Hypothesis

Competitive inhibition of nicking–closing enzymes may explain some biological effects of DNA intercalators

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Intercalating agents cause varied and multiple biological effects. These include the inhibition of RNA and DNA synthesis, frameshift mutations and protein-associated DNA breaks. However, some non-intercalating analogs of intercalating compounds behave similarly. The model of DNA intercalation does not adequately explain all these biological effects. It is suggested here that intercalators and similar compounds may competitively inhibit the closing reaction of some nicking–closing enzymes. Hypothetical mechanisms built on this suggestion are presented for the formation of protein associated DNA breaks, frameshift mutation, inhibition of macromolecular synthesis, and recombination.

Nicking–closing enzyme *DNA-intercalating agent* *Protein-associated DNA break*
Macromolecular synthesis inhibitor *Frameshift mutation* *Recombination*

1. INTRODUCTION

Over 20 years ago Lerman put forward a hypothesis to explain the interaction between aminoacridine and DNA [1]. Aminoacridine was assumed to intercalate between adjacent DNA basepairs. Many different compounds were subsequently shown to form intercalative complexes (see fig. 1, I–VII; for review see [2–6]). The main contribution to the binding energy of the complex usually comes from the interaction between the Pi electronic system of the intercalated molecule, which is invariably aromatic, and the neighbouring DNA bases. Ionic interactions with DNA phosphates may provide additional stabilization if the molecule of intercalator is positively charged, while oligopeptide or oligosacharide sidechains may form hydrogen bonds with DNA constituents. The conformation of DNA changes substantially as a result of intercalation. The distance between the DNA basepairs adjacent to the intercalator increases by 1.8–4.5 Å while the helix becomes tilted and unwound by 11–26° per intercalating molecule. The complex usually has a higher melting temperature than uncomplexed DNA, reflecting an increase in the stability of the double helix.

The addition of intercalating drugs to cells in culture causes many cellular effects including cell death, inhibition of cell growth, and transformation. Explanations of these effects are commonly based on the assumption that the intercalator-induced changes in the DNA structure disturb correct functioning of some DNA-dependent enzymatic systems. For example, the antibiotic actinomycin D is thought to inhibit ribosomal RNA synthesis by forming complexes with the DNA which obstruct the movement of RNA polymerase (review [5, 6]).

However, even in this most studied case, it is not clear why actinomycin selectively inhibits rRNA synthesis or why some cell lines are resistant to actinomycin treatment. There are other biological phenomena which are even more difficult to explain only on the basis of intercalator-induced changes in DNA conformation. These include the formation of protein-associated DNA breaks (PADB) in bacterial plasmids [7] and in mam-

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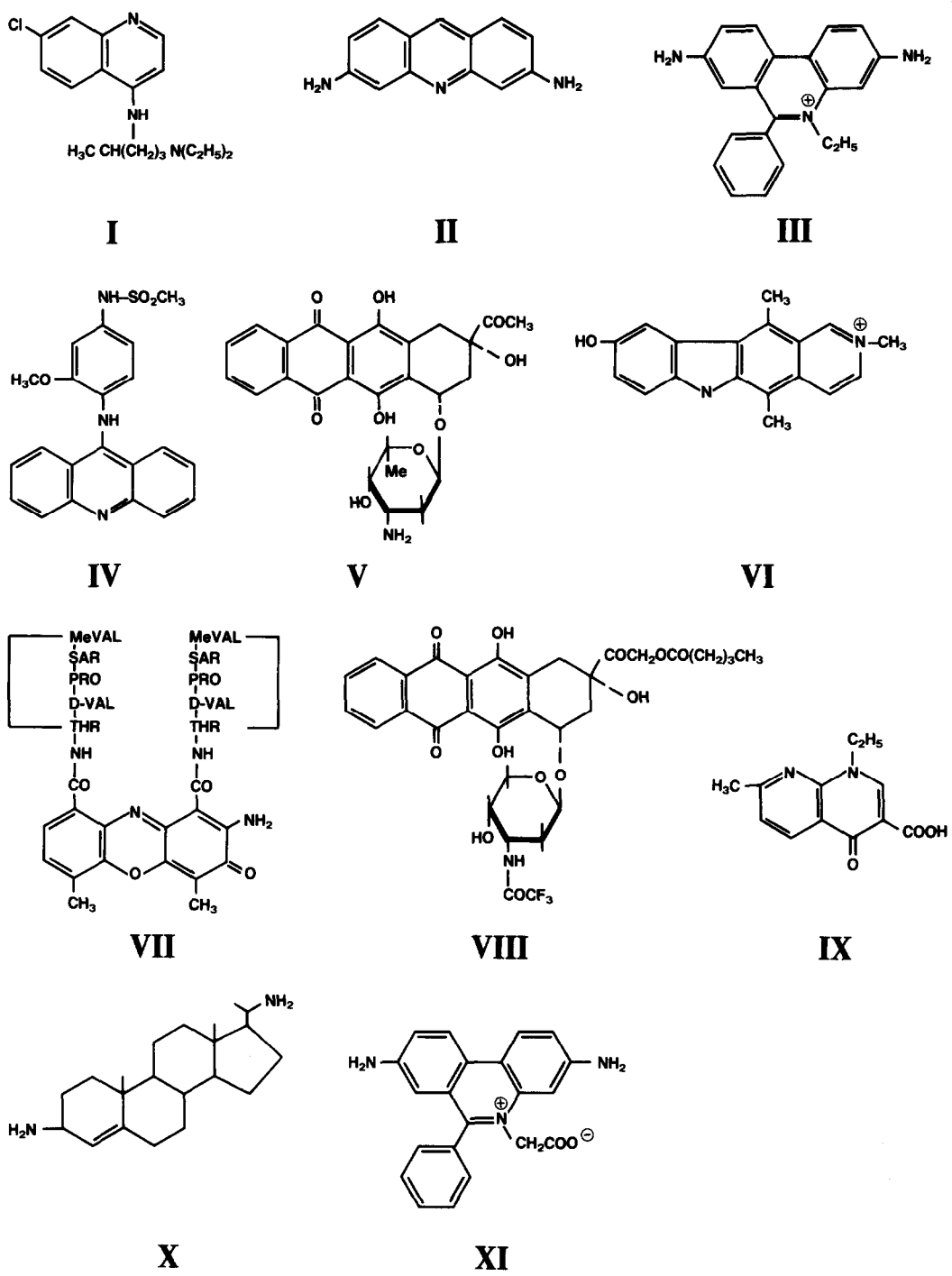

Fig. 1. Intercalating (I–VII) and nonintercalating (VIII–XI) compounds causing biological effects typical for intercalators: (I) chloroquine; (II) proflavine; (III) ethidium; (IV) 4′-(acridinylamino) methanesulfon-*m*-anisidine, (m-AMSA); (V) daunamycin; (VI) 2-methyl-9-hydroxyellipticinium; (VII) actinomycin D; (VIII) *n*-nitrofluoroacethyl-adriamycin-14-valerate (AD 32); (IX) nalidixic acid, (X) irehdiamine A; (XI), 3,8-diamino-5-carboxymethyl-6-phenyl-phenanthridinium.

malian cell nuclei [8–11], and various genetic effects such as frameshift mutation [12,13] and recombination [14,15].

I propose that these phenomena might be more readily explained by supposing that the 'intercalator' binds not only to DNA but also to a protein of the relevant DNA-dependent enzymatic system. Many DNA-dependent cellular processes involve enzymes which are able to introduce a single strand break or nick into the DNA. These enzymes bridge the break, binding covalently to one end of the nicked DNA and noncovalently to the other end. The reaction is reversible, thus all these proteins could be called 'nicking–closing' enzymes.

More specifically, I propose that some molecules, including, but not limited to intercalators, competitively inhibit this 'closing' reaction by blocking the site on the enzyme normally occupied by the non-covalently bound end of the nicked DNA.

2. COMPETITIVE INHIBITION OF THE 'CLOSING' REACTION

There are many examples of nicking–closing enzymes. These include the bacterial plasmid DNA-relaxing proteins, topoisomerases I and II, lambda phage integration (*int*) protein, *E. coli* synaptase, adenovirus 5 terminal protein, and ϕX174 *cisA* protein (reviews [16–19]). The proteins responsible for PADBs in mouse L 1210 nuclei [10,11] and for illegitimate recombination in *X. leavis* oocytes [20] may also be 'nicking–closing' enzymes. Fig. 2 illustrates the possible mechanism of inhibition of the closing reaction which may apply to nicking–closing enzymes in general. Features of the model include the following:

(i) Nicking of the DNA with simultaneous formation of a covalent bond between the enzyme and either the 3′- or 5′-end of the nicked DNA (fig. 2A–D).

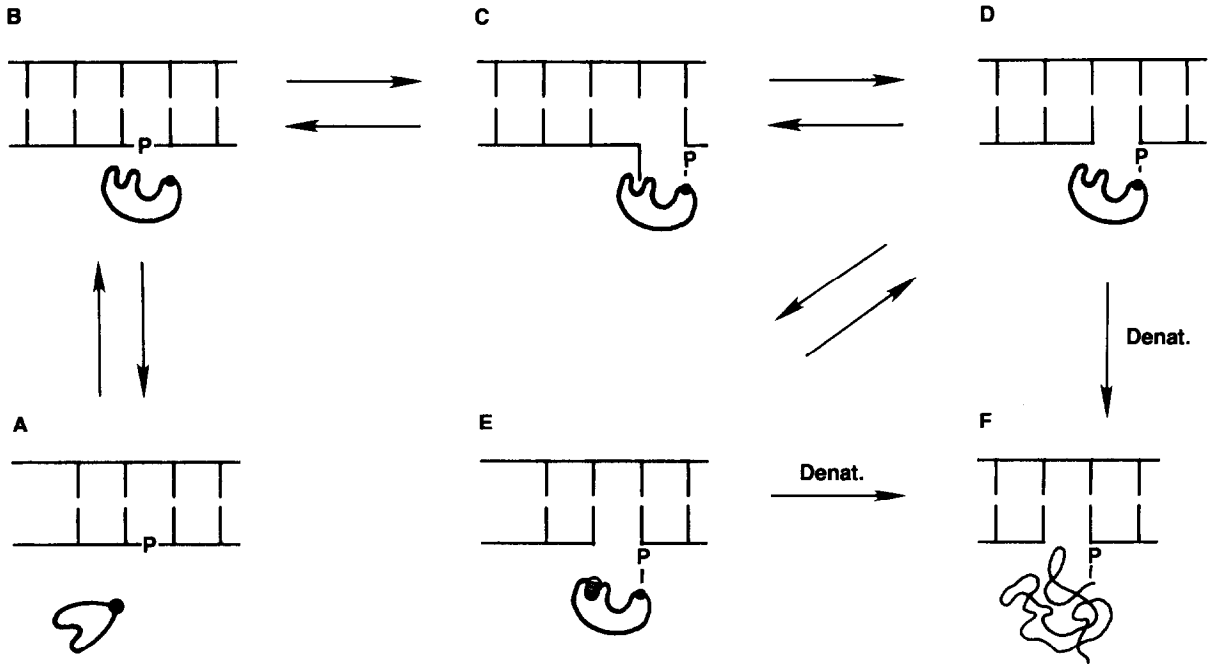

Fig. 2. Reactions of 'nicking–closing' enzyme with DNA and intercalator: (A) unreacted components; (B) weak DNA–enzyme complex; (C) the enzyme forms a 'linker' with one covalent and one non-covalent protein–DNA bond. This structure is in equilibrium with structure (D), in which the protein is bound to DNA by the covalent bond only; (E) addition of an inhibitor blocks the noncovalent DNA-binding site on the protein; (F) denaturation of the enzyme irreversibly binds it to the DNA.

(ii) Formation of a non-covalent bond with the other end of the nicked DNA; (Pulleyblank and Ellison [21] postulated a hydrogen bond.) The model presented here suggests that other interactions between the DNA bases and the enzyme may also be involved (fig. 2C).

(iii) The phosphodiester bond shuttles between the enzyme (fig. 2C,D) and the ribose of the DNA backbone (fig. 2B). There is an equilibrium between these states (postulated also by Clevell and Helsinki [22]).

(iv) Denaturation by detergents, alkali or acid of the enzyme leads to permanent PADB in a fraction of the molecules by destroying its catalytic site and 'freezing' the phosphate bond (fig. 2F).

(v) The non-covalently bound end of the DNA oscillates between its complementary strand (fig. 2D) and the binding site on the enzyme (fig. 2C). This equilibrium may depend on the DNA base sequence.

(vi) Various compounds may disrupt the non-covalent enzyme–DNA binding (fig. 2C). Some of these compounds may act as competitive inhibitors displacing the DNA from its binding site and thereby making it impossible for the nick to be resealed. The result is a shift of the equilibrium towards the complex 2E.

Intercalating compounds (fig. 1, I–VII) might be expected to be effective inhibitors of the closing reaction of some of these enzymes. Since they are able to replace the DNA bases in the base–base stacking interactions, it is likely that they can also substitute for the DNA bases in the protein–DNA interaction. Because these inhibitors of the 'closing' reaction may bind primarily to the protein site rather than to the DNA, one might predict that:

1. The extent of inhibition of the closing reaction caused by intercalating agents would not correlate with their DNA binding parameters;

2. In the extreme case the inhibition of the closing reaction could be caused by intercalator-like molecules which are unable to intercalate because of some structural features such as a net negative charge, a bulky substituent or a nonplanar conformation [examples of such molecules are shown in fig. 1 (VIII–XI)];

3. Another piece of single stranded DNA can replace the noncovalently bound end of the nicked DNA molecule and, may act as an inhibitor of the closing reaction or may even be joined to that DNA.

There are several reports in the literature which are consistent with the above predictions.

With **1**:

- The strong intercalator EtBr produces a much lower frequency of PADB in L 1210 cells than the weak intercalator m-AMSA [9,11];
- The strong intercalator ellipticinium does not produce PADB in isolated L 1210 cell nuclei, while ellipticine and other weaker intercalators produce a high frequency of PADB in this system [10, 11].

With **2**:

- A derivative of the strong intercalator adriamycine, AD 32, (fig. 1, VIII) fails to bind to DNA; however, it produces a high frequency of protein associated DNA breaks in L 1210 cells [23];
- Nalidixic (fig. 1,IX) and oxolinic acids intercalator-like molecules do not bind to DNA, inhibit the resealing reaction of some type II topoisomerases (reviews [16–19]).
- Intercalator-stimulated nucleolytic activity from yeast mitochondria is stimulated also by a carboxyl-substituted analogue of EtBr (fig. 1, XI) which does not intercalate into DNA [24]. One may suspect that this intercalator-stimulated fragmentation of DNA is caused, at least partially by a nicking–closing enzyme.

With **3**:

- Topoisomerase activity of lambda *int* protein is inhibited by a single-stranded DNA [25];
- Poly(dGdC) inhibits the action of calf thymus topoisomerase on supercoiled DNA resulting apparently in the formation of nicked DNA molecules [26];

- Synaptase (review [27]), lambda *int* protein [25], and proteins from *X. laevis* oocytes [20] exchange ends from two different DNA molecules, a process which results in branched, Holliday-type, DNA structures.

3. BIOLOGICAL EFFECTS OF THE INHIBITION OF THE CLOSING REACTION

3.1. *Inhibition of macromolecular synthesis*

I will discuss three possible biological consequences of the inhibition of the resealing reaction of the nicking–closing enzymes. The first is linked to the fact that the nicking–closing enzyme, unable to finish the closing reaction, becomes immobilized and crosslinked to the DNA. Enzymatic systems, such as DNA or RNA polymerases, the function of which depends upon (unobstructed) movement along the DNA are likely to be inhibited. This effect was postulated [28] to explain the inhibition of the replication of T4 phage by nalidixic acid, a gyrase inhibitor, even though replication of this phage does not require a functional gyrase. By inhibiting the resealing reaction of gyrase, nalidixic acid causes gyrase molecules to become linked to DNA, thereby impeding polymerase movement.

A similar effect may be responsible for the selective inhibition of mitochondrial DNA replication by some intercalating agent (e.g., [29]). Rat liver mitochondrial topoisomerase is sensitive to EtBr while the nuclear enzyme is not [30].

3.2. *Frameshift mutation*

The second biological consequence of the inhibition of the resealing reaction results from the possible release of the noncovalently bound end of the DNA from its binding site. Addition or removal of bases from the free end might lead to frameshift mutations. A model of such an effect is presented on fig. 3. In the first step, the nicking–closing enzyme produces a nick. A molecule of intercalator binds to the enzyme. This simultaneously stabilizes the nick and displaces the noncovalently bound end of the DNA, which may then be exposed to exonucleases. A second molecule of intercalating agent may stabilize the loop-out in the DNA, as postulated [12]. If the loop is close to a region of repetitive DNA sequence, partial base pairing may be restored at both sides of the loop (step II). Dissociation of the intercalator enables the nicking–closing enzyme to finish its closing reaction (step III). Replication of such DNAs would result in a frameshift mutation.

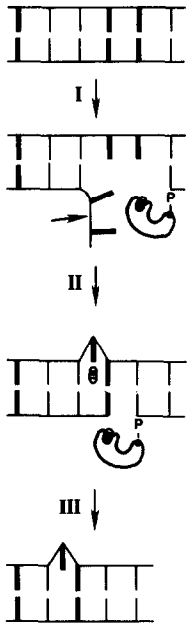

Fig. 3. Proposed mechanism for the formation for frameshift mutations: (I) 'nicking–closing' enzyme nicks the DNA, the noncovalent DNA binding site is blocked by an intercalating agent, and an exonuclease trims the displaced DNA end; (II) the complementary DNA strand forms a loop which is stabilized by another molecule of intercalator; (III) after the dissociation of intercalator, the 'nicking-closing' enzyme reseals the break and dissociates.

In contrast to the classical Streisinger model [12], this model predicts that more than one molecule of intercalator is involved in each frameshift mutation event, a prediction which is in good agreement with experimental results (review [31]). Another prediction is that mutagenic activity does not necessarily correlate with the DNA binding parameters of various intercalating agents. In fact attempts to find such a correlation have failed (review [32]). One may suppose on the basis of the model presented here that the reason for the lack of the correlation is the requirement of the binding of the mutagen to both DNA and the protein. In

addition proteins from different sources may have different affinities for a given intercalator. This may explain why proflavine causes frameshift mutations in a T4 phage system but not in *Salmonella* [32].

The model presented here suggests that sequence specificities may be involved at several steps in the formation of a frameshift mutation:

(1) The DNA sequence specificity may be involved in the positioning of the nicking–closing enzyme on the DNA;
(2) The base sequence close to the nick should be repetitive in order to allow slippage of the DNA end;
(3) The intercalator has to have an affinity towards the mismatched sequence;
(4) The affinity of the end of the nicked DNA towards the noncovalent binding site on the nicking–closing protein should not be much higher than the affinity of the intercalator to the same binding site, otherwise the intercalator would not interfere in the nicking–closing equilibrium. A DNA base sequence fulfilling these requirements might be a mutational 'hot spot'.

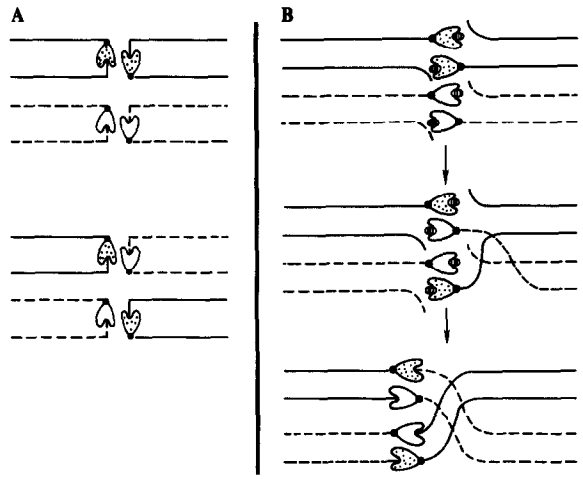

Fig. 4. Mechanisms of oxolinic acid-stimulated recombination. (A) mechanism proposed [33]. Gyrase subunits from interstrand bridges at a DNA double strand scission. Gyrase subunits then exchange. (B) Mechanism proposed here. Gyrase subunits form intrastrand bridges at a DNA double strand scission. Oxolinic acid displaces the noncovalently bound DNA ends (top). Gyrase subunits exchange (middle). Displaced DNA ends may rebind with different gyrase subunits, leading to recombination (bottom).

3.3. *Recombination*

The third biological consequence of the inhibition of resealing results when the displaced ends of the nicked DNA are exchanged between two different protein–DNA complexes. An example of this effect is the increase of the recombination frequency in a cell-free lambda phage packaging system in the presence of oxolinic acid [33]. This effect was interpreted as resulting from the subunit exchange between two gyrase–DNA complexes fixed by the inhibitor (fig. 4A). This hypothesis implicitly assumes that a subunit of gyrase (each subunit consists of one protomer A and one protomer B) forms an *interstrand bridge*, with one covalent and one noncovalent bond, at the site of the DNA scission. This assumption is rather unlikely since it is known that oxolinic acid at low concentrations causes mainly single-stranded DNA breaks [34], a fact which cannot be explained by the model in fig. 4A. More consistent with the known properties of the gyrase is the formation of an *intrastrand bridge* by each subunit (fig. 4B). Both the gyrase subunits and the noncovalently bound DNA ends are able to exchange, resulting in the recombinational event (fig. 4B, bottom). Oxolinic acid would be expected to stimulate this process by displacing the noncovalently bound DNA ends from their binding sites on the protein. Compared to the other model (fig. 4A), this model suggests a specific role for oxolinic acid. Similar processes may be involved in recombination in yeast caused by daunamycine [14] or in sister chromatid exchanges caused by intercalating compounds in mammalian cells [15].

4. CONCLUDING REMARKS

The model presented here suggests that some biological consequences of many intercalator and intercalator-like compounds may result not from their binding to DNA but from their binding to nicking–closing enzymes. The verification of this model will depend on the isolation and characterization of the appropriate enzymes. This model may be useful in explaining the biological effects of intercalators and similar compounds which are not easily explained otherwise.

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