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Isolation and Analysis of Inhibitors of Transposon Tn*3* Site-Specific Recombination

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We have constructed a genetic bioassay for inhibitors of site-specific recombination by transposon Tn*3* resolvase. Of 6,000 compounds tested, 26 inhibited in vivo, and 5 of these 26 inhibited in vitro. At least two inhibitors also inhibit the topoisomerase of resolvase. We have also identified analogs of A1062 which inhibit.

Transposon Tn*3* encodes a site-specific recombination system that is part of its transposition process (3). Tn*3* transposition is usually a two-stage event. In the first stage, Tn*3* is duplicated, and the donor and acceptor replicons fuse to form a cointegrated molecule in which a Tn*3* is at each junction between the replicons (2). Resolution of this fusion into individual molecules occurs by site-specific recombination carried out by the Tn*3* resolvase (5, 9). In addition to catalyzing site-specific recombination, resolvase contains a site-specific type 1 topoisomerase activity and represses synthesis of itself and the *tnpA* gene (1, 6). Tn*3* resolvase has been purified to homogeneity and will perform site-specific recombination in vitro, using a model substrate with two directly repeated *res* sites, pRR51 (6).

Since there were no known inhibitors of site-specific recombination, our first step was to establish a genetic assay. We employed λXJS845, which was isolated by Muster et al. (8). This bacteriophage is a λCM derivative that is defective and which replicates as a plasmid inside the cell. This phage carries a streptomycin resistance (Sm^r) gene that is flanked by two directly repeated copies of TnA ΔAp. TnA ΔAp is a deletion that still retains a recombination site but is deleted for its resolvase gene (2). Upon recombination by resolvase, the Sm^r gene is deleted and quickly lost because it does not have an origin of replication. The process of resolution is so fast and efficient that cells containing resolvase infected with λXJS845 are all Sm^s by the time they become colonies. Muster et al. (8) found that the equilibrium ratio of recombined/unrecombined molecules is greater than $10^5/1$ in cells in which the resolvase gene is on a high-copy plasmid. The rationale of the assay is that if resolvase is present but inhibited, then cells infected with λXJS845 will be Sm^r, whereas cells with uninhibited resolvase will be Sm^s. Inhibitors of resolvase would produce Sm^r colonies if the inhibitors are taken up into the cell and inhibit resolvase more than cell growth. The assay uses two LB soft agar (0.8%) overlays on top of a regular LB plate containing chloramphenicol (40 μg/ml) and streptomycin (25 μg/ml) (7). The first overlay contained 10^4 transducing particles of λXJS845 in 2.5 ml of LB soft agar. The second overlay contained LB soft agar with 10^9 cells carrying the resolvase gene on a high-copy plasmid. The strain used was *Escherichia coli* K-12 C600 with the plasmid RSF1050 which carries the resolvase gene (6). Potential inhibitors were dissolved in dimethyl sulfoxide, spotted onto a sterile filter disk, and placed on the topmost layer. The plates were then incubated at 32°C for 48 h (Fig. 1).

We searched at random through a collection of chemicals maintained by Abbott Laboratories. We screened over 6,000 compounds for their ability to produce Sm^r colonies, and 26 of these were positive in this assay (Fig. 1). A20832 was the best inhibitor that we found in this assay (Fig. 1). Our next step was to test these 26 compounds for inhibition of site-specific recombination in vitro, using the purified system of Reed, with homogeneous Tn*3* resolvase substituted for γδ resolvase (6, 10). This system measures intramolecular recombination with the pRR51 plasmid which contains two

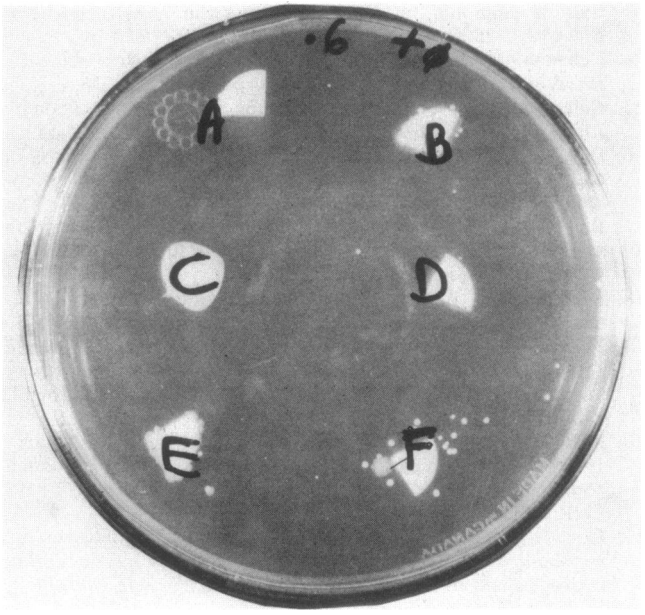

FIG. 1. The resolvase inhibitor assay. This assay involves two soft agar (0.8%) overlays, one which contains λXJS845 and one which contains the cells with resolvase. The regular agar (1.5%) layer is LB with chloramphenicol at 40 μg/ml and streptomycin at 25 μg/ml. The first soft agar overlay is 2.5 ml of LB soft agar with 10^7 transducing particles of λXJS845. The second soft agar overlay contains LB soft agar with the cells carrying *tnpR* on a high-copy plasmid. The chemicals were dissolved in dimethyl sulfoxide at 100 mg/ml and spotted onto a sterile filter disk which was placed on top of the second layer. The plates were incubated at 32°C for 48 h. Letters: A, dimethyl sulfoxide alone; B, A1062; C, A9387; D, 18947; E, A20812; and F, A20832.

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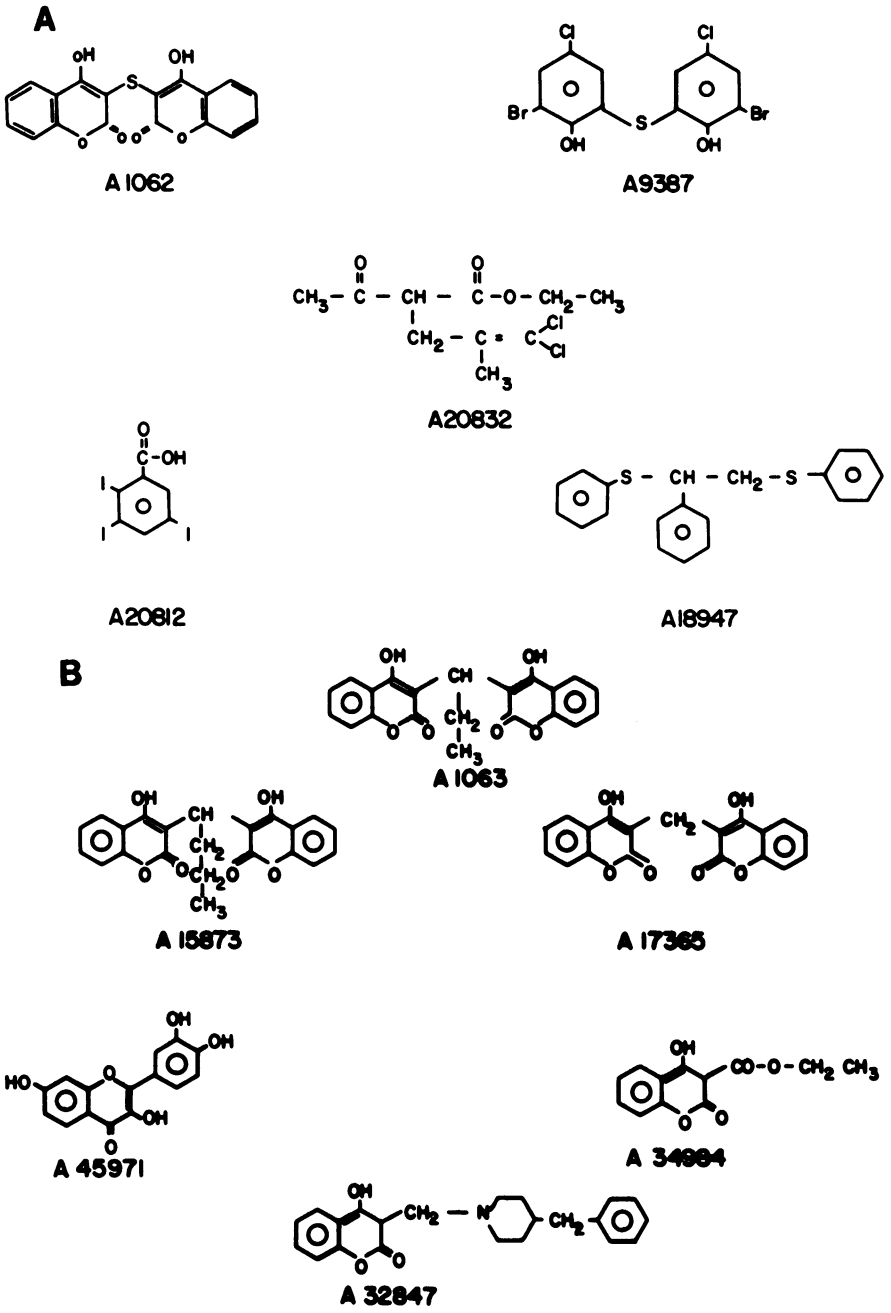

FIG. 2. (A) Structures of the five inhibitors of Tn*3* site-specific recombination. (B) Structures of compounds related to A1062. The numbers under the compounds refer to the catalog number of Abbott Laboratories. These structures have recently been reconfirmed by scientists at Abbott.

directly repeated *res* sites. This reaction has unusual stoichiometry in that little or no recombination occurs until a ratio of 20 resolvase monomers to one DNA molecule is achieved and no protein turnover has been demonstrated. These unusual features have made conventional K_i measurements impractical. Instead, we have determined the amount of inhibitor needed to inhibit recombination 50% at a set level of resolvase and DNA. As a control for nonspecific inhibition, we also tested inhibition of the restriction enzymes *Eco*RI and *Bam*HI. Of 26 compounds, 5 (Fig. 2) inhibited site-specific recombination in vitro more than they inhibited *Eco*RI or *Bam*HI. Three of these five (A1062, A9387, A18947) have structural similarities, but the other two (A20812 and A20832) are distinct from the others. The other compounds did not show any inhibition of resolvase at 300 μg/ml or inhibited *Eco*RI and *Bam*HI as strongly as site-specific recombination. Even though these other 21 compounds did not inhibit in vitro, we do not maintain that these compounds cannot be inhibitors. It could be that these compounds must be metabolized in vivo to a true inhibitor or that we are not assaying resolvase under the proper reaction conditions to elucidate inhibition.

The 50% inhibition point for A20832 was ca. 100 μg/ml (Fig. 3). Resolvase also contained topoisomerase activity

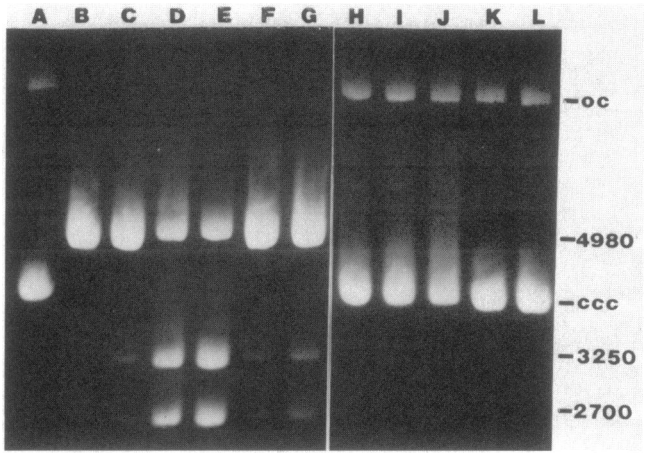

FIG. 3. Inhibition of resolvase by A20832. Shown are the supercoiled starting pRR51 DNA (ccc) and the open circular pRR51 DNA (oc). 4980 represents 4,980 base pairs, the size of a fragment of unrecombined pRR51 cut with *Eco*RI. 3250 and 2700 are the products of site-specific recombination that have been cleaved with *Eco*RI. Tn*3* resolvase was incubated with 0.2 μg of pRR51 substrate DNA in 20 mM Tris (pH 7.5)–50 mM NaCl–10 mM $MgCl_2$–1 mM dithiothreitol–1.5 mM EDTA–1% dimethyl sulfoxide for 25 min at 37°C. Then, 5 U of *Eco*RI was added to lanes B through G, and the reaction was continued for another 10 min. Lanes: A, no resolvase, no *Eco*RI; B, no resolvase, *Eco*RI; C, 26 ng of resolvase, *Eco*RI; D, 52 ng of resolvase, *Eco*RI; E, 104 ng of resolvase, *Eco*RI; F, 52 ng of resolvase, 200 μg of A20832 per ml, *Eco*RI; G, 52 ng of resolvase, 100 μg of A20832 per ml, *Eco*RI; H, 26 ng of resolvase; I, 52 ng of resolvase; J, 104 ng of resolvase; K, 52 ng of resolvase, 200 μg of A20832 per ml; L, 52 ng of resolvase, 100 μg of A20832 per ml.

that was detected as a topoisomerase ladder on an agarose gel (Fig. 3, lanes I and J), and A20832 inhibited this topoisomerase activity in parallel with inhibition of site-specific recombination (Fig. 3, lanes K and L). For A1062, the 50% inhibition point was ca. 10 μg/ml. A1062 also inhibited the topoisomerase activity of resolvase. To try to establish which groups in the molecules are important for inhibition, we have begun to examine analogs of the compounds discovered in vivo. Figure 2 gives the structures of six compounds related to A1062 that we have tested for inhibition of site-specific recombination. Three of these (A1063, A15873, A17365) inhibited site-specific recombination, two (A32847, A34974) did not inhibit, and one (A45971) inhibited *Eco*RI as well as site-specific recombination. These results suggest that the bridged, double-ring structure of A1062 is important for inhibition, with the bridging group having some variability. Unexpectedly, one of these analogs (A17365) was the drug dicumarol. We have not yet determined the site of inhibition of these compounds, but they did not cause single-or double-stranded breaks in DNA nor did they intercalate DNA. These results suggest that the inhibitors are affecting the resolvase protein. We are in the process of studying their effect on other site-specific recombination systems and their mechanism of inhibition.

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