Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

Kurt W. Kohn, MD, PhD
Scientist Emeritus
National Cancer Institute
Bethesda, Maryland
kohnk@nih.gov

Chapter 9: The DNA filter elution story.

In 1977, we made some surprising observations about how DNA strands of different lengths pass through membrane filters. Those findings allowed us to develop a set of methods for measuring DNA damage and repair in cells treated with various anti-cancer drugs, carcinogens, and x-rays. The filter methods were more sensitive and precise than other methods of the time and allowed us to measure several types of DNA damage at pharmacologically relevant dosage. Perhaps the most notable result of our experiments using the filter methods was the discovery that some anti-cancer drugs blocked topoisomerases (although the term 'topoisomerase' was not yet in vogue). That story, however, will be told in the next chapter (Chapter 10), which is about drug actions on DNA topoisomerases.

A research breakthrough in a long desired direction sometimes happens unexpectedly during studies aimed in a different direction. In 1973, we were following up on a then current idea that DNA replication occurred in a part of the chromatin that was attached to the surface membrane of the nucleus. We thought we could filter out the nuclear membrane-bound chromatin and test whether it mostly contained newly replicated DNA. We therefore deposited cell nuclei on a membrane filter and measured how much newly replicated DNA was retained on the filter after passing various solutions through the filter to wash away any DNA not attached to nuclear material large enough to be retained. We saw some selectivity for newly replicated DNA in the nuclear material retained on the filters and tried to optimize this selectivity by using various solutions to wash away the unbound DNA. When we tested various detergent solutions to dissolve the nuclei and letting the unbound DNA drip out, we found that our hypothesis was at least partly correct: an increased amount of newly replicated DNA was retained on the filter.

However, when we tested a solution made alkaline with sodium hydroxide, the opposite happened: the newly replicated DNA quickly ran out through the filter, instead of being retained on it, whereas the bulk of the DNA was retained (Kohn and Grimek-Ewig, 1973) (Figure 9.1). I soon realized that the alkaline solution would have caused the paired DNA strands to separate and would have release the short newly replicated DNA segments, allowing them to pass freely through the filter.

It seemed that this might be a way to measure DNA damage that produced strand breaks yielding relatively short DNA single-strands that would pass relatively easily though the filter. I had been trying for a long time to think of how to measure DNA breaks in a more sensitive way than the problematic centrifugation methods then in use. It seemed that all we had to do to accomplish that goal would be to increase the sensitivity of the alkaline filter phenomenon by a factor of 10. Was it possible to do that? A dictum attributed to Thomas Edison came to mind, to the effect that one could often improve a new phenomenon by a factor of 10 by systematically varying the conditions used. Together with my assistant, Regina A. G. Ewig, we followed that recommendation and systematically examined the effects of varying the conditions -- such as pH, flow rate, filter type, and composition of the alkaline solution.

First, we controlled the outflow from the filter by means of a peristaltic pump, thereby allowing us to control the flow rate of the alkaline solution through the filter. That turned out to be important: slowing the flow to several hours duration increased the sensitivity of the assay. After optimizing the other conditions, the method became sensitive enough to measure DNA damage in mammalian cells at therapeutically relevant dosage, which no other method of the time had achieved (Kohn and Grimek-Ewig, 1973) (Kohn et al., 1976). During several years of experience and further development the method acquired a sound quantitative basis for measuring several types of DNA damage, including single strand breaks, double-strand breaks, inter-strand crosslinks, and DNA-protein crosslinks (Kohn, 1996).

A condition that had worried me was the number of cells loaded onto the filter. Our procedure was to lyse (dissolve) the cells on the filter with a detergent solution, in order to free the DNA from other cell constituents before starting the controlled flow with the alkaline solution. I was afraid that the measurements would be sensitive to the number of cells loaded, since that would affect the viscosity of the lysed cell material. It was a great relief (also a surprise and somewhat of a puzzle) to find that the DNA elution rates were completely unaffected by number of cells loaded, as long as the number of cells was not so large as to clog the filter and stop the flow.

A condition I had not thought to consider was room light. Our early experiments had an annoying variability of the control measurements with undamaged cells. Eventually, I suspected that the room fluorescent lighting might cause some DNA strand breakage and raise the background measurements. What happened next may be of some interest or at least amusing. I asked Reggie Ewig, my assistant who carried out most of the assays, to arrange covers for the funnel units that had the filters with the lysed cells, in order to block the light coming from the ceiling. Reggie was skeptical and proceeded to obey my direction by preparing dunce-like conical caps to put over the funnels, and she pasted labels on them marked “Kohn’s folly.” That was an example of Reggie’s independent way of thinking that I treasured. Of course, when she saw the results, which indeed reduced the background, she always insisted that there be only enough light to allow the work to be done. She taped the room’s light switch in the off position and used only a single desk lamp turned away from the counter on which the work was done. Later, we had yellow lights installed, which solved the problem. A few years later, Matt Bradley,

a post-doctoral fellow in our laboratory, showed that fluorescent light caused DNA single-strand breaks in cells (Bradley et al., 1978).

In addition to the method for measuring single-strand breaks, we worked out variations, whereby we could measure other types of DNA damage, such as double-strand breaks, inter-strand crosslinks, and DNA-protein crosslinks (Kohn, 1996). For nearly two decades, the filter elution methods were the methods used in most laboratories for measuring DNA damage and repair.

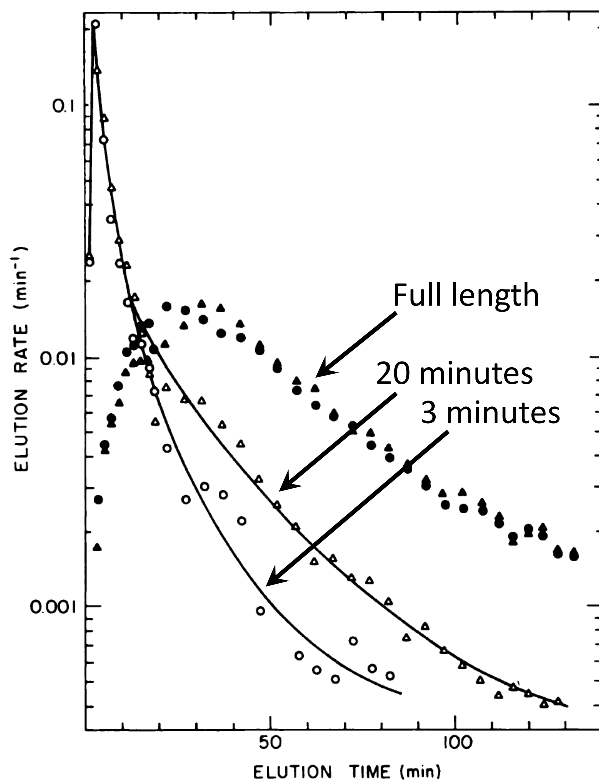

Figure 9.1. One of our first DNA alkaline elution experiments. Newly replicated DNA (after 3 or 20 minutes of replication) eluted more rapidly than the cell's full-length mature DNA. Our procedure was to deposit cells on a membrane filter and lyse them with a detergent-containing solution that would disrupt the cells and loosen proteins that were bound to the chromatin. We then pumped a solution containing 0.1 M sodium hydroxide (pH 12.0) slowly through the filter. The solid black symbols show the rate at which full-length DNA strands eluted from the filter as a function of time. The lower two curves show the higher rates of release of newly replicated DNA from the filter (Kohn and Grimek-Ewig, 1973). Note that the DNA that eluted during the first 10 minutes was mostly newly replicated DNA.

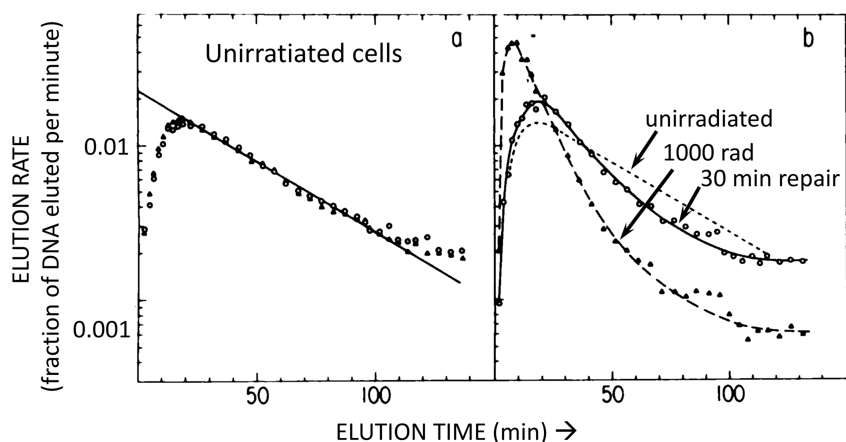

Figure 9.2. Proof that DNA single-strand segments elute from filters at rates inversely related to the length of the strands (Kohn and Grimek-Ewig, 1973). Cells were x-rayed to produce random breaks in their DNA. The cells were lysed on a filter and the DNA was eluted with 0.1 M sodium hydroxide as described in Figure 9.1. **a)** Unirradiated cells. **b**) Cells irradiated with a 1000 rad dose of x-rays: their DNA eluted more rapidly than DNA from unirradiated cells. After 30 minutes, the cells repaired most of the damage, and the elution rate of their DNA reverted close to that of unirradiated cells.

How the DNA filter elution phenomenon works.

The likely reason that the method works may be that long strands of DNA enter multiple pores in the filter, which retards their passage through the filter (Figure 9.3). The experiments were carried out by essentially the following procedure. Cells whose DNA damage was to be measured were deposited on a membrane filter and lysed by adding a detergent-containing solution. An alkaline solution (pH > 12.0) was then applied that caused the paired strands of DNA double helix to separate. As the alkaline solution was slowly pumped through the filter, the shorter DNA stands came through the filter more rapidly than longer strands. The reason that the longer strands took longer to pass through may be that each strand entered a larger number of pores simultaneously, which made it more difficult for the strand to pass through (Figure 9.3). The rate at which the DNA strands eluted from the filter told us the average length of the strands, which told us the frequency of drug-caused single-strand breaks in the cell's DNA.

We calibrated the elution rate by irradiating the cells to produce known frequencies of DNA strand breaks. In the early studies, we carried samples on ice from our laboratory in building 37 to the basement of the Clinical Center where there was a special pair of x-ray machines that irradiated the sample from above and below, thereby delivering a uniform dose over the sample. Each radiation dose produced a known frequency of DNA single-strand breaks in the cells. By comparing the elution rate of DNA from the x-rayed cells with the elution rate of DNA

from drug-treated cells, we calculated the DNA break frequency in the drug-treated cells (Figure 9.4).

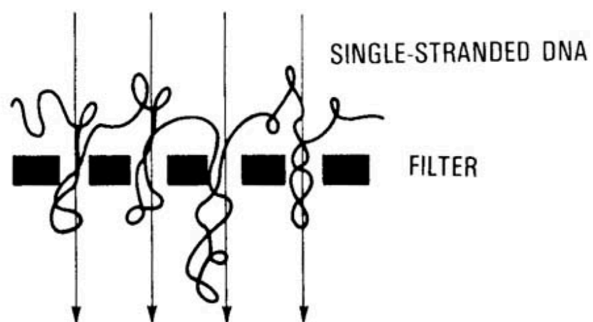

Figure 9.3. How a filter may impair a long DNA strand from passing through as an alkaline solution is pumped through. Since the DNA strands are long enough to enter several pores at once, longer strands would take longer to get through (Kohn, 1996).

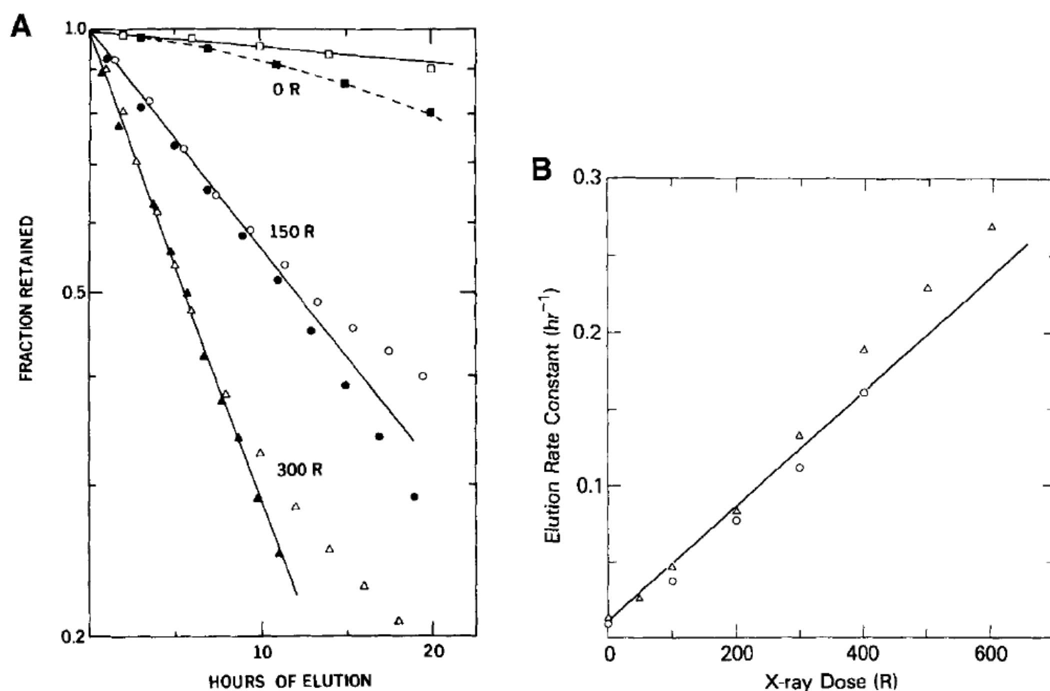

Figure 9.4. The rate at which DNA single-strands pass through the filter depends on the average length of the strands. The DNA was cut at random places by subjecting the cells to various doses of x-rays that produced known frequencies of strand breaks. Panel A shows the rates of DNA elution from cells irradiated with 0, 150, or 300 rad of x-rays. The vertical axis indicates the fraction of the DNA remaining on the filter as a function time as an alkaline solution (pH12.1) was slowly pumped through the filter. (The open and closed symbols were from experiments using alkaline eluting solutions of different pH, which showed that beyond a critical alkaline pH, raising the pH further had no effect, which was in accord with the theory of DNA strand separation developed by Paul Doty.) Panel B shows that the DNA elution rate increased linearly with x-ray dose, thus linearly with the frequency of DNA strand breaks (Kohn et al., 1976). (Note that the vertical axis in panel A is a logarithmic scale and that the elution rates were log-linear, which indicated that elution of the randomly cut DNA strands was random in time.)

We discovered that another property of the filters we used was that proteins tended to adhere to them. Therefore, a single-stranded piece of DNA that was linked to a protein did not pass through the filter, regardless of the length of the strand. To avoid this complication, we applied an enzyme to digest away most of the protein. Also, we used filters that had less tendency to bind proteins. Those two measures together eliminated protein binding to the filters.

However, we took advantage of the ability of certain types of filter bind proteins to create a quantitative assay for DNA-protein crosslinks. The procedure was to subject the cells to a

relatively large dose of x-ray (3000 rad) to produce relatively short DNA strand segments and then carry out an alkaline elution. The strand segments that had a DNA-protein crosslink in it stuck to the filter and did not pass through, whereas the other strand segments were able to pass through. The fraction of the DNA that could not pass through the filter allowed us to calculate (with a little bit of algebra) frequency of DNA-protein crosslinks. We validated the procedure by using the *trans*-isomer of cisplatin, which we had determined produced exclusively DNA-protein crosslinks (Chapter 3), as shown in Figure 9.5.

At that point, we were able to quantify both DNA single-strand breaks and DNA-protein crosslinks in drug-treated cells, which was to lead us to the conclusion that some drugs cause certain enzymes (later identified as topoisomerases) to produce DNA strand breaks and to become linked to the strand ends (Chapter 10).

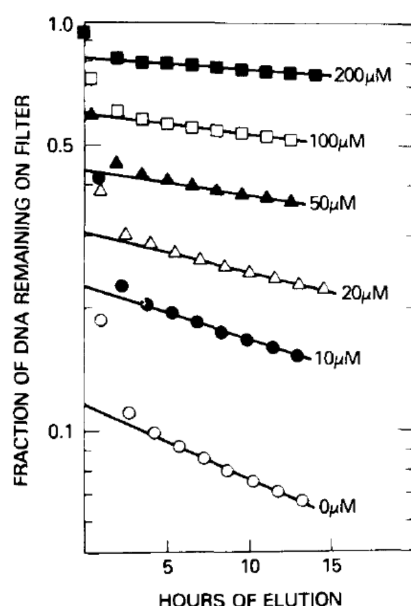

Figure 9.5. How we measured DNA-protein crosslinks. We treated cells with various concentrations of the *trans* isomer of cisplatin, which we had found produced exclusively DNA-protein crosslinks (see Chapter 3). We then subjected the cells to a relatively high dose of x-ray (3000 rad). The bottom curve (marked 0µM) shows that, for cells that received no *trans*-platinum treatment, about 90% of the DNA eluted rapidly. With increasing doses of *trans*-platinum, progressively less DNA eluted rapidly – which was the fraction of the DNA that was protein-linked. In order to get a true measure of the fraction of the DNA that was protein-linked, the curves were extrapolated back to zero time (Kohn and Ewig, 1979).

I was pleased that my propensity for quantitative studies, engendered by my undergraduate background in physics and experience in Paul Doty's laboratory (see Introduction), yielded these quantitative measures of DNA single-strand breaks and DNA-protein crosslinks. However, I had no idea that those quantitative measurements would lead us to discover that certain

drugs produce protein-linked DNA strand breaks that were caused by enzymes that came to be known as DNA topoisomerases. That story will be told in the next chapter (Chapter 10).

It was an exciting time for us as additional results poured in from our DNA filter experiments. Here are some of our most memorable findings: In 1974, we were able to measure the rate of DNA chain growth during replication (Kohn et al., 1974). Then, in 1979, Len Erickson carefully investigated intermediate elution pH values and succeeded in fractionating and measuring the growth rates of newly replicated DNA chains of different lengths (Erickson et al., 1979).

In 1975, Al Fornace used the filter methods to investigate the DNA repair defects in cells of the light-sensitive genetic disease xeroderma pigmentosum. He showed that ultraviolet light produced DNA single-strand breaks and DNA-protein crosslinks and measured the repair of those DNA lesions. When he tested cells from the different subtypes ("complementation groups") of the disease, he noted different degrees of DNA repair deficiencies in the various subtype (see Chapter …) (Fornace and Kohn, 1976; Fornace et al., 1976).

In 1977, we worked out how to measure DNA inter-strand crosslinks by means of the filter methods. We investigated the production and repair of those DNA lesions in cells treated with nitrogen mustard (HN2), BCNU and related drugs (Ewig and Kohn, 1977, 1978) (Figure 9.6). HN2 produced inter-strand crosslinks rapidly and then repaired them over a period of several hours (*left* panel of Figure 9.6). BCNU, on the other hand, produced crosslinks slowly, as monoadducts were slowly converted to crosslinks (*right* panel of Figure 9.6), and there was no evidence of repair, perhaps because those crosslinks, which are between paired guanine and cytosine (see Chapter 3) are more difficult to repair.

We developed a quantitative model to estimate the frequencies of strand breaks, inter-stand crosslinks and DNA-protein crosslinks, even when these DNA lesions are all present at the same time. Figure 9.7, for example, shows that the calculated lesion frequencies were proportional to the BCNU concentrations and therefore were suitable for quantitative studies of drug actions on DNA (Ewig and Kohn, 1978).

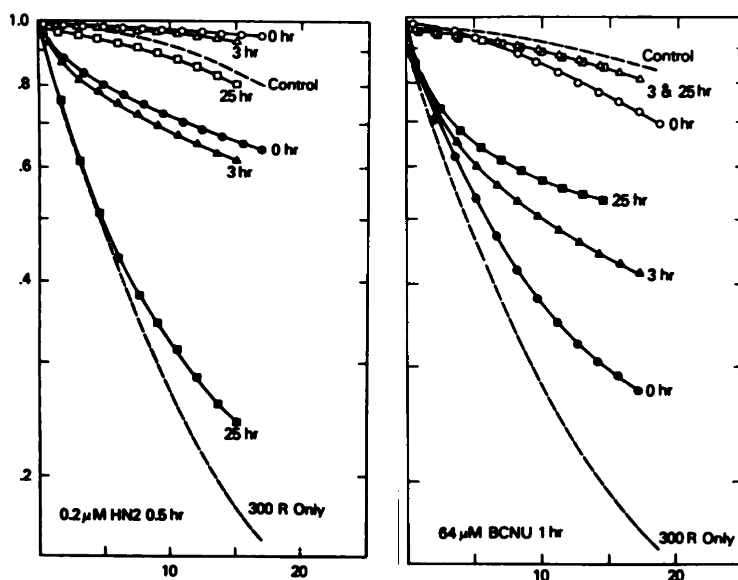

Figure 9.6 DNA inter-strand crosslinking by nitrogen mustard HN2 (*left*) and BCNU (*right*) (Ewig and Kohn, 1977).

After treating the cells with HN2 for 0.5 hours, there was a marked reduction of elution in 300 rad irradiated cells. (The cells were irradiated on ice just before lysis and elution. The 4 curves near the top are controls.) The reduced elution after the 300 rad irradiation was a measure of inter-strand crosslinking. The curve marked "3 hr" indicated a small degree of repair. The curve marked "25 hr" indicated nearly complete repair (the curve was nearly back to the "300 rad only" curve.)

After treating the cells with BCNU for 1 hr, 300 rad of irradiation produced less elution than 300 rad only. The three curves marked 0 hr, 3 hr, and 25 hr were for cells treated with BCNU for 1 hour and then incubated for 0, 3, or 25 hours before irradiation, lysis , and elution.

Thus, whereas HN2 showed repair after the rapid production of crosslinks, the BCNU results showed delayed production of crosslinks with no evidence of repair.

DNA-protein crosslinks contributed to these results, but carrying out similar experiments with and without digesting the lysed cells with a proteinase, allowed us to separate the effects of the two types of crosslinks (Ewig and Kohn, 1978).

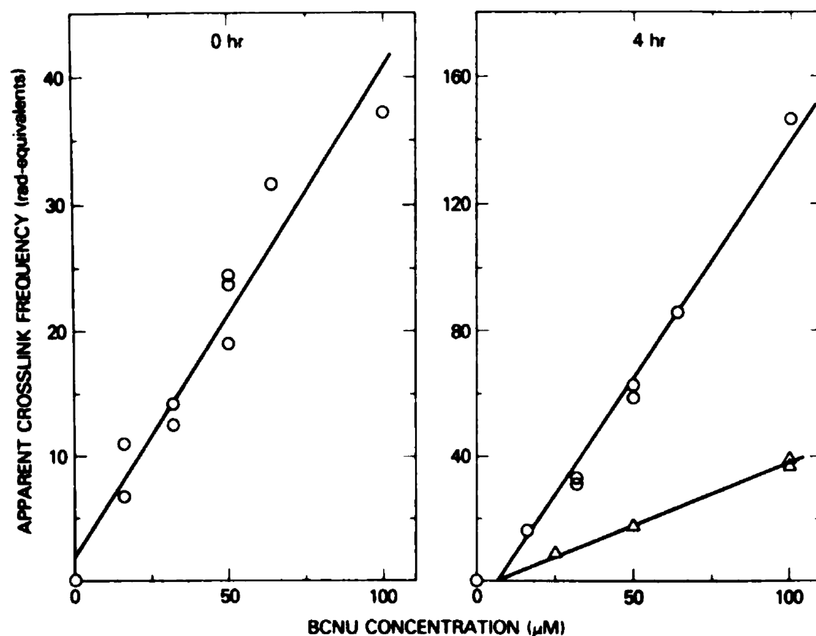

Figure 9.7. A quantitative model for inter-stand crosslink and DNA-protein crosslink production gave estimates that were proportional to drug concentration. Cells were treated with BCNU for 1 hour and were then, either assayed immediately (*left*) or incubated for 4 hours to allow time for more crosslinks to form. *Circles*, assay combination of inter-strand and DNA-protein crosslinks (without proteinase). *Triangles*, inter-strand crosslinks, which developed slowly. At zero time (immediately after 1-hour treatment with BCNU) almost no inter-strand crosslinks had yet formed; hence the results showed DNA-protein crosslinks only. After 4 hours (*right*), inter-strand crosslinks appeared (*triangle*) (Ewig and Kohn, 1978).

In 1978, Matt Bradley, as already mentioned, discovered that fluorescent lights caused DNA single-strand breaks in cells illuminated in culture medium (Bradley et al., 1978). Tissues, however, normally have enzymes that destroy the free radicals that are the likely cause of the observed DNA damage -- the cells in the experiments were illuminated in solutions that lacked those enzymes.

In 1979, Warren Ross worked out how to measure DNA double-strand breaks by using solutions of neutral pH for elution from filters (Bradley and Kohn, 1979). Using that method , he together with Warren Ross later observed double-strand break production in cells treated with doxorubicin or other DNA intercaltors, and inferred that the double-strand DNA breaks were produced by a trapped topoisomerase (Ross and Bradley, 1981).

In 1985, Neil Gibson and Len Erickson found that a new drug related to the chloroethylnitrosoureas (see Chapter 2) produced inter-strand crosslinks and that the ability of cells to survive the treatment was related to their ability to repair or prevent the formation of those crosslinks (Gibson et al., 1985). Then, in 1987, we found that brain cancer cell lines

grouped according to whether or not they were able to prevent DNA crosslink formation by chloroethylnitrosoureas and that this was related to the ability of the cells to survive (Sariban et al., 1987). As explained in Chapter 2, the ability of the cells to survive these treatments was dependent on whether or not the cells produced an enzyme (MGMT) that quickly removed the chloroethyl groups from the DNA before they could go on to form inter-strand crosslinks.

The next chapter will relate what was for us the most exciting finding: the discovery that a DNA topoisomerase enzyme was the target of action of several anti-cancer drugs.

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