATTINGER and C. J. KENSLER, *Biochem. Pharmac.* **15**, 1225 (1966).
16. C. J. KENSLER, P. E. PALM, H. M. DAY, S. P. BATTISTA, W. I. ROGERS, D. W. YESAIR and I. WODINSKY, *Cancer Res.* **25**, 1622 (1965).
17. D. W. YESAIR, F. A. KOHNER, W. I. ROGERS, P. E. BARONOWSKY and C. J. KENSLER, *Cancer Res.* **26**, 202 (1966).
18. L. SIMPSON and J. GOLDIN, *Proc. Am. Ass. Cancer Res.* **4**, 62 (1963).
19. A. SIVAK, A. J. MAHONEY, JR. and W. I. ROGERS, *Biochem. Pharmac.* **16**, 1821 (1967).
20. D. W. YESAIR, J. T. FUNKHOUSER, W. I. ROGERS and C. J. KENSLER, *9th Int. Conf. Biochem. Lipids, Phospholipids*, abstract No. 4 (1965).
21. H. A. LARDY, J. L. CONNELLY and D. JOHNSON, *Biochemistry, N.Y.* **3**, 1961 (1964).
22. G. HOLLUNGER, *Acta pharmac. Tox.* **11**, 1 *suppl.* (1955).
23. M. L. MELONI, W. I. ROGERS and C. J. KENSLER, *Fedn. Proc.* **25**, 572 (1966).
24. G. H. HOGEBOOM, W. C. SCHNEIDER and G. E. PALLADE, *J. biol. Chem.* **172**, 619 (1948).
25. M. E. PULLAMN and G. C. MONROY, *J. biol. Chem.* **238**, 3762 (1963).
26. H. A. LARDY and J. L. CONNELLY, *Biochemistry, N.Y.* **3**, 1969 (1964).
27. A. GOMEZ-PUYOU, M. TUENA, J. GUZMAN-GARCIA and A. PENA, *Biochem. Pharmac.* **12**, 669 (1963).
28. M. A. SPRITES and P. S. GUTH, *Biochem. Pharmac.* **12**, 37 (1963).
29. A. SCOTT, J. WEINSTEIN and F. E. HUNTER, *Fedn. Proc.* **22**, 450 (1963).
30. E. RACKER, in *Mechanisms in Bioenergetics*, p. 187 Academic Press, New York (1965).
31. E. RACKER, *Biochem. biophys. Res. Commun.* **10**, 435 (1963).
32. A. L. LEHNINGER, *Physiol. Rev.* **42**, 467 (1962).
33. D. W. YESAIR, A. SIVAK, W. I. ROGERS and C. J. KENSLER, *Proc. Am. chem. Soc.* abstract 96-C (1963).
34. H. TABOR, C. W. TABOR and S. M. ROSENTHAL, *A. Rev. Biochem.* **30**, 579 (1961).
35. A. SIVAK, W. I. ROGERS, I. WODINSKY and C. J. KENSLER, *Cancer Res.* **25**, 902 (1965).
36. D. W. YESAIR, W. I. ROGERS, J. T. FUNKHOUSER and C. J. KENSLER, *J. Lipid Res.* **7**, 492 (1966).
37. W. I. ROGERS, D. W. YESAIR and C. J. KENSLER, *J. Pharmac. exp. Ther.* **152**, 139 (1966).