Evidence for the Intercalation of Thalidomide into DNA: Clue to the Molecular Mechanism of Thalidomide Teratogenicity?

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The intercalation of thalidomide (α-phthalimidoglutarimide) into nucleic acids was studied by spectrophotometric titrations, displacement of known intercalators, viscosity changes of DNA solutions, and by phase partition analysis. Specific binding of thalidomide was found to DNA of various specimens (human placenta, calf thymus, salmon sperm), but not to RNA (from bakers yeast, torula yeast).

Almost a quarter of a century elapsed already since the thalidomide disaster in the late fifties, and yet the molecular mechanism of action of this 'classical teratogen' has not been elucidated completely. Numerous speculations have been brought forward during the years in order to explain how this agent may exert its teratogenic action, the majority of them quickly turned out as false, and there is no need to discuss them any more. However, two hypotheses have gained a higher degree of probability to meet the actual process which takes place in the developing embryo and finally causes the malformations known as the 'thalidomide syndrome'. These are the so-called i) *intercalation hypothesis* and ii) the *arenoxide hypothesis* of thalidomide action, respectively [1–3].

The available biological data suggest that thalidomide interacts in a certain manner with nucleic acids, and by this way disturbs the normal embryonic growth process. Because of its structural similarity with nucleic acid bases, it was proposed that thalidomide intercalates between the base pairs of the DNA helix and causes depurination of the nucleic acid [4]. Three structural requirements have been found to be essential for this interaction with DNA: the flat aromatic phthalimide ring system, one reactive carbonyl group of this system, and the ionizable glutarimide hydrogen or a comparable structural element in an at least four members containing side chain of the phthalimide moiety; this was believed to be the 'minimum teratogenic structure' as a prerequisite for teratogenic activity in such compounds [4, 5].

The second hypothesis, which was originated by the senior author [3], postulates that thalidomide causes birth defects only after biotransformation to a toxic metabolite, probably a reactive arene oxide, which then reacts with the DNA and denaturates the nucleic acid through concomitant formation of a covalent bond with a guanine base. Others who had the same idea independently, shortly thereafter presented convincing experimental evidence for this assumption [6].

The 'intercalation hypothesis' has long been favoured by ourselves [7, 8] and others [9], but later it was abandoned and replaced by the 'arenoxide hypothesis' [3]. Now we reach to the conclusion that *both* mechanisms are valid, and the truth must be searched somewhere in a combined 'intercalation-bioactivation mechanism' of thalidomide action. The target, anyway, is the embryonic and/or placental DNA.

In this paper, we present the experimental evidence for the intercalation of thalidomide into various specimens of nucleic acids, among them human placental DNA, calf thymus DNA, either of the low molecular weight and of the high polymerized type, and salmon sperm DNA. All these nucleic acids interacted with thalidomide, clearly demonstrating that this sort of binding of the drug is a common feature of DNA. No intercalation, however, was found with torula yeast and baker's yeast RNA, thus demonstrating that RNA might not be the target of thalidomide's action.

Abbreviations: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; EDTA, ethylenediaminotetraacetic acid; DMF, dimethylformamide.

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Material and Methods

Spectrophotometric titration

1.0 mg DNA (nucleic acids were from Sigma Chem. Co, Dorset, U.K.; all other chemicals were 'analytical' grade) was dissolved and swelled for 24 h in 0.5 ml buffer (0.01 M EDTA in aq. bidest., pH 6.6) at 4 °C. Samples of 1 ml buffer were mixed with 0.1 ml of a thalidomide solution (1.0 mg/ml in the same buffer plus DMF, 4:1, v/v) and increasing amounts (0.02 ml added step by step) of the DNA solution at 22 °C. UV/VIS-spectra were recorded in the range between 250 and 350 nm (diode array spectrophotometer HP-8451A, Hewlett-Packard, Vienna) using a 1 cm light path. The fraction of drug bound by intercalation into the DNA (R) was calculated from the respective absorption value (ABS) of thalidomide alone (TH), the nucleic acid alone (DNA), as well as of the complex of both (DNA-TH), respectively, according to the equation:

$$R = \frac{ABS_{TH} - (ABS_{DNA-TH} - ABS_{DNA})}{ABS_{TH}} .$$

The absorption at 300 nm was converted into amount of bound molecules (R) versus the ratio of bound to free intercalator (R/C), and the resulting data plotted according to the method of Scatchard [10]. From these plots, the numbers of binding sites, N1 and N2, and the association constants of the complexes, K1 and K2, were calculated. Further on, the stability of the intercalation complex was monitored spectrophotometrically over a period of 60 minutes; no spectral changes were found during this interval.

Displacement of known intercalators

A second set of experiments comprised of the displacement of known intercalators, such as methylene blue ('basic blue 9', Serva, Heidelberg), methyl green ('basic blue 20', Merck, Darmstadt), ethidium bromide (Serva, Heidelberg), and acridine orange (AO, Merck, Darmstadt), from the nucleic acid which had been saturated with the respective intercalator prior to the treatment with thalidomide.

A typical experiment was as follows (others were performed in similar manner): 0.1 ml of a solution of salmon sperm DNA (250 mg dissolved and swelled for 24 h in 200 ml 0.01 M tris-HCl buffer pH 6.7 at 4 °C) was mixed with 0.1 ml of an acridine orange solution (7.2 mg/5 ml in the same buffer plus DMF, 1:3, v/v). To this mixture, 0.1 ml of solutions with increasing amounts of thalidomide (3.7, 5.2 and 6.5 mg/ml, resp.) in the same solvent were added and the HPLC was carried out, selectively measuring the decrease (AO-DNA) of the fluorescence at 22 °C and 540 nm with excitation at 450 nm.

HPLC system: Kontron 414 pump, Rheodyne 7125 injection system 20 µl loop, spectrofluorimeter SFM 23 (Kontron), Anacomp 220 control and integration unit, column: Spherisorb ODS, 5 µ, 125 × 4.6 mm ID, eluent: water/*n*-propanol (90:10, v/v) [14].

Viscosity measurements

100 mg salmon sperm DNA was dissolved and swelled in 25 ml buffer (0.01 M EDTA in aq. bidest., pH 6.7) at 4 °C for 24 h. 0.1 ml of a fresh solution of thalidomide (1.0, 5.0 and 20 mg/ml, resp.) in the buffer plus DMF (1:1, v/v) was added instantly to 2.0 ml of the DNA solution. In addition, each sample contained 0.1 ml of a solution of 0.1 mg ethidium bromide (Merck, Darmstadt) per ml in the same solvent. The viscosity was measured at 26 °C in a flow-through capillary Ostwald-viscosimeter (Haake, Vienna) and put into relation to the viscosity of the DNA solution (buffer plus DMF, 1:1, v/v).

Phase partition analysis

This set of experiments was carried out in order to study the distribution of thalidomide between *n*-octanol-(1) and tris buffer pH 7.4 in the presence of increasing amounts of DNA in the aqueous phase and in controls without any DNA, respectively. This technique, the so-called solvent or phase partition analysis, has been proposed as a general method for studying the interaction of small molecules with macromolecules [12]. Octanol was selected, because partition between this water-immiscible solvent and buffers at best correlates with the distribution between the biological membrane lipids and the aqueous body fluids [13].

Samples with increasing concentrations (see Fig. 5) of calf thymus DNA (low mol. type, pretreated as usual) in tris buffer (0.05 M, pH 7.4) were mixed with equal amounts of a saturated aqueous solution of thalidomide. This mixture (2 ml) was equilibrated by shaking at 37 °C with octanol (2 ml, saturated with water). Analytical samples (20 µl) were withdrawn every 10 minutes, and the

thalidomide concentration determined by means of a sensitive HPLC method [14].

Results and Discussion

Ample evidence results from the four types of experiments reported above that thalidomide is an effective intercalator into DNA of various sources. It could be excluded, however, that thalidomide also interacts with RNA in a similar manner.

Spectral titration of thalidomide with solutions of DNA of various kind leads to significant hypochromism in every case, and there are, although less significant, slight spectral shifts to longer wavelengths. The absorption spectra show isosbestic points and were used to estimate the binding affinities and the numbers of binding sites per nucleotide in the DNA samples used. DMF as solvent was devoid of any influence upon the spectral properties or interactions of thalidomide and DNA.

Fig. 1 displays representative absorption spectra of the combinations of thalidomide and DNA from human placenta; similar spectral shifts (not displayed here) were obtained also with the other DNA samples. Fig. 2 shows the Scatchard plot of the data given in Fig. 1; a summary of the binding behaviour of thalidomide towards all used nucleic acid specimens is given in Table I.

Table I. Binding ratios and dissociation constants of thalidomide with various specimens of nucleic acids.

Sources of nucleic acids	Binding ratios N1	N2	Constants $\times 10^{-4}$ M K1	K2
Human placenta DNA	0.192	0.326	37.4	6.4
calf thymus DNA, low mol. weight	0.129	0.480	58.2	12.8
calf thymus, high polymer	–	0.375	–	64.1
salmon sperm DNA	0.106	0.390	1254	72.2
torula yeast RNA	no binding observed			
baker's yeast DNA	no binding observed			

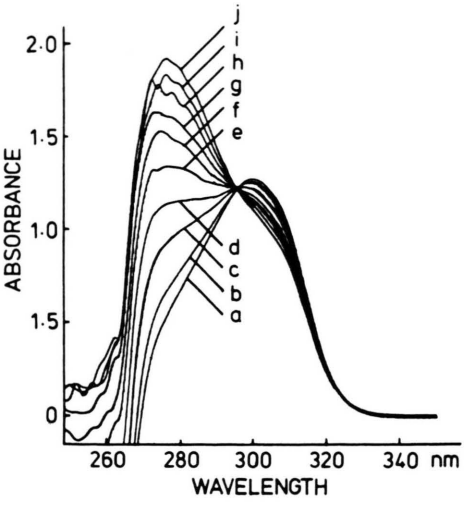

Fig. 1. Spectrophotometric titration of thalidomide with DNA from human placenta. The curves show the hypochromic and bathochromic shifts produced by increasing concentrations of DNA in buffer pH 5.6: a = thalidomide alone, from b to j = stepwise addition of 0.02 ml DNA solution (reference: DNA solution).

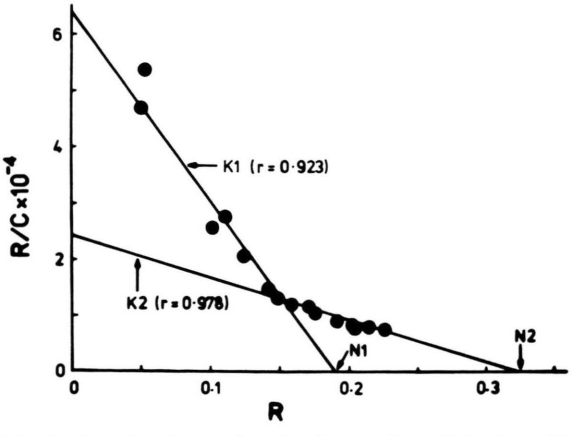

Fig. 2. Scatchard plot for the interaction of thalidomide into DNA from human placenta. R bound fraction, C free thalidomide [M]; N1 = 0.192 and N2 = 0.326 binding ratios per nucleotide; K1 = 37.4×10^{-4} M and K2 = 6.4×10^{-4} M association constants of the complexes; r, correlation coefficient of regression line.

The existence of strong and weak binding sites is indicated by spectrophotometry, since the Scatchard plot of the data clearly distinguishes two sets of data points giving straight lines of different slope. This suggests that at least two forms of complex are present at equilibrium. It may be speculated that the complexation of thalidomide with different nucleic acid bases, presumably guanine and adenine, occurs with variable strength. It is obvious that the flat aromatic ring is positioned between two adjacent base pairs, the glutarimide moiety (which may have been split hydrolytically, too) must lie in the major or minor groove of the DNA double helix.

However, *in vivo* the situation might be somewhat different: Besides the original thalidomide molecule, presumably a metabolically activated form of it, for instance an *arene oxide*, may intercalate into the nucleic acid. As soon as the complex has been formed, the extremely active metabolite will react with a nucleic acid base, forming a covalent bond, thus making the denaturation of DNA definitive. This seems to be the prerequisite for a persisting disturbance of the DNA template. Since the relatively weak intercalation complex would easily dissociate, or the drug would be replaced by other intercalators, as we have seen, there is no reason to assume that intercalation *alone* would have such severe consequences as for instance thalidomide teratogenicity.

Table II. Displacement by thalidomide (TH) of various intercalators (IC) from salmon sperm DNA.

Molar ratio TH:IC	Per cent displacement of methyl green	ethidium bromide	acridine orange
0:1	0	0	0
1:1	2.8	1.4	2.0
2:1	4.0	2.1	3.4
3:1	7.8	2.6	4.0
4:1	7.9	2.2	4.7
5:1	11.4	2.4	6.9
6:1	11.7	2.5	7.1

The further studies on the nature of the binding of thalidomide to DNA were conducted by measuring the changes of the viscosity in the solutions of the nucleic acid, as influenced by the intercalating thalidomide molecules. In case of real intercalation of a drug into the DNA, the viscosity of the solution increases due to partial unwinding of the helix. It is known that one molecule of intercalator extends the helix by approximately 3 Å [11].

The results (Fig. 3) strongly indicate that the nature of the binding of thalidomide to DNA is by true intercalation and not simply by aposition to the surface of the DNA helix at the minor groove. The viscosity changes can only be explained by a base pair unwinding and a length increase of the DNA filament. The effect is clearly concentration-dependent and in obvious analogy to the effect that is induced by the archetypical intercalating drug ethidium bromide. The unwinding angle of the DNA

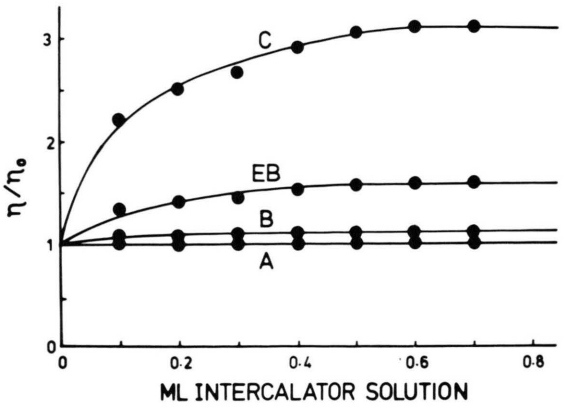

Fig. 3. Representative viscosimetric titrations of salmon sperm DNA with thalidomide (TH) and ethidium bromide. The abscissa shows the stepwise addition (each time 0.1 ml) of intercalator solutions of the following concentrations: A = 3.9×10^{-3} M TH, B = 19.0×10^{-3} M TH, C = 77.0×10^{-3} M TH, EB = 3.0×10^{-3} M ethidium bromide; Ordinate: ratio of the reduced specific viscosity of the DNA-ligand complex (σ), and the reduced specific viscosity of DNA alone (η).

under thalidomide was calculated as approximately 4 deg, as compared with 26 deg for ethidium bromide.

Further evidence for an intercalative binding of thalidomide comes from the displacement studies. One representative example (acridine orange) is given in Fig. 4. Since all the known intercalators cho-

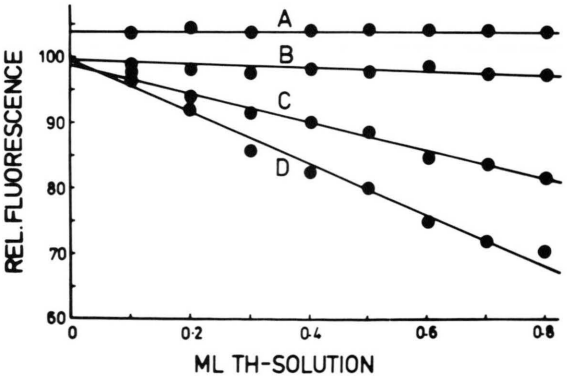

Fig. 4. Displacement of acridine orange (AO) from salmon sperm DNA by increasing amounts of thalidomide (TH). The abscissa shows the stepwise addition (each time 0.1 ml) of TH solutions of the following concentrations: A = none, B = 2.9×10^{-3} M, C = 4.0×10^{-3} M, D = 5.2×10^{-3} M; Ordinate: per cent relative intensity of AO-DNA fluorophor.

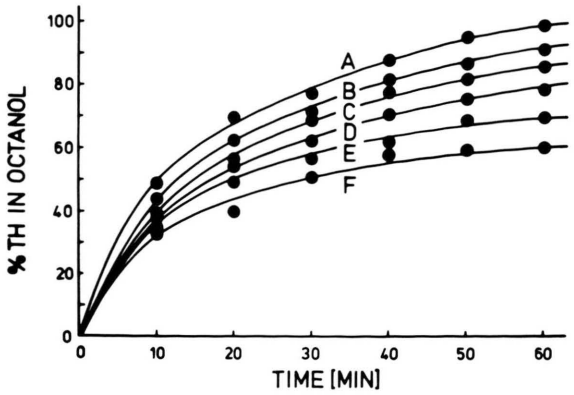

Fig. 5. Concentration-time curves of thalidomide (TH) in the organic phase of the system *n*-octanol/tris buffer pH 7.4 at 37 °C and increasing concentrations of calf-thymus DNA in the aqueous phase. A = none, B = 0.14, C = 0.28, D = 0.56, E = 2.25 and F = 4.50 mg DNA per ml.

sen get replaced from the DNA by increasing concentration of the drug, this must also bind to the DNA through the same mechanism.

Additional support for the intercalation model is provided from solvent partition experiments. Fig. 5 depicts the time dependence and equilibrium concentration of thalidomide in the octanol phase under the experimental conditions chosen. The thalidomide concentration is expressed as per cent of the maximal concentration reached at equilibrium in the octanol phase in absence of DNA. These and other similar curves (not shown here), clearly demonstrate the correlation between the increasing DNA concentration in the aqueous phase and the falling amount of thalidomide in the organic layer, another fact that clearly demonstrates the binding by the nucleic acid of the teratogen.

The idea of an intercalative binding to DNA of thalidomide has been raised many years ago, however, true experimental evidence for such an interaction has never been presented so far, although some earlier findings [15–17] pointed to this possibility (for a more thorough description of the whole problem see ref. [2] and [3]). In this study, the experimental proof for the complexation of the drug with nucleic acids is presented by several methods *in vitro*, but there is no doubt about it that this interaction might occur *in vivo* as well.

The question remains open why thalidomide, among many intercalators, provides that high specifity of teratogenic action that has been known that long time. The answer might be that the drug also undergoes concomitant metabolic activation *in vivo*, *i.e.* to an arene oxide intermediate which can be covalently bound to the target ('arenoxide hypothesis', see ref. [3]). Another suggestion that has received its experimental proof in the meantime [6].

In the view of these facts, we propose as a novel 'working hypothesis' a combination of the intercalation and the bioactivation concept as the basis for the further investigations into thalidomide's molecular mechanism of action as a teratogenic agent. This might be the clue to the whole thalidomide problem which, hopefully, will arrive at its definitive resolution in the not too distant future.

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