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STUDIES OF THE MECHANISM OF ACTION OF THE TEREPHTHALANILIDE ANTAGONISTS

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Abstract—The antagonistic action of some terephthalanilide congeners with terminal acidic groups on the antileukemic activity *in vivo* and *in vitro* of the original terephthalanilide derivatives and related compounds has been correlated with the effects on the ultraviolet absorption spectra. In many cases this antagonistic activity is based on a complex formation between the two groups of compounds. The structure of the molecules indicates an interaction of the terminal basic groups of the antileukemic terephthalanilides with the terminal sulfate or phosphate groups of the antagonists.

THE TEREPHTHALANILIDES[1] and related compounds have received considerable attention for extraordinary effectiveness in experimental mouse leukemias.[2–10] Of some 800 synthesized compounds, approximately 100 have shown such antileukemic activity. Since acute and chronic toxicity has resulted in humans,[10–14] clinical tests with five members of this group of compounds have not been pursued.

Figure 1 shows the structure of some representative compounds.† Characteristically, they are composed of a central aromatic ring, either unsubstituted or substituted with a chloro or amino group. This central ring is connected to another benzene ring on either side by an amide, ureido, or amidine bridge. In turn, these benzene rings are substituted in the para-position by an imidazolin ring or an amidine group. These basic compounds are mostly used in the form of their hydrochlorides or other salts. Our studies with NSC 38280, 57155, and 66761 in animals and humans demonstrated no breakdown or metabolic alterations of the molecule.[15–17] No metabolic

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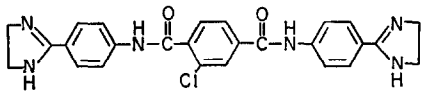
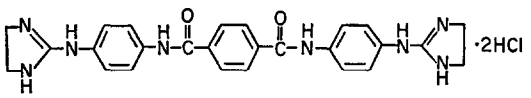
† Reference compounds:

NSC 38280 = 4′,4″-bis(2-imidazolin-2-yl)-2-chloroterephthalanilide, dihydrochloride.
NSC 50469 = 2-amino-4′,4″-bis(2-imidazolin-2-yl)terephthalanilide.
NSC 53306 = 4′,4″-bis(2-imidazolin-2-yl-amino)terephthalanilide, dihydrochloride.
NSC 55156 = *N*,*N*′-terephthaloyldisulfanilic acid, disodium salt.
NSC 57155 = *N*,*N*″-bis[*p*-(*N*′-methylamidino)phenyl]terephthalamidine, tetrahydrochloride.
NSC 60339 = 4′,4″-bish(2-imidazolin-2-yl)-2-chloroterephthalanilide.
NSC 60349 = *N*,*N*′-terephthalamido-4′,4″-bis naphthalenesulfonic acid, disodium salt.
NSC 66761 = 2-amino-4′,4″-bis(4-methylimidazolin-2-yl)terephtyalanilide dihydrochloride (AMIT).
HR 2504 = *N*,*N*′-terephthalamido-4′,4″-bisbenzenephosphoric acid, disodium salt.
HR 2289 = 6,6′ (terephthaloyldiimino)bis[1-methylquinolinium]chloride.

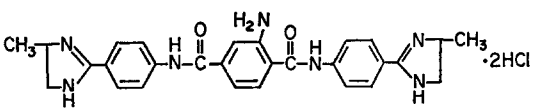
change of the fluorescent compound NSC 50469 after incubation of tissues with this terephthalanilide derivative was reported by Booth *et al.*[18] The stability and toxic effects of the hydrolysis products of the related ureido compounds have been described by Rogers *et al.*[19] There is, however, indication in the literature suggesting an unidentified metabolic alteration of the deschloro analog of NSC. 38280.[20]



NSC 60339 (free base)



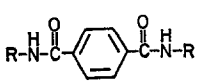
NSC 53306

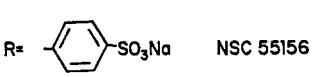


NSC 66761

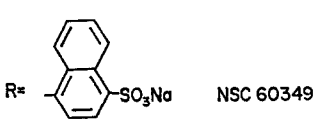
FIG. 1. Structures of three terephthalanilide derivatives which have shown antileukemic activity.

Studies of the reaction mechanism by Pine *et al.*[21] and Mahoney *et al.*[22] revealed an inhibition of the oxidative phosphorylation *in vitro* in various systems, and Meloni *et al.*[23] reported NSC 60339 to be equivalent to oligomycin in its effects on mitochondrial swelling. The affinity of these compounds to phospholipids[24, 25] and to DNA and RNA *in vitro*[26, 27] seems to be of considerable importance for their reaction mechanism. The aim of our study was to investigate further the DNA/drug complex and the mechanism of inhibition of such complex formation by the so-called terephthalanilide antagonists. In general, these antagonists, developed primarily to find ways to prevent the considerable toxic side effects of the terephthalanilides while retaining at least part of the antileukemic activity, are compounds similar to the active terephthalanilides, e.g. NSC 55156, NSC 60349, and HR 2504 (Fig. 2).[10] As a

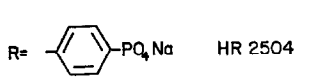




NSC 55156



NSC 60349



HR 2504

FIG. 2. Structures of three antagonists of the terephthalanilide derivatives.

rule, the terminal basic components (e.g. imidazolin rings or amidine groups), shown earlier to be essential for the antileukemic activity,[9] are replaced by sulfate or phosphate substitutions of the benzene or naphthalene rings. They are biologically inactive in our mouse leukemias.

EXPERIMENTAL

Ultraviolet studies

One million P815 cells[28] injected i.p. into BDF_1 mice produced ascitic leukemia. On day 7 after inoculation, the mice were killed. DNA was then extracted from the harvested ascites by the procedure of Kay *et al.*[29] The following combinations were dissolved in 1/100 M phosphate buffer at pH 7·4: DNA at 33·75 μg/ml and NSC 66761‡ at $2{\cdot}5 \times 10^{-5}$ M; NSC 53306‡ at $2{\cdot}5 \times 10^{-5}$ M and NSC 55156‡ at $2{\cdot}5 \times 10^{-5}$ M; NSC 53306 at $2{\cdot}5 \times 10^{-5}$ M and NSC 60339‡ at $1{\cdot}25 \times 10^{-5}$ M; NSC 66761 at $1{\cdot}25 \times 10^{-5}$ M and NSC 55156 at $1{\cdot}25 \times 10^{-5}$ M; NSC 66761 at $1{\cdot}25 \times 10^{-5}$ M and NSC 60349‡ at $1{\cdot}25 \times 10^{-5}$ M. A Beckman DB spectrophotometer was used for the u.v. measurements, and the spectra were recorded on a Photovolt Varicord, model 40.

Distribution studies of radioactivity

The effects of an antagonist and an active terephthalanilide, NSC 55156 and NSC 60339, respectively, were studied on the distribution of ^{14}C-NSC 53306§ in various tissues of normal mice. Two or three animals were used for each experiment. The controls received only a single dose of the labeled drug i.p. 2·5 mg/kg. NSC 55156 at 80 mg/kg and NSC 60339 at 40 mg/kg, respectively, were administered subcutaneously in a single dose 30 min. before the labeled compound. The animals were killed 30 min, 4 hr, and 24 hr after injection of ^{14}C-NSC 53306. The distribution of radioactivity in kidney, liver, and spleen was studied by the dry-combustion technique of Kalberer and Rutschmann.[30] The F values[31] represent the mean values of two or three determinations:

$$F = \frac{\text{specific activity of tissue} \times \text{body weight}}{\text{total activity administered}}$$

Cell culture studies

Leukemic cells (P815Y) adapted to cell culture by Schindler *et al.*[32] were grown in liquid medium containing 10 per cent horse serum, according to the Fischer technique.[33] The experiments were set up as described previously.[34] NSC 53306, in concentrations ranging from 0·03 to 3·0 μg/ml, and the antagonist NSC 55156, ranging from 10 to 60 μg/ml, were then added either simultaneously or the antagonist added 2, 4, 8, or 24 hr after the active compound. NSC 53306 and the antagonist, NSC 60349, were given simultaneously in concentrations ranging from 0·03 to 0·01 μg/ml and from 10 to 120 μg/ml respectively. NSC 66761, in concentrations from 0·1 to 3 μg/ml, was added simultaneously with either NSC 55156 or NSC 60349

‡ Synthesized by Dr. R. Hirt of the Research Division of Dr. A. Wander, AG Berne, Switzerland, and supplied through the Cancer Chemotherapy National Service Center, National Cancer Institute.

§ The radioactive substance ^{14}C-NSC 53306 was synthesized by Dr. J. L. Greene of the Southern Research Institutue, Birmingham, Ala. Its specific activity was 2·75 μc/mg.

at concentrations ranging from 60 to 120 and from 10 to 60 μg/ml respectively. Cells were incubated at 37° and counted in an electronic cell counter (Coulter, model A) 72 hr after addition of the compounds to the cell culture. The inhibitory effect of the terephthalanilide on cell growth was expressed as treated/control per cent. In Table 1, no blocking of the antileukemic activity of the terephthalanilide was designated by a minus sign; strong blocking was designated plus.

TABLE 1. INTERACTION OF "ACTIVE" AND "BLOCKING" DERIVATIVES OF THE TEREPHTHALANILIDE SERIES

	Blocking compounds					
	NSC 55156			NSC 60349		
Active compounds	Spectrum, u.v.	Leukemia P815Y *in vitro*	Leukemia P815 *in vivo*	Spectrum, u.v.	Leukemia P815Y *in vitro*	Leukemia P815 *in vivo*
NSC 53306	+	+	+	−	−	−
NSC 66761	−	−	−	+	+	+

+, Strong inhibition by blocking agent of the antileukemic activity of terephthalanilide.
−, No inhibition by blocking agent of the antileukemic activity of terephthalanilide.
In the u.v. specta plus means 40–60% hypochromicity.
In the u.v. spectra minus means less than 10% hypochromicity.

Studies in vivo

BDF_1 mice were inoculated i.p. with one million cells of leukemia P815[28] in saline suspension. The resultant ascitic leukemia later progressed to generalized disease. Treatment was begun 24 hr after inoculation of the leukemic cells with the terephthalanilide alone or in combination with the antagonist and was continued for ten daily doses. NSC 53306 was given at 2·5 and 5 mg/kg i.p. or s.c., and the antagonists, NSC 55156 and NSC 60349, at 40 mg/kg i.p. or s.c., 30 min before or at the same time as the active compound. The dosage for NSC 66761 ranged from 2 to 6·7 mg/kg i.p. or s.c. NSC 55156 was administered i.p. in doses ranging from 10 to 80 mg/kg either 30 min before or at the same time as the terephthalanilide. In the experiments with NSC 60349, NSC 66761 was given at 2 mg/kg i.p. and at 2, 4, and 6·7 mg/kg s.c., and the antagonist at 10, 20, and 40 mg/kg i.p. or s.c. either simultaneously or 30 min before. The treated/control per cent values were then compared. Complete prevention of the antileukemic activity of the terephthalanilide by the antagonist administered over a wide range of doses was recorded as plus in Table 1.

RESULTS AND DISCUSSION

Strong affinity of NSC 66761 for selective tissues in rats and dogs was reported by Philips *et al.*[35] These authors found the highest concentrations in the kidney cortex in dogs, and in the submaxillary salivary gland in rats. Significant amounts were detected in these tissues of dog and rat respectively as long as 2 months after treatment. Most of the fluorescence observed was limited to the nuclei, indicating a strong affinity of the drug to this part of these cells. An almost selective affinity of the compound NSC 66761 for chromosomes of P815 leukemic cells *in vivo* could be

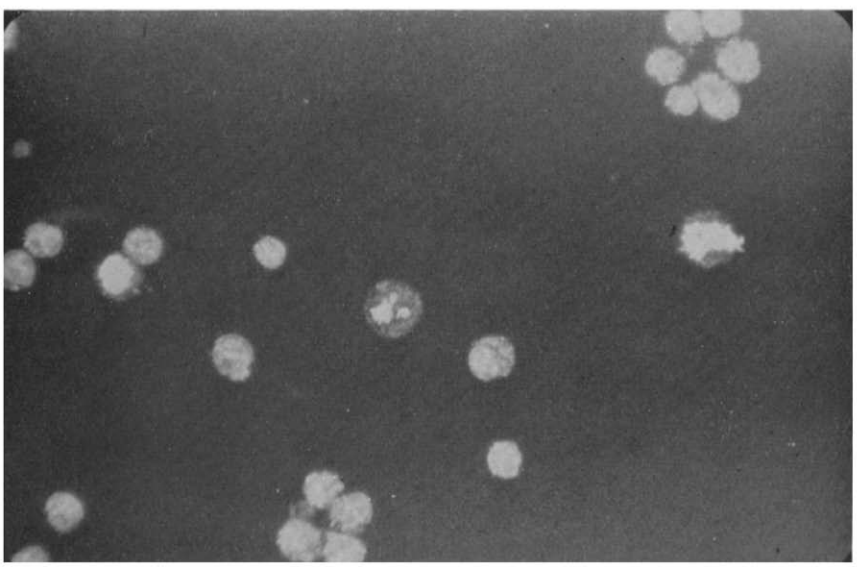


FIG. 3. Nuclei of P815Y leukemic cells *in vitro* showing bright. fluorescence after 24-hr incubation in 3 μg NSC 66761/ml.

demonstrated 24 hr after a single i.p. injection of the drug (Fig. 3).[36] Figure 4 shows the considerable bathochromic and hypochromic effect of DNA on this compound *in vitro*. Titration of DNA with NSC 66761, following the method of Sivak *et al.*,[37] resulted in a value of 1·2 μg DNA/μg NSC 66761, which compares well with the

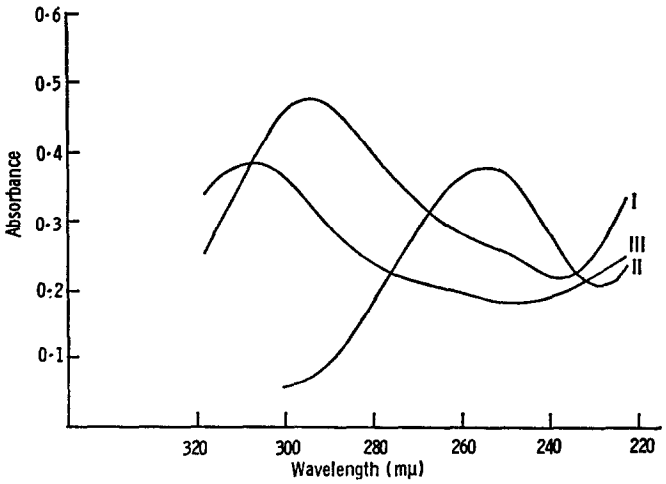


FIG. 4. Interaction of NSC 66761 and DNA in the ultraviolet. A pronounced bathochromic shift of 13 mμ is seen in the absorption of the compound when combined with DNA. I: NSC 66761 (AMIT), $2{\cdot}5 \times 10^{-5}$ M. II: DNA, 33·75 μg/ml. III: Combination of I and II vs. DNA.

findings of Sivak for several terephthal derivatives and DNA of salmon sperm and P388 leukemic cells.[37] This affinity for DNA suggests an interaction of the negatively charged DNA with the positively charged terephthalanilide derivative. As with the interaction of the planar acridine and DNA,[38] it is assumed that the coplanar terephthalanilide derivatives also bind predominantly by hydrogen bridges. Whether the molecule is situated tangentially or perpendicularly to the axis of the helix of DNA remains to be determined.[27]

The affinity of this group of compounds for phospholipids[24, 25] and DNA and RNA constituents *in vitro*[26, 27] is not specific for tumor cells. The considerable accumulation in various tissues of animals[15, 35] and humans, as expressed by slow excretion rates,[16] probably contributes to the toxicity of these compounds.

In contrast to the biologically active terephthalanilides, the few antagonists investigated showed no complex formation with DNA in the u.v. spectrum. However, when these compounds were combined with one of the active compounds *in vitro*, hypochromicity in the u.v. spectrum was observed, e.g. with the combination of NSC 53306 and NSC 55156, as shown in Fig. 5. When the theoretically expected absorption (Fig. 3) is compared with the actual findings (Fig. 4), a considerable hypochromic effect is demonstrated. In some cases the complex or salt formation resulted in an insoluble substance which precipitated out within 5–10 min after mixing. Combinations of active terephthalanilides *in vitro* consistently showed an addition of their u.v. absorption spectra, indicating *no* further interference with each other. The same result was obtained when two antagonists were combined.

From these findings it is evident that in many cases the cause of the blocking or antagonistic phenomenon is a direct physicochemical interaction of the two groups of compounds. The consequences of such an interaction might be a decreased ability of the reaction product to penetrate through capillaries, reduced or inhibited permeation through the cell with accumulation of the resulting products in the intercellular spaces, or accelerated excretion.

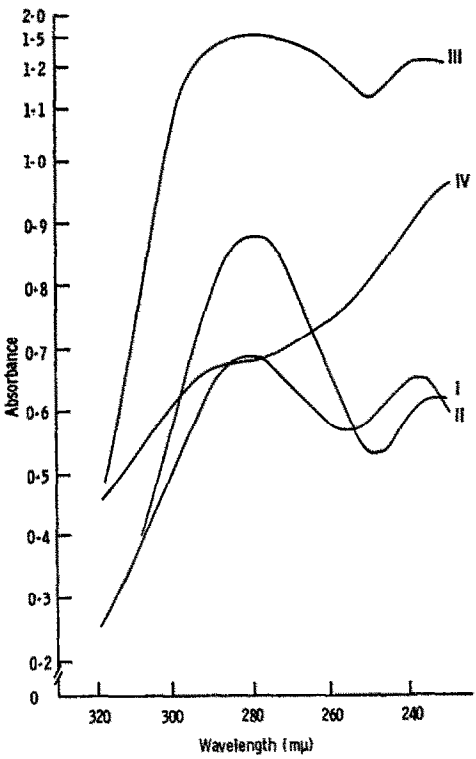


FIG. 5. Ultraviolet spectra of combination of active terephthalanilide (NSC 53306) and antagonist (NSC 55156). A strong hypochromic effect is found, indicating an interaction of the two compounds. I: NSC 53306, $2{\cdot}5 \times 10^{-5}$ M. II: NSC 55156, $2{\cdot}5 \times 10^{-5}$ M. III: I and II, theoretical values. IV: I and II, experimental values.

In the study of the distribution of ^{14}C-NSC 53306, injected i.p. 30 min after NSC 55156 s.c., a significant reduction of the accumulation factor of radioactivity was noted in the kidney and liver, but not in the spleen (Table 2). When another active agent, NSC 60339, was given instead of the antagonist, the radioactivity was comparable to the control levels in kidney, liver, and spleen. This experiment demonstrated that significantly less ^{14}C-NSC 53306 is found in the tissues of liver and kidney under the influence of a previous injection of the antagonist NSC 55156. The product of their interaction, however, is accumulated principally in the spleen.

The results of the u.v. studies correlate with the corresponding experiments *in vivo* and *in vitro* shown in Table 1, where combinations of two active compounds

and two antagonists are compared. Obviously not all combinations of the two groups interacted. While NSC 66761 and the antagonist NSC 60349 suggested a complex formation in the u.v. and a corresponding interaction, *in vitro* and *in vivo*, NSC 66761 and NSC 55156 showed no, or only slight, complex formation and negligible interaction in the biological systems. The difference in the behavior of the two pairs of compounds is probably steric and could possibly be demonstrated with

TABLE 2. EFFECT OF NSC 55156 AND NSC 60339 ON DISTRIBUTION OF ^{14}C-NSC 53306 IN VARIOUS BODY TISSUES OF NORMAL MICE

Time after injection of control	Kidney			Liver		Spleen		
	30 min	4 hr	24 hr	4 hr	24 hr	30 min	4 hr	24 hr
Control*	11·0	14·4	15·2	6·41	9·0	1·53	1·8	1·9
Control + NSC 55156†	1·91	1·7	6·2	1·32	2·0	6·18	3·4	5·3
Control + NSC 60339‡	9·86	21·96	14·37	8·13	11·19	1·2	2·3	2·15

Figures represent accumulation factors. F.

* ^{14}C-NSC 53306 at 2·5 mg/kg i.p. stat.

† NSC 51556 at 80 mg/kg s.c. stat 30 min before control.

‡ NSC 60339 at 40 mg/kg s.c. stat 30 min before control.

molecular models. We assume that the places of interaction are the terminal groups, i.e. the basic imidazolin or amidine substitutions in the active phthalanilide, and the sulfate or phosphate groups in the antagonist. We also hoped that the antagonists would deplete the active compounds from the cells and thus reverse the accumulation and with it the toxic manifestations of the original antileukemic agents. Apparently, however, once these compounds enter the cells they cannot be removed from their binding sites. When the antagonists were given 30 min prior to the active phthalanilides in the experiments *in vivo*, an inhibition of the antileukemic activity was observed; however, when they were given 1 or 2 hr afterwards, the inhibitory effect on the antileukemic activity was partially diminished. When they were given more than 4 hr later, their inhibitory effect had disappeared.[39] Similar results were obtained in experiments with P815Y leukemic cells in cell culture.[40] Indications for a definite specificity among NSC 55156, 60349, and HR 2504 and among NSC 38280, 53306, and HR 2289 had been reported earlier.[41]

The present experiments show that the antagonists are selective as to the terephthalanilides blocked. This specificity probably results from a sterically determined affinity of the active and blocking agent, and an interference of the opposite charges of the terminal groups of the two compounds. This interaction is expressed in the u.v. as a hypochromic effect, suggesting a complex formation, and in the biological experiments as an inhibition of the antileukemic activity of the terephthalanilide. Separation of antileukemic activity and undesirable side effects of these terephthalanilides caused by these particular antagonists could not be expected from their behavior on the molecular level. The interaction of the two compounds presumably occurs at those sites of the active molecules which are essential[9] for antileukemic activity. Further antagonist–phthalanilide combinations are under study.

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