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STUDIES OF THE EFFECTS OF 4'4''-BIS-(1,4,5,6-TETRAHYDRO-2-PYRIMIDINYL)-TEREPHTHALANILIDE DIHYDROCHLORIDE* ON OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA OF RAT KIDNEY AND LIVER†

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Abstract—The effects of 4',4''-bis(1,4,5,6-tetrahydro-2-pyrimidinyl)-terephthalanilide (NSC 57153) on oxidative phosphorylation in liver and kidney mitochondria of rat have been determined after daily intravenous administration of the drug for various periods up to 27 days, and after exposure to the drug *in vitro*. In all cases, phosphorylation and the oxidation of succinate were inhibited in proportion to drug concentration up to a limiting value. Inhibition of phosphorylation never exceeded about 60%, but it was always greater than the inhibition of oxidation. Although NSC 57153 caused equivalent inhibition of mitochondria from kidney and liver when added *in vitro*, the effects on liver mitochondria after exposure to drug *in vivo* were minimal because little drug was localized in the liver. It is estimated from the data that a higher drug concentration is required to produce an equivalent inhibition of oxidative phosphorylation after brief exposure to drug *in vitro* than after days of exposure to drug *in vivo*. Possible reasons for this finding are discussed.

PREVIOUS studies of the physiological disposition of two substituted terephthalanilides NSC 57153 and NSC 60339,‡ have shown that the highest concentrations of drug were in the kidney after administration to dogs and monkeys; liver and spleen also contained significant concentrations.[1, 2] Histopathological examination of kidney and liver tissues revealed varying degrees of damage depending upon dose and route of administration.[3, 4] It was also found that the drug was equally distributed between the nuclear and mitochondrial fractions of kidney, liver, and lymphocytic leukemia cells[5, 6] and that it binds to DNA[7] *in vitro* and a new class of phospholipids *in vivo*.[2, 8, 9] Several ATP-dependent biosyntheses were inhibited by the substituted terephthalanilides.[5, 10–12] We have previously reported that both phthalanilides inhibited oxidative phosphorylation at low concentrations when added *in vitro* to mitochondria from various sources.[4–6] Because of the possible application of these findings to the understanding of the chemotherapeutic and toxic natures of the terephthalanilides, detailed comparisons of the effects of the substituted phthalanilides on mitochondria from P388 lymphocytic leukemia cells[6] and rat liver and kidney have been made after

* NSC 57153.

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‡ NSC 60339 is 2-chloro-di(2-imidazolin-2-yl)terephthalanilide.

short exposure to drug *in vitro* and after prolonged exposure to drug *in vivo*. The studies of the effects of NSC 57153 are reported in this paper.

MATERIALS AND METHODS

Preparation of mitochondria

Rat liver mitochondria were prepared by the method of Schneider and Hogeboom.[13] The mitochondrial pellet, washed once instead of twice, was suspended for use in 0·25 M sucrose. The protein content of the suspension was determined by the method of Lowry *et al.*[14]

Assay methods

Oxidation was observed with standard manometric techniques.[15] Reaction temperature was kept constant at 25°. The reaction was stopped by the addition of cold 10% TCA and immediate centrifugation in the cold. Inorganic phosphate was determined on an aliquot of the deproteinized supernatant by the method of Fiske and SubbaRow.[16]

Determination of the NSC 57153 content of the mitochondria was made on an identical set of vessels to which no TCA was added. The mitochondria were quickly centrifuged down in the cold at 13,000 *g*. Alcohol extraction of the drug resulted in protein-free solutions which were chromatographed on cellulose citrate according to the method for terephthalanilides of Sivak *et al.*[17]

In all cases, the Warburg vessels contained mitochondria (1·5–2·2 mg protein) in 0·25 M sucrose; potassium phosphate buffer (pH 7·4), 50 μmoles; $MgCl_2$, 20 moles; NaF, 30 μmoles, sodium succinate, 150 μmoles; ADP, 10 μmoles; cytochrome *c*, 1 mg; the side arm contained 60 μmoles glucose and 40 KM units yeast hexokinase; total volume 2·8 ml; the center well contained 10% KOH, 0·2 ml. The drug was added about 15 min prior to the addition of glucose and hexokinase.

Inhibition of phosphorylation or oxidation in mitochondria which had been exposed to drug *in vitro* or *in vivo* is shown in the figures as Δ μmoles P_i or Δ μatoms oxygen uptake (control minus treated). These primary data are presented rather than per cent inhibition so that the actual magnitude of the uptakes can be visualized directly. The numbers increase in proportion to the per cent inhibition because control − treated/control × 100 = per cent inhibition.

Drug treatment in vivo

Four hundred μg of NSC 57153 in 0·5 ml water was injected daily into the tail veins of female Sprague-Dawley rats weighing an average 94 g on day 1. Any higher dose level produced such violent acute reactions that only a small percentage of animals survived the first one or two injections. A daily record of individual weights was kept and average values plotted. The amount of drug administered to each rat was constant during the experiment so that the gradual weight loss up to 35% effectively increased the relative dose on a mg/kg basis.

Reagents

NSC 57153 was supplied by the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health. All other reagents were the best grades commercially available.

RESULTS

Effects on oxidative phosphorylation produced by exposure of mitochondria to NSC 57513 in vitro

The effects of exposure of mitochondria to NSC 57153 *in vitro* were examined over a concentration range of 10^{-6} to 2×10^{-4} M, approximately the limit of solubility. Drug was added to each flask about 15 min prior to the addition of hexokinase and glucose, to start the measured reaction. Oxidative phosphorylation was then measured for approximately 1 hr. At the end of that hour, direct analysis showed that $15 \pm 3\%$ of the drug available in the flask was associated with the mitochondrial pellet over the concentration range of 3×10^{-5} M to 10^{-4} M.

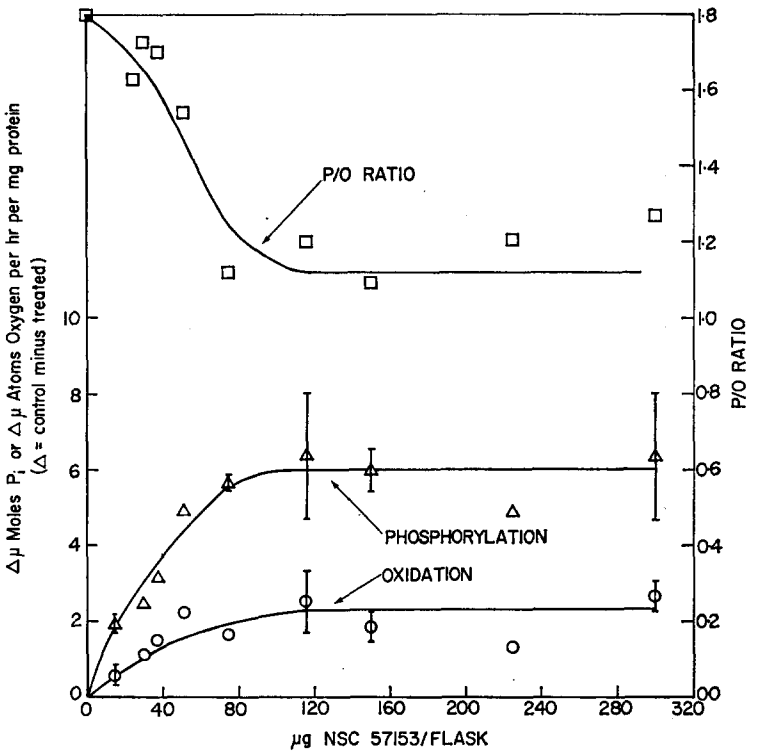

FIG. 1. Effect of NSC 57153 on oxidative phosphorylation in liver mitochondria treated *in vitro*. Flask contents are given in the Methods section. Per cent inhibition = data in figure/control value × 100. Mean control values with S.D. (four experiments): 10·04 ± 0·8 μmoles P_i/hr/mg protein; 5·6 ± 0·4 μatoms oxygen/hr/mg protein.

Figure 1 shows that the absolute decrease in both phosphorus uptake and oxygen consumptions between control and treated liver mitochondria was proportional to drug concentration up to approximately 90–100 μg* of drug per flask which contained an average of 2·19 ± 0·17 mg mitochondrial protein. Further increase in drug concentration produced no further increase in inhibition. With 90–100 μg drug per flask, about 15 μg drug was found to be bound to the mitochondria at 60 min. Under these conditions, the maximal inhibition observed, based on mean control values for five

* One μmole = 551 μg.

experiments, was about 60% of phosphorylating capacity and about 40% of the capacity to oxidize succinate.

Similar observations were made in kidney mitochondria (Fig. 2). In the case of phosphorylation by kidney mitochondria, the plateau for inhibition was reached at about 120 μg drug per flask, i.e. about 18 μg bound to the mitochondria. Each flask

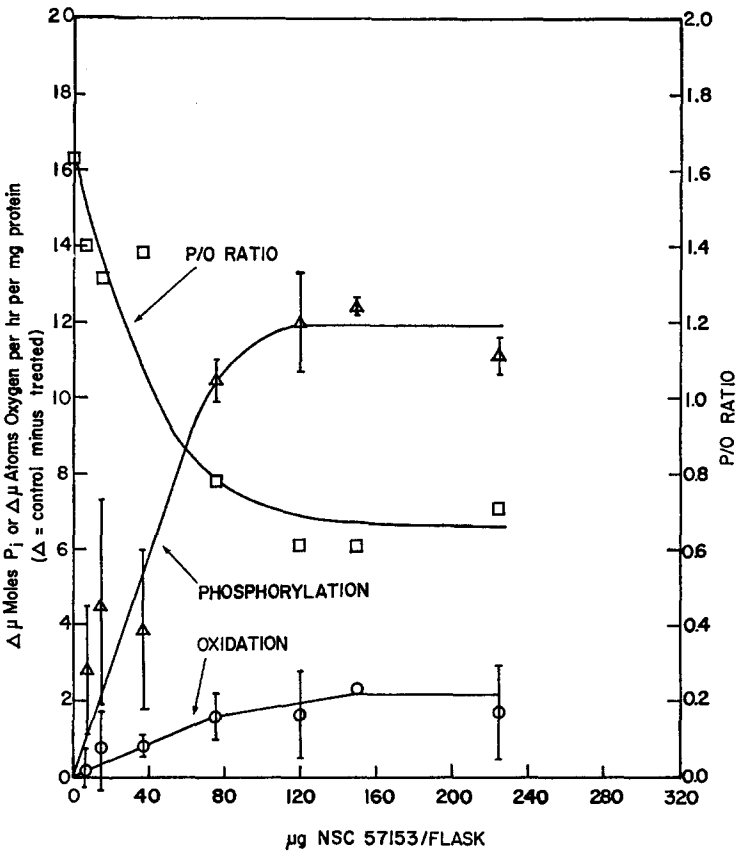

FIG. 2. Effect of NSC 57153 on oxidative phosphorylation in kindey mitochondria treated *in vitro*. Flask contents are given in the Methods section. Per cent inhibition = data in figure/control value × 100. Mean control values with S.D. (three experiments): 17·6 ± 2·2 μmoles P_i/hr/mg protein; 10·8 ± 0·4 μatoms oxygen/hr/mg protein.

contained an average of 1·55 ± 0·11 mg mitochondrial protein. Based on mean control values from four experiments, maximal inhibition of phosphorylation was about 60% with kidney as with liver mitochondria, but inhibition of succinate oxidation was only about 25%. Although kidney mitochondria showed slightly lower P/O ratios, their greater oxidative capacity resulted in greater total phosphorylating capacity than liver mitochondria per milligram protein under the conditions of these experiments. The slopes of the inhibition curves were identical: loss of 1 μmole P_i/hr per 6 μg NSC 57153/flask.

Also shown in Figs. 1 and 2 is the decrease of P/O ratio which resulted from exposure to increasing concentrations of NSC 57153 *in vitro*. The inhibition of oxidation was

proportional to drug concentration up to the point of maximal inhibition, but oxidation was less affected than phosphorylation. It is of interest that only 25–40% inhibition of oxidation and only 60% inhibition of phosphorylation could be attained even by doubling (Fig. 2) or tripling (Fig. 1) the drug concentration required to reach maximal inhibition. This is in contrast to 2-chloro-di(2-imidazolin-2-yl)-terephthalanilide dihydrochloride, which caused 100% inhibition of phosphorylation by rat liver mitochondria and only 50% inhibition of coupled succinate oxidation.[4]

Effects on oxidative phosphorylation produced by exposure of mitochondria to NSC 57153 in vivo

NSC 57153 was injected into the tail vein of Sprague-Dawley rats at an average daily dosage of 4 mg/kg. At various stages during the experiment, groups of 4–6 rats were chosen randomly for determination of drug content of the kidneys and liver, and for determination of the oxygen and phosphorus uptake by the isolated mitochondria. The yield of mitochondria was constant throughout the experiment and was not affected by drug (Table 1). Other details are described in the Methods section.

TABLE 1. YIELD OF MITOCHONDRIA AFTER REPEATED ADMINISTRATION OF NSC 57153

	Kidney*	Liver†
Control	36 ± 3‡	41 ± 8
Treated	35 ± 3	40 ± 11

* Mean value ± S.D. from days 18, 20, 25, and 28.
† Mean value ± S.D. from days 20, 25, and 28.
‡ Mitochondrial protein isolated, mg/g tissue.
Details of the isolation are given in the Methods section.

It was characteristic that the substituted phthalanilide caused a profound physiological response (including convulsions) when administered intravenously to animals for the first few days. After that, the animals tolerated the drug with much less acute response until sufficient numbers of doses had been given to cause substantial weight loss (Fig. 3) and physical weakness.

The concentration of drug in the tissues increased as a function of the number of daily doses, as seen in Fig. 3. It is noteworthy that the kidneys accumulated significant quantities of drug before any was found in the liver. This is characteristic for NSC 57153 after intravenous or oral administration.[2]

The data in Fig. 4 summarize the effects on oxidative phosphorylation of NSC 57153 administered *in vivo*. The effects were determined up to day 28. By this time several animals had succumbed, and the remaining animals were moribund. The marked increase in inhibition of oxygen uptake and phosphorylation on the last day (160 μg drug/g kidney) may have been due to the extremely poor condition of the animals. There was no change in the P/O ratio. However, no histopathological damage was found in either kidneys or liver.*

* Dr. Paul E. Palm, personal communication.

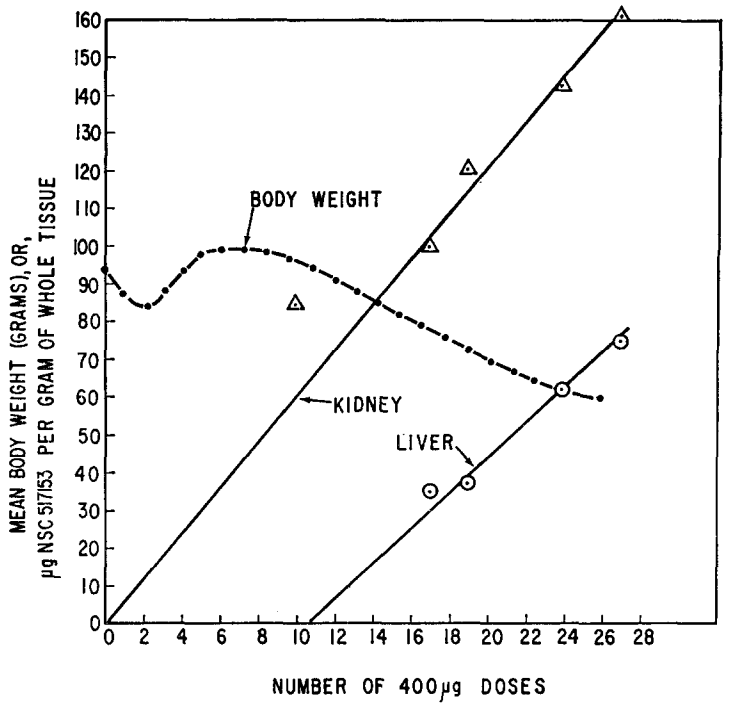

FIG. 3. Body weight and concentration of drug in liver and kidney of rats after repeated intravenous injections of NSC 57153; 400 μg NSC 57153 in 0·5 ml water was injected daily into the tail veins of female Sprague-Dawley rats. Forty-four animals were started; four to six were sacrificed for each drug assay and the measurement of oxidative phosphorylation. Determination of drug concentration is described in the Methods section. The record of body weight comprises the mean values of individually determined daily weights of all surviving rats.

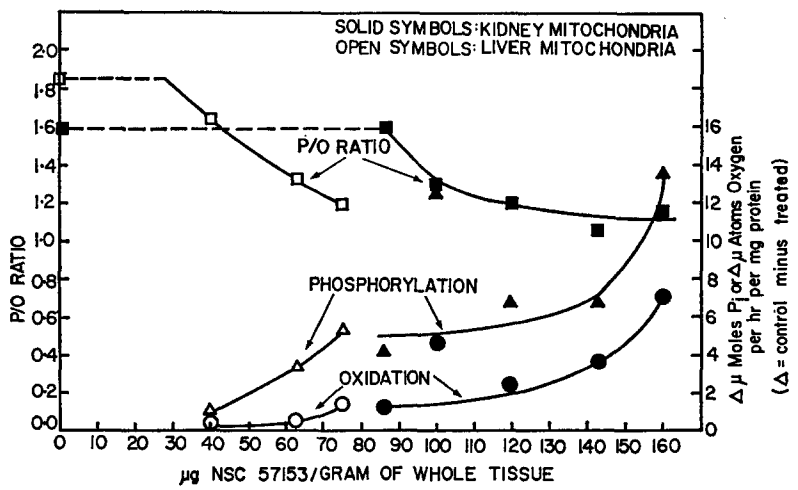

FIG. 4. Effect of NSC 57153 on oxidative phosphorylation in mitochondria treated *in vivo*. Flask contents are given in the Methods section. Per cent inhibition = data in figure/control value × 100. Mean control values with standard deviation (four experiments): Kidney, 17·5 ± 2·3 μmoles P_i/hr/mg protein; 10·3 ± 1·2 μatoms oxygen/hr/mg protein; liver, 9·7 ± 0·5 μmoles P_i/hr/mg protein; 5·3 ± 0·3 μatoms oxygen/hr/mg protein.

Although the data of Figs. 1, 2, and 4 provide estimates of the relative effects of NSC 57153 on oxidative phosphorylation after exposure to drug *in vitro* and *in vivo*, it was important to know if the assay system affected the results calculated after 60-min incubation *in vitro*. It is seen in Fig. 5 that inhibition of oxygen uptake by drug added to isolated mitochondria *in vitro* increased with time. This increase paralleled the uptake of drug by the mitochondria. The uptake and inhibition did not reach a maximal value until about 30 min, even though the mitochondria had been pre-exposed to the drug for 15 min in all cases. The shape of the curves indicates that per cent inhibition at 60 min is a good approximation of the maximal inhibition for drug added *in vitro* (open symbols, Fig. 5).

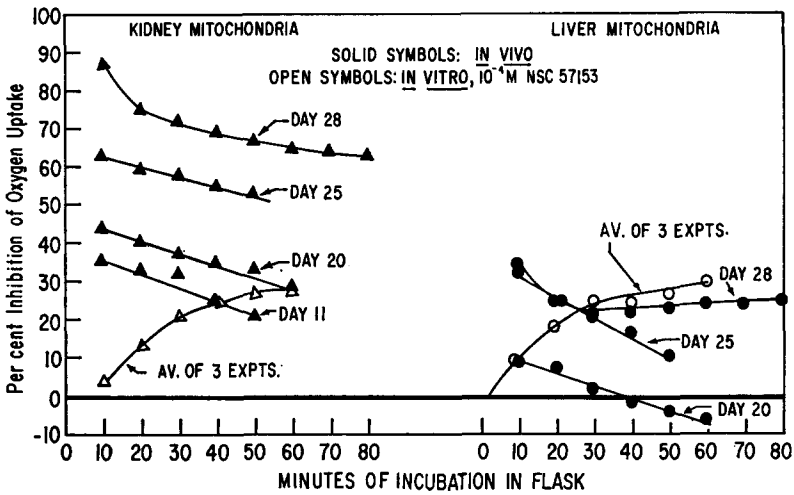

FIG. 5. Inhibition of succinate oxidation by NSC 57153. Flask contents are given in Methods section. Oxygen uptake was determined at intervals for 90 min. Freshly prepared untreated mitochondria were used as controls in each experiment. Per cent inhibition was calculated at each time period by comparison of the treated mitochondria and appropriate controls.

When drug was administered to animals and the mitochondria isolated and washed before assay *in vitro*, there was opportunity for elution of drug during isolation and during incubation at 25° with possible loss of the inhibition which was present before the animals were killed. The data of Fig. 5 suggest that this was the case, particularly with mitochondria from tissues which had relatively low drug concentration (e.g. days and 20, Fig. 3). By extrapolating the 60-min values to zero time, it is seen that some per cent inhibition values might be only half the actual values. The degrees of inhibition apparent in the early time periods and from the extrapolation to zero time suggest that loss of drug from the mitochondria occurred almost entirely during incubation at 25° and not during isolation at 0–2°. Other data support this conclusion.*

* Addition of control nuclei or control mitochondria during the isolation of drug-containing mitochondria or nuclei from cell homogenates at 0–2° did not change the amount of drug found in the mitochondrial fraction (in the presence of control nuclei) or in the nuclear fraction (in the presence of control mitochondria).

DISCUSSION

Studies of the distribution of NSC 57153 in tissues of dogs and monkeys after repeated administration indicate that highest drug concentrations are in the kidney, with significant amounts in the liver.[2] The affinity of the drug for kidney is consistent with the toxicological studies which have revealed that kidney tubular necrosis is one of the major damages that occur after repeated administration of the compound to dogs and monkeys.[3, 4] Typical high drug contents of kidney are in the order of 200 μg NSC 57153/g frozen tissue.[2] It was not possible to attain such high concentrations of drug in the kidneys of Sprague-Dawley rats after repeated intravenous administration over the course of 27 daily intravenous injections of about 4 mg/kg. Maximal drug concentration in rat kidney was 160 μg/g tissue. Intracellular drug distribution studies indicated that about one half this quantity is associated with the mitochondrial pellet.[5,6]

It was found that phosphorylation in rat kidney tissue which contained 105 μg drug/g tissue after long-term exposure *in vivo* was inhibited about 50% when the isolated mitochondria were assayed in Warburg flasks for 1 hr. This concentration of drug is equivalent to about 2×10^{-7} moles/g whole tissue and, since about one half the intracellular drug is localized in the mitochondria, 1×10^{-7} mole drug would be found in the mitochondria from 1 g kidney.

To obtain about a 50% inhibition of phosphorylation after 60-min exposure of isolated mitochondria to NSC 57153 *in vitro*, about 2×10^{-7} mole drug was required in the flask. Fifteen per cent of this, or about 3×10^{-8} mole, was associated with the 2 mg mitochondrial protein. Since about 36 mg mitochondrial protein could be isolated from 1 g kidney, about $5{\cdot}4 \times 10^{-7}$ mole drug would be associated with the mitochondria from 1 g tissue.

Thus, $5{\cdot}4 \times 10^{-7}$ mole is required *in vitro* for 50% inhibition of phosphorylation, while, as shown above, 1×10^{-7} mole is required for 50% inhibition of phosphorylation in an equivalent amount of mitochondria extracted from the kidneys of treated rats This fivefold differential would be even larger if the extrapolation to "zero-time inhibition" of oxidation by the mitochohdria exposed to drug *in vivo*, as suggested by the data in Fig. 5, was also true for inhibition of phosphorylation. The apparently greater sensitivity of the mitochondria treated *in vivo* could be due to structural damage to the mitochondria caused by exposure to drug for a long period. It is possible that such long-range damage may be different from, or at least additional to, the short-range effects found during the experiments when drug is added *in vitro*.

The substituted phthalanilides have been shown to interact with phosphatides in model systems.[18] The substituted phthalanilides are localized in mitochondrial (and nuclear) fractions of cells[5, 6] and have been isolated as specific drug–phospholipid complexes.[8, 9] These drug–phospholipid complexes can be cleaved by protons (pK is about 3·6 for NSC 57153 complex) or in neutral solution by Ca^{++} or Mg^{++}, but not by comparable quantities of Na^{+} or K^{+}. Therefore, it is possible that the drug could displace Ca^{++} or Mg^{++} from binding sites in certain mitochondrial lipids. The involvement of Ca^{++} and Mg^{++} with mitochondrial function and the role of phospholipids in electron transport, ion transfer and swelling[19] suggest that there may be a relationship between the drug–phospholipid complexes and the mode of inhibition of oxidative phosphorylation by the drug. The congener, 2-chloro-di(2-imidazolin-2-yl)terephthalanilide, which also binds to mitochondrial lipids,[20] is equivalent to oligomycin in its effects on rat liver mitochondrial swelling and ATPase activity.[21] The

lack of complete inhibition at high drug concentrations *in vitro* and the possible loss of inhibition during incubation of mitochondria which were exposed to drug *in vivo* suggest that the site of phthalanilide action can be saturated and that the binding may be reversible. The maximal 60% inhibition of phosphorylation suggests that only one of the two phosphorylating sites which are active with succinate as a source of electrons may be affected initially. Other damage could occur during long-term exposure to the drug *in vivo*.

In terms of toxicity of the drug, the data indicate that the inhibition of oxidative phosphorylation found *in vitro* also occurs *in vivo*. The low drug concentrations in liver result in only marginal inhibition of oxidative phosphorylation. Concentrations of NSC 57153 in kidneys after daily administration of the drug are high enough to cause significant deleterious effects.[3, 5]

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