

## THE CHALLENGE AHEAD

Regardless of our early confidence in **phenoxodiol**, I don't believe that any of us in those early days, least of all myself, under-estimated the huge mountain that we were about to have to climb. We were all only too well aware of the appallingly high failure rate that experimental anti-cancer drugs faced in eventually making it onto the market. The small number of successful anti-cancer drugs on the market today in light of the effort that has gone into anti-cancer drug development over the past 50 years puts into perspective just how much the odds are stacked against successfully making it through the process.

In very broad terms, the figures usually quoted are that for every new drug that looks promising in terms of its ability to kill cancer cells in the test-tube

- ✚ only 1 in 1000 will make it through all the preliminary laboratory and animal testing and get to be tested in humans
- ✚ of those drugs that get to be tested in humans, less than 1 in 100 eventually will be approved for use in humans
- ✚ the time it takes to bring a new anti-cancer drug from the concept stage to being approved for marketing is between 10-12 years.

In other words, the statistics facing us were that the chances of **phenoxodiol** making it through all the testing and getting approval for being used in cancer patients were about 1 in 10,000....not a statistic to inspire confidence. Another sobering statistic was that the average cost of bringing a new anti-cancer drug to market was in the order of \$350 million. Perhaps more sobering still was the thought that there was no guarantee that the drug would ever get anywhere near the stage of being approved, but that reaching that point of abandonment could still involve the expenditure of anything between \$25 million and \$100 million of investors' money. These are not statistics that anyone needs to dwell on to any extent when starting out lest they dissuade you from even starting. Patience, perseverance, blind faith, risk-taking and very deep pockets are essential characteristics of scientists and investors alike in this business.

If there is any re-assurance about starting out on the development of an anti-cancer drug it is that it is a well-worn path where the mistakes and experiences of many before have led to the creation of well-established procedures. These procedures have been codified into guidelines

issued by the principal regulatory authorities such as the United States Federal Drug Administration (FDA). These guidelines spell out key questions, mainly about safety and efficacy that must be asked about any new anti-cancer drug before it can be considered for approval as a therapeutic. That is, you know up front what you have to do to get a drug to market. Importantly from a practical point of view, these questions are posed in a step-wise fashion with each step essentially having a ‘STOP-GO’ outcome. ‘STOP’ means that you need to go back to the drawing-board to try and correct the problem, or if insurmountable, that you abandon the project and thereby save yourself any further unnecessary cost or effort. ‘GO’ means that you are free to go to the next level, reasonably secure in the knowledge that you have put one more key foundation stone in place.

Drug development is conducted in two distinct parts. The first part, the so-called *pre-clinical* phase, involves testing the new drug in the test-tube and in animals. This phase addresses some fundamental questions about safety and the appropriate target cancers before taking the major step of using the drug in humans, the so-called *clinical* phase.

Before you get to test out a new, experimental anticancer drug in humans, there are two important questions to be answered – first, does it work in animals, and second, how safe is it in animals? These questions are non-negotiable because no drug regulator, hospital administrator, or doctor is going to allow anything to be put into a patient on a trial basis, even if that patient has a life-threatening condition with no other therapeutic options, without having some confidence that the drug is likely to work, and some understanding of just how safe or unsafe it is likely to be. No matter how sophisticated laboratory testing gets, there really is no alternative to answering these two critical questions in animals. The studies involved in answering these two questions constitute the bulk of the pre-clinical testing phase.

The easier scenario in the pre-clinical setting is having a drug that is highly targeted. Usually this has come about as a result of rational drug design – drugs such as **herceptin** and **avastin** – where you know from the outset what the clinical target is, where you generally also know how the drug is working, and where existing therapies have set benchmark outcomes in both the test-tube and animal models against which the new drug can be measured. Under these circumstances, the pre-clinical testing program can be highly focused in terms of the key objectives.

The harder scenario involves drugs such as **taxol** and **phenoxodiol** that have no readily identified clinical target because the drug is working across a range of clinical options. Plus, the mechanism of action is usually unknown to start with and this may take quite a few years to work out, clouding any decision on whether the new drug is going to be more appropriate as a monotherapy or in combination therapy, and, if the latter, then what type of other drugs is it more likely to work best with. All of this simply means that the pre-clinical phase generally goes on for several years longer compared to a drug produced by rational drug design before the program becomes focused on a particular clinical target or set of targets.

If there is any solace in having a drug fitting into the harder scenario, it is that rationally-designed drugs have proved to be far less effective than drugs discovered by accident such as **taxol**, **cisplatin** and **methotrexate**. There is nothing particularly surprising about this...cancer is almost certain to be such a multi-headed Medusa-like beast involving a

multitude of underlying survival mechanisms, that a blunt instrument capable of delivering a crude but fatal injury to the heart of the beast (namely, the nucleus), has to be more effective than a precise strike against just one of the many heads. That harsh reality is reflected in the fact that drugs such as **herceptin** and **avastin**, for all their sophistication and smart computerised design, do little more than provide short-term benefit to sub-sets of patients with particular forms of cancer. Blunt-acting drugs such as the platinum and the taxanes and alkylating agents still are the heavy-lifters of cancer chemotherapy.

So let's assume that the test-tube studies have reached a point where you are satisfied that you have a drug that is killing cancer cells to an extent at least, if not greater, than existing drugs, and that its toxicity against cancer cells is sufficiently greater than that against non-cancer cells to give you confidence to proceed. The next step is to see if the drug can still kill cancer cells or at least slow their growth in a setting more akin to the human body than a test-tube. The normal way of testing this is to use a strain of mouse that will tolerate the growth of a human cancer. The mice in question are special mice – they have been bred to have a defective immune system as a result of lacking a thymus gland (so-called *athymic* mice). They are like the so-called 'bubble' children who are born with a defective immune system and who need to spend their lives in isolation in a plastic bubble because their immune systems lack the ability to fight infections. Everyday organisms that surround us that you and I have learnt to live with, almost certainly would prove to be fatal in these children. The particular strain of mice used in this process, like the 'bubble' children, need to be kept under sterile conditions in order to survive. Just as the immune system of these mice cannot fight foreign organisms such as bacteria or viruses, so it is unable to fight foreign tissue such as human cancer cells. When human cancer cells are injected into the body of these mice, they grow and flourish just as though they were in a human body. Most forms of human cancer can be grown in these mice, making them an ideal model to test a new anticancer drug.



**An athymic mouse bearing a tumor stemming from human prostate cancer cells injected under the skin on the shoulder.**

Human cancer cells injected under the skin of these mice usually will grow over a period of 3-4 weeks into solid tumours that ultimately would kill the mouse in the same way that it would the human patient from which it came. This model provides as close to the real thing that it is possible to get in order to test a new drug's anti-cancer ability. Typically the mice are treated daily with the test drug for about 7-10 days, and the effect of treatment on the rate of growth of the cancer determined by directly measuring the size of the tumour mass as it appears under the skin. There are no hard and fast rules with this model, but in general terms new test drugs are only considered to be worthy of taking further if they reduce the rate of

growth of the human cancer in the mouse by at least 60% compared to mice where no drug treatment is given. Any new drug delivering at least a 60% reduction in tumour growth in this model generally is considered as an important and highly promising new anti-cancer agent. That is not to say that any drug delivering, say, a 40% reduction in tumour growth is unlikely to make it any further, but experience has shown that the odds of success in humans where that is the case lengthen considerably.

It is still a huge leap for a drug to go from providing an anticancer effect in mice, to working in humans, and the fate of most experimental anticancer drugs is that never successfully bridge that gap. Cupboards in medical research laboratories and pharmaceutical companies around the world contain hundreds, if not thousands, of long-forgotten experimental drugs that have successfully stopped the growth of human cancers in mice. And the reason they are long-forgotten is that they didn't deliver any meaningful anticancer effect when they finally got to be tested in humans.

There are a couple of good reasons for this species gap. One is a simple practical issue that has to do with the drug gaining access to the cancer cell. The drug is never going to get better access to the individual cancer cell as it will in the test-tube, where the drug is presented with a single layer of cancer cells to which it has unlimited access for as long as it takes to work. The same cancer cells grown in a mouse present a significant step-up for the test drug, but a relatively small mass of a single tumour in a mouse is a considerable way from the human situation where the drug usually is presented with multiple, large, often impenetrable masses of cancer cells.

The main reason for the gap, however, has to do with the purity of the cancer. Human cancers are heterogenous populations. That is, within the one cancer, there are multiple strains of the same cancer...they all look the same, and are all closely related, but they do display different behavioural characteristics such as sensitivity to different anticancer drugs. When a cancer cell line is established and perpetuated in tissue culture, that derived cell line comes from a single sub-population of cancer cells. When that cell line is injected into a mouse, the ensuing tumour contains a single population of cancer cells.

We don't know for certain what creates this heterogeneity within single human cancers, although a likely explanation is that it is the result of the same original insult (eg. a chemical or a virus) coincidentally causing different cells to become cancerous in different ways. Cancer is rarely the result of a single insult or event. Generally it happens over years and represents a progressive accumulation of damage by the initiating cause such as a chemical, virus, radiation etc. It is difficult to believe that exposure of any tissue containing billions of cells to such continuous insult over years is only going to result in cancer arising in a single cell. It is far more likely that it will arise coincidentally in a number of cells. And while it is true that all in an individual body are genetically identical, cancer cells are the exception to the rule.

Cancer arises as the result of damage to a cell's DNA, and when you consider the complexity of DNA, it is not too difficult to imagine that different cells are going to suffer different types of DNA damage, resulting in discrete populations of cancer cells within the one cancer. The different cancer cells that go to make up the overall cancer are all of the same tissue type and all will look the same – eg, they are all prostate cells or breast cells – but they can be different in subtle ways. One important way is their capacity to respond differently to drug treatment. For example, within the same cancer, some of the cells may respond very well to a particular

drug or to radiotherapy, while others less so, and others not at all. This variation in sensitivity to therapy appears to be tied up to the type of DNA damage that the original cells incurred.

This heterogeneity is why cancers can respond well in humans in the first instance to anticancer therapy and virtually shrink away almost to nothing, only to return months or years later with renewed vigour and often showing far less responsiveness to repeat therapy. All that has happened is that at the time of therapy the cancer was populated mainly with cells that happened to be sensitive to that particular therapy. The death of those sensitive cells led to the cancer shrinking dramatically, but leaving a residual tumour composed of some minority cells that survived because there were insensitive to the therapy. With the dominant cells gone, the remaining cells are now free to grow unhindered, resulting in a new cancer with a quite different drug-response profile.

Getting back to the predictive value of mice, the cancer facing the test drug in the mouse is a single, homogenous population (because all the cancer cells were derived from single cell line) that will show a uniform response to the drug, with that response ranging from no response to a highly sensitive response. That is in contrast to the challenge facing the drug in humans, where there will be a mixture of populations, some unresponsive and some highly responsive.

Given these shortcomings, it might seem strange to put so much faith in animal models such as the athymic mouse, but the reality is that no-one has come up with a system that is a more reliable predictor of efficacy in humans. Most new drugs that show some efficacy in mice don't pass the grade when eventually tested in humans, and conversely, there are sure to be some test drugs confined to the trash bin because they failed to work in mice but which may have delivered the goods if they had ever got to be tested in humans. But we work with what we have, and for the moment, the athymic mouse remains the best model.

Before embarking on the crucial make-or-break step of seeing if the drug will show anticancer activity in the athymic mouse, you want to be confident that the test drug is reaching the cancer at levels that will be likely to work. The test-tube studies will have provided some idea of the level of drug required to kill all cancer cells, but that is under the most ideal conditions available, and so for the considerably greater challenges facing the drug in the body, the general rule of thumb is that you will need to aim for about 10-times that level in the blood. With that target in mind, preliminary dosing studies are conducted in mice without tumours to see if achieving those sorts of blood levels is practical. These studies are referred to as *pharmacokinetic studies*, meaning that you are following the level of the drug inside the body over time.

The first matter to think about is *how to get the drug into a mouse?* You hope it will be straightforward, and mostly it is. Usually it is no more difficult than dissolving the drug in water or some other suitable solution and injecting it under the skin of the mouse. All you need to do then is to take a blood sample from the mouse 30 minutes or so later and show that the drug is present in the bloodstream. Unfortunately for a small proportion of drugs, it is not as simple as this. Problems can arise when the drug is almost completely insoluble in water or another suitable solvent, making delivery by injection virtually impossible, because if you can't dissolve the drug in water, then it is not going to enter the bloodstream. For these drugs, the oral route is generally a suitable alternative. There is still a fair bit of work required to work out how to get an exact amount of drug down the throat of a mouse, but that is not insurmountable. Again, a blood sample collected about 30 minutes after dosing to

demonstrate the presence of the drug in the bloodstream is all that is needed to confirm this as a suitable method of dosing.

Once you know that you can get the drug into the bloodstream, the next issues are *can you get it there at levels that you think might have a chance of working*, and *will the drug stay there long enough to work?* The first of these issues is simple enough (10x the optimal test-tube dose), and after that, it is a relatively simple matter of working out a suitable dose.

It is one thing to achieve the sort of drug levels in blood that you think might give the drug a chance to work, but the second part of that issue is how long those levels will last. The term used to describe this behaviour is a drug's *half-life*, or the time that it takes for half the drug present in the body to disappear. Some drugs have a half-life measured in minutes, usually the result of the body vigorously attacking the foreign chemical and actively breaking it down. In those cases there would be little point in going on. The usual experience with anti-cancer drugs in the test-tube is that they need to be present for at least several hours in order for them to work their magic, so it is difficult to see any drug with a half-life of an hour or less having any meaningful effect.

The more usual situation with anti-cancer drugs is that they remain intact in the body and are eliminated from the body by excretion in the urine or other body fluids (faeces, perspiration etc). The half-life of these drugs usually is measured in hours, typically ranging from about 4 hours to 24 hours. With drugs such as antibiotics where it is important to maintain fairly constant levels of drug in the bloodstream in order to keep a constant barrage up against ever-dividing bacteria, and where the typical half-life is half-life is 4-6 hours, it is necessary to take them 3-4 times daily. With anti-cancer drugs, it is not quite so important to maintain constant blood levels over a 24-hour period, and in fact with some of the more toxic anti-cancer drugs, a pulsing approach (yielding peaks and troughs) is far more preferable than a constant body level. Generally you just need to ensure that you get reasonably good levels for about 8-12 hours each day. In the case of mice being used for a preliminary test of anti-cancer efficacy, providing that the drug has a half-life of at least 4 hours, then a single, large dose usually is enough to see if it is going to work or not.

Once you have confidence that you can get the drug into the bloodstream at appropriate levels and to keep it there for an adequate time, then the third and final issue to think about is *what cancer type you are going to use*. With drugs such as **herceptin** (breast cancer), **tamoxifen** (breast cancer) and **gleevec** (chronic myeloid leukaemia) that were designed for a specific cancer target, there isn't any choice to be made. But drugs such as **cisplatin** or **methotrexate** or **phenoxodiol** discovered without any particular cancer type in mind can present something of a dilemma. These sorts of anti-cancer drugs are so broadly acting across a wide range of cancer types that you inevitably fall back on a degree of educated guessing at this early stage. The point of the initial animal studies is that it is nothing more than a signal as to whether or not the test drug has anti-cancer activity in a far more complicated environment than a test-tube. The decision about the ultimate cancer target to pursue in humans is a long way off, probably several years. All you want to know at this point is that the drug can kill cancer cells, any kind of cancer cells, in the whole body.

A harsh reality is that having a drug that is killing, say, 18 out of 20 different types of cancer cells in the test-tube, doesn't necessarily mean that it will be just as effective against the same broad range of cancer cells when those cells are growing in mice. So this is where the educated guessing comes in. It is usual to choose a small number (typically 3 or 4) of

different cancers to start with, eg. a prostate, a lung and an ovarian cancer, just to see if there is any anti-cancer effect in mice. The educated guessing lies in assuming those types of cancer that you think might be most responsive, based partly on the test-tube results and partly on how you think that the drug might be working. It is entirely possible that you might get it horribly wrong and pick 3 cancers that are completely insensitive to the drug in mice, and overlook the one cancer that might be exquisitely sensitive. That is unlikely where a drug shows broad-ranging activity in the test-tube, but it still is a risk that you run.

Assuming that the preliminary mouse studies are successful, and that you achieve at least a 50-60% reduction in the growth of at least one cancer, and assuming that the mice have survived the treatment, then you can allow yourself the luxury of believing that you have a promising anti-cancer drug. You now have the justification to take the project to the next stage, which is to conduct more extensive animal testing, the primary reason being to establish the drug's toxicity profile.

Toxicity is the next potential major road-block facing the drug. The mice have survived the challenge of being treated with the new drug to the point where they survived long enough to show that it slowed down cancer growth, but that hardly qualifies it as being an 'acceptably safe' drug. The initial animal studies designed to test the drug's anti-cancer properties, usually involves treating the mice for only about 7 – 10 days. With longer courses of treatment, such as those likely to be used in humans, significant safety issues might well emerge. The purpose of the next step is to characterise the safety profile of the drug as much as possible in animals, as an essential step in being allowed to proceed into humans. This so-called *animal toxicity* program will take approximately 2 years, and if done in full, cost several million dollars.

There is an expectation that all anti-cancer agents will be toxic. The drug has been designed to kill human cells, and anything with that capacity is likely to have some unpleasant side-effects. It is just a matter of degree ... where a drug is killing both cancer and normal cells in equal measure, or where the difference is reasonably small, then the drug is not likely to go any further in its development. The challenge is to find a drug that delivers a meaningful anti-cancer effect without life-threatening consequences.

A drug's toxicity traditionally is determined in the first instance in animals such as mice. Instead of using nude mice as before, this process uses normal, healthy mice. The drug is injected into the mice, usually daily for about 6 weeks, in order to characterise the nature of the toxicity and the amount of drug that will prove to be fatal. Providing that that amount is greater than what is needed to kill cancer cells both in the test-tube and in the nude mice, then the outcome is a 'GO'. If not, the drug is unlikely to proceed any further.

Before any experimental drug is allowed to progress into human testing, most health authorities around the world insist on a complete safety profile of the drug in 3 animal species. This is a comprehensive and exhaustive process that requires giving the drug on a daily basis for up to several months to animals, recording any changes in their behaviour and health over that time, and conducting extensive post-mortems at the end of the study to determine what side-effects the drug is having on the body. The time and cost of this are so considerable that it is only done for those drugs where the decision has been made to go into human studies. The first animal species to be tested usually is the rat. The second species usually is the rabbit. The third usually is the dog, although sometimes the pig or monkey.

The purpose of this entire animal testing is to give us an idea of the sort of toxicity that we will need to be aware of when the drug finally is given to humans, as well as guidance to the sort of dosage that we need to think of starting with in humans. All animal species have sufficiently different constitutions that we can expect them to react differently to different drugs. A drug that proves toxic in a rat, doesn't necessarily have to prove toxic in a rabbit or dog or even in a human. Equally a drug that proves to be well tolerated by rats, rabbits or dogs, is no guarantee of its safety in humans. As imperfect as this system is, it is the best we have and is a prudent preliminary step to taking the drug into humans.

The pre-clinical phase, running from the time a promising new drug is identified in the laboratory, through to the conclusion of the animal toxicity studies, typically takes 4-5 years. By the time this is over, it is expected that we would have a pretty good idea about:

- ❖ the type (or types) of cancer that we are likely to want to target in humans;
- ❖ the types of toxicity that we might expect to encounter in humans;
- ❖ the dosage that we might be aiming to use in humans;
- ❖ how we are going to administer it to humans (eg. by injection or orally; given once or three times a day);
- ❖ how we are going to use it as an anti-cancer agent (eg. on its own or in combination with other anti-cancer drugs).

There are a few other pre-clinical questions still to be addressed, not the least being how the drug works, but answering that question is not a precondition to moving the drug into the clinical phase of testing. Plenty of drugs, anti-cancer drugs and others, have only had their mechanism of action fully understood after being on the market for some years and after having been used in some tens of thousands of patients.

The clinical stage of testing marks an exciting time for any team developing a new drug. No matter what level of confidence that you might have in the new drug, and what level of understanding that you think you have about how to use the drug, the move into humans represents a huge leap into the unknown, carrying with it a blend of fears, hopes, uncertainties and self-doubt.

Clinical testing is conducted in four distinct steps, designated Phases 1, 2, 3 and 4. There are no hard-and-fast rules governing these phases, and they tend to blur around the edges and overlap to a certain degree, so the following is a general description of these four steps.

**Phase 1** This first step is mainly about

- understanding how the new drug behaves in the body and how the body responds to it
- gaining a basic understanding of its safety and being confident that toxicity is not an insurmountable problem
- learning how to use the drug and determining an appropriate dose rate
- generally familiarising the investigators with its use.

Usually Phase 1 is sub-divided into two separate steps – *Phase 1a* and *Phase 1b*.

A Phase 1a study, being the first tentative step into humans, consequently is conducted in a very cautious and conservative manner. Its main purpose is to look at the pharmacokinetic profile of the new drug. The mouse pharmacokinetic studies will have provided some guidance to what to expect, and it would be unusual for the two species to be radically different, but nevertheless it is vital to be sure that it will be possible to get the drug into the

bloodstream in potentially meaningful amounts, and to understand how quickly the body is eliminating the drug and how it is eliminating it.

The amount of drug given at this stage normally is well below the level suspected to have any toxicity based on the animal studies. For example, it is common to give the first-time use of the drug in humans at a dosage one-tenth (on a body weight basis) of the dose deemed in animals to be non-toxic. That normally is a remarkably small dose, usually given over no more than 1 or 2 days. For that reason, a Phase 1a study often is conducted in healthy volunteers with no more than about 3-6 individuals being used.

The Phase 1b study represents a much bolder step. The main purpose of this step is to define the safety profile of the drug, with the drug being given exactly as it would be if it was being used to treat cancer. Based on the overwhelming experience with anti-cancer drugs that they come with substantial safety issues, the main function of the Phase 1b step traditionally has been to determine the highest dose of the drug that can be administered before the drug becomes unsafe to use. This means starting with a low dose of the drug, and progressively increasing the dose until a safety ceiling is reached. The dose just below that ceiling is known as the *maximum tolerable dose* (or MTD) and generally becomes the dose to be used in all subsequent testing. It doesn't mean that the MTD is without side-effects – it simply means that the side-effects are manageable and considered sufficiently tolerable to not be life-threatening.

Phase 1b studies are conducted in cancer patients. Where a drug is being developed for a very specific type of cancer (eg. **gleevec** and chronic myeloid leukaemia, or **herceptin** and breast cancer), the patients used in the study normally have the target cancer. However, for most other new drugs that show broad anti-cancer activity, it is usual for Phase 1b studies to be conducted in patients with any type of cancer. Phase 1b studies typically involve about 30-40 patients, and involve the range of cancers normally seen in any busy oncology ward. The patients who volunteer for Phase 1b studies typically have terminal cancer and have failed all forms of conventional therapy. For them, there is little to lose by participating in a trial of a new drug, even though they are made well aware that there probably will be no direct benefit for them. It is a mark of the unselfish altruism of such patients that they are happy to contribute to the possibility of a new drug being developed that leads them to subject themselves to the unknown. Of course there is always the possibility that the test drug may deliver a marked anti-cancer effect in some Phase 1b patients, but the odds are stacked against that for a couple of reasons. One is that the main purpose of a Phase 1b study is to determine the maximum tolerable dose of the new drug, which means starting very low and gradually working the dosage higher. That means that most of the Phase 1b study will involve dosages likely to be too small to be effective.

The other reason is that most Phase 1b study patients have advanced, well-established and aggressive cancers that have failed all standard forms of chemotherapy, and to expect a new drug to have any meaningful effect in such a situation is unrealistic, more so when you have yet to understand how best to use it.

It is uncommon for drugs to fail at the Phase 1 step. Unexpected super-quick elimination of the drug from the body or unexpected severe toxicity would be two reasons for failure at this stage, but it would be highly unusual to encounter either problem after an extensive animal testing program.

At the conclusion of Phase 1 studies, you should have a good understanding of

- the best way to administer the drug (oral or intravenous)
- the frequency of dosing (eg. once a day, or every 4 hours, or continuously)
- to what extent the drug is broken down by the body
- how the drug is excreted from the body and how quickly that happens
- the main side-effects to expect
- the maximum tolerable dose.

Armed with this basic information, and satisfied that the drug is not too toxic to use, you then are able to proceed into Phase 2.

**Phase 2** The main objectives in this step are

- to answer the key question ..... *does the drug provide any meaningful anti-cancer effect?*
- to decide on the clinical target (type of cancer, stage of disease etc)
- to learn more about the drug's side-effect profile and whether there are any adverse interactions with other commonly-used drugs
- to answer as many questions as possible about the design of the all-important Phase 3 study.

The harsh reality is that the fate awaiting most experimental anti-cancer drugs that reach Phase 2 is failure. Mostly because they are ineffective, or they don't provide enough additional benefit over existing therapies to justify going any further, or the exposure of more people to higher doses of the drug reveals an unacceptably high level of life-threatening side-effects not encountered in Phase 1.

This means using the drug in a way that is intended to treat the cancer, and to use it in patients with the specific form of cancer for which it is intended that the drug will receive marketing approval. The number of patients required for a Phase 2 study varies enormously depending on the type of cancer and the degree of benefit expected. For example, in the case of **gleevec**, a phase 2 study was conducted in only 37 patients with chronic myeloid leukaemia, of who 34 went into remission as a result of treatment. With other drugs where the benefit to patients with breast or lung or prostate cancer might mean an extension of survival of 6-8 weeks over an 18-month period, Phase 2 studies might mean having to use hundreds of patients in order to detect that small benefit.

One of the hardest challenges for a drug developer can be deciding on the clinical target. If, like **gleevec**, the drug has been designed with a particular cancer in mind, then that is one challenge you don't have to face. Or if you have a drug that in pre-clinical studies proves to be head and shoulders better against a particular type of cancer, then that drug preselects its clinical target. However, for drugs like **taxol**, **cisplatin**, **methotrexate** and **phenoxodiol**, the represent the great bulk of anti-cancer drugs either developed or being developed, they are active across a wide range of cancers with no single cancer being a stand-out, and this is where the challenge arises.

Commercial imperatives (eg. size of potential market, number of competitive products) and regulatory issues (eg. ease of gaining marketing approval) come into play in any decision-making process as much as scientific factors. And even when you select a particular type of cancer, you then have to decide whether you propose to use it as a first-line therapy or as a

last-line form of salvage therapy, or whether it will be used on its own or in combination with other drugs, or what the nature is of any anti-cancer benefit that you are expecting to achieve (eg. tumour shrinkage or something less dramatic such as delaying progression of the disease). Once on the market and freely available, doctors are entitled to use an anti-cancer drug *off-label*, meaning that it can be used in any way that the doctor believes will benefit the patient. But in order to get the drug on to the market in the first place, it needs to be approved for use in a particular cancer in a very specific way. That approval will rest on the outcome of a Phase 3 study, but back here in Phase 2, some hard decisions have to be made that will guide the design of any future Phase 3 study.

It is not uncommon for drugs to undergo a number of different Phase 2 studies as part of the process of coming to a decision on the preferred clinical target. And the Phase 2 step can be the longest and most expensive part of the whole development path for that reason. This is all part of fine-tuning an understanding of, if not how best to use the drug, then how best to achieve marketing approval.

Having bitten the bullet and made a decision on the likely clinical target (in the full knowledge that that decision might change 2-3 times over the course of the next couple of years), an initial Phase 2 study (called a *Phase 2a* study) is started, usually with a single major objective .... to see if there is any clinical benefit with the new drug. Usually *benefit* in this case is measured empirically, meaning that clinicians rely on their clinical experience with such patients to determine whether or not there has been any tumour response. Often a spread of two or three different dose rates is employed, with a dose-response effect (increasing benefit from increasing dosage) providing even further evidence of efficacy.

Safety is another key observation at this stage, with a significantly larger number of patients (50-200) providing the first opportunity to determine the drug's side-effect profile when being used at its maximum dose rate. Drug interactions also are an important observation, checking to see whether the new drug blocks or accentuates the effects (both positive and negative) of other drugs (such as painkillers, antibiotics etc) commonly used in patients with advanced disease.

If a Phase 2a study is about getting an indication of the presence of an anti-cancer effect, a Phase 2b study is about quantifying that effect and being certain of that positive effect through a statistical comparison against a control arm of patients receiving either no treatment or standard treatment.

**Phase 3** This phase has a single key objective

- to generate the data to support an application to regulators for marketing approval.

The design of the Phase 3 study represents the sum total of the knowledge and experience of the drug gained to date. In reality for most anti-cancer drugs, that knowledge is remarkably elementary. The great bulk of our understanding of the most appropriate way to use any anti-cancer drug comes later, once it is approved and freely available on the market and following the collective experience from its use in thousands of patients across a wide range of clinical conditions.

Again, the number of patients involved in Phase 3 studies varies enormously depending on the degree of benefit expected. About 400 patients would be close to a minimum number,

with trials involving 5,000 patients not uncommon. As a general rule, the smaller the clinical benefit, the greater number of patients is required.

Phase 3 studies normally are conducted in a controlled, double-blind manner, thereby removing any element of bias from the outcome. This usually means that the new drug needs to be compared to standard therapy, with neither patients nor medical staff knowing which therapy individual patients are receiving. This requires extraordinary lengths to ensure that everyone remains 'blinded' to the outcome.

Phase 3 studies usually are designed in collaboration with the regulatory authorities. Given that the same authorities are going to be reviewing the Phase 3 data and making a decision to approve the drug, their input into the way the study is designed and conducted is essential. Agreement normally is obtained beforehand on the numbers of patients to be involved in the study, as well as the minimum clinical benefit that will need to be obtained.

At the conclusion of the Phase 3, all of the clinical data (and for several thousands of patients this typically is measured by the semi-trailer load) is collated and sent for review by a body such as the FDA. The crucial factors for consideration by a body such as the FDA are that the new drug be delivering a clinical benefit that is meaningful and sufficiently superior to current therapies to warrant being allowed on the market, and that any anti-cancer benefit be gained without significant side-effects.

Each country has its own regulatory approval process, although the basis requirements in relation to Phase 3 trial design and the interpretation of the data are essentially common to all national jurisdictions. Most drug developers elect to run their Phase 3 study under the auspices of the US Food and Drug Administration (FDA), in part because the US represents the largest single market for an anti-cancer drug, and in part because the FDA generally is recognised as the gold standard in terms of rigour, and FDA approval, while not an automatic guarantee of approval in other jurisdictions, remains an important reference point for other countries.

Phase 3 studies typically are conducted as multi-national studies. In part this is driven by the need to open as many recruitment sites as possible in order to achieve rapid enrolment of patients. But also it can be part of a deliberate strategic step to ensure the involvement of patients across a number of major jurisdictions.

Approval, as we have noted earlier, is for a particular application in a particular group of patients. That is the indication that must appear in all literature relating to how to use the drug. Companies cannot make claims for its use in any other circumstance, and insurance companies are entitled to limit reimbursement to that particular application. Despite this, doctors are free to prescribe and use the drug in any way that they see fit, including its use in non-cancer patients.

**Phase 4** The main objective of this phase is  
■ to extend knowledge of the drug's safety profile.

This is a post-marketing phase. It represents the collection of data from health professionals on an ongoing basis for as long as the drug remains on the market. The use of the drug in many tens of thousands of patients, across a wide range of circumstances and in uncontrolled

conditions, provides an opportunity to observe the drug's behaviour in ways that were not possible to reproduce in clinical testing. This is a regulatory requirement ... the collection of safety issues and drug interaction issues that over time allow regulators and doctors to build up a comprehensive safety profile of the drug.

## GETTING TO KNOW PHENOXODIOL

The horizon for any drug developer seems a long way off at the start of the journey..... somewhere between 10 and 12 years in fact. And that's if everything goes smoothly. Anti-cancer drugs can take slightly less time to develop than other drugs because the life-threatening nature of the disease and the absence of treatment options for many cancers mean that they are allowed to take some short-cuts. Some anti-cancer drugs have gone from bench top to market in a matter of 7-8 years, but that is highly unusual. Most anti-cancer drugs take the more usual longer time of about 10 years, and that has proved to be the case for **phenoxodiol**.

**Phenoxodiol** turned out to have a very significant advantage over virtually every other anti-cancer drug in that it is without any detectable toxicity. That feature alone should have shortened its development program considerably, but perversely that very benefit also became something of a burden. Its highly selective toxicity meant that it had a unique mechanism of action, and that uniqueness made making a decision about how best to use the drug particularly challenging.

Our knowledge of **phenoxodiol** in its first year of life was limited to three factors – first, that it was broadly effective (in the test-tube) against a wide range of human cancer cells; second, that it was killing those cancer cells by the process of *apoptosis*; and third, that it was highly selective against cancer cells. As sketchy as this was, it at least represented the three key factors that most people would consider fundamental to a successful anti-cancer drug.

The fact that **phenoxodiol** was able to kill a wide range of different types of cancer cells in the test-tube was highly unusual. At that time there was no such thing as a universally effective anti-cancer drug that would work effectively across most, let alone all, forms of cancer. Even the most potent anti-cancer drugs are enormously variable in their activity against different types of cancer. **Cisplatin**, for example, is highly active in the test-tube against ovarian cancer cells, and for that reason was the drug of choice to treat ovarian cancer for many years. In contrast, its activity in the test-tube against melanoma cells or bowel cancer cells is so weak that it is rarely if ever used in such patients. It takes about 1000-times more **cisplatin** to kill melanoma cells in the test-tube than it does to kill the same number of ovarian cancer cells. Translating that effect into a patient with cancer means that to have any effect against melanoma, the dose of **cisplatin** that would need to be given to have any meaningful anti-cancer effect would be fatal to the patient. For this reason, oncologists have

learnt by experience that only certain anticancer drugs are worth using for each type of cancer. They also know that for some cancers such as melanoma and cancer of the brain or gall-bladder, standard chemotherapy is virtually ineffective because the cancers are so insensitive to the amount of drug that can be used safely.

This wasn't the case with **phenoxodiol**. The drug showed activity against a broad range of different cancer types, and more significantly was active against these different cancers within a fairly narrow dose range. Whereas a drug like **cisplatin** showed a 1000-fold spread of activity across a range of cancer types, the spread with **phenoxodiol** was only about 10-fold.

Our preliminary test-tube studies had used a panel of different types of human cancers including ovarian, prostate, breast, pancreatic, leukaemia, lung, colorectal, mesothelioma, glioma, cervical and rhabdomyosarcoma cancer cells. Those cell lines had been selected on the basis that they represented a cross-section of human cancers from widely different tissues and with widely different causes. Breast, prostate, ovary and colorectal cancers were 4 of the 5 so-called 'Western' cancers, which is where this story had its beginnings, and which are thought to be associated with lifestyle risk factors. Lung cancer and mesothelioma were selected because they are associated with carcinogenic chemicals (nicotine and asbestos). Cervical cancer is known to have a viral (papillomavirus) association. In the case of leukaemia (blood), pancreatic (pancreas), glioma (brain) and rhabdomyosarcoma (muscle), the cause of the cancers are largely unknown, which in itself was a relevant reason to include them because it brought into play the possibility of causes unrelated to lifestyle factors, chemicals or viruses.

While this panel of cancer cell lines was by no means a comprehensive representation of the full range of human cancers, it was sufficiently diverse both in terms of the types of tissues involved and the causes (diet, viruses, chemicals) of the cancers to suggest that **phenoxodiol** was a likely to be a relatively comprehensive anti-cancer drug irrespective of where the cancer arose or what caused it. Subsequent test-tube studies conducted in the years ahead confirmed this thought. **Phenoxodiol** ultimately proved to be active against all forms of human cancer against which it was tested in the test-tube, including cancers such as melanoma and cholangiocarcinoma (gall-bladder cancer) which as we noted earlier are notoriously resistant to standard anti-cancer drugs. What we didn't understand at the time was why **phenoxodiol** was able to kill all forms of cancer when most other anticancer drugs couldn't. That understanding took another 5 years and is an intriguing aspect of the story that we will look at shortly.

The second important piece of information that we had at the start of the **phenoxodiol** programme was that the drug was killing cancer cells by the process of *apoptosis*. When **phenoxodiol** was added to cancer cells in the test-tube, the first thing noticed (within 20 minutes) was an inability of newly divided cells to enlarge. When a cancer cell divides, the two daughter cells are about one-third the size of the parent cell, and take a few hours to reach full size. In the presence of **phenoxodiol**, newly produced daughter cells failed to enlarge. At this early stage, all cancer cells also displayed signs of undergoing respiratory distress. This was followed within 12 hours by cells being unable to divide, and within 24-48 hours by those same cells then undergoing apoptosis.

The apoptotic effect was relevant because this is the way that almost all anticancer drugs as well as radiotherapy kill cancer cells. Apoptosis is the process by which all of our cells,

cancer as well as healthy, die. It is a natural process that goes on in our bodies every second of our lives. It is the way that all of our cells die in due course and is a remarkable design of Nature to ensure that all of our cells are replaced on a regular basis. The main reason for wanting this regular turn-over of cells is because of the normal background level of potential DNA damage that our cells are exposed to from the time of birth. Viruses, chemicals, and simple wear-and-tear are just part of living, all capable of inflicting damage to the genetic apparatus of our cells. If our cells were not replaced regularly, the steady accumulation of that damage over a lifetime would almost certainly ensure that we would suffer far greater risk of getting cancer than we already do. And this is where the cleverness of Nature (or intelligent design if you prefer). Imagine having to design a self-destructing system where you want a cell to die after, say 3 months as in the case of a white blood cell, because you were worried about it suffering potentially serious damage to its DNA in that time. The problem then becomes, how do you rely on a self-destruction mechanism working when the damage to the cell might result in the self-destruction mechanism itself being disabled, leading to the cell going on to live well past its use-by date? Such an outcome would almost guarantee the steady accumulation of potentially cancer-forming cells in the body. So the system that has evolved is one where the self-destruct mechanism will still operate in the face of any fatal damage to the cell, and in fact will be triggered because of such fatal damage. The process of apoptosis is what Nature cleverly came up with to get around this problem.

Apoptosis relies on the fact that our cells have to work continuously from the moment they are created to stop from dying. Life is a long way from the passive process that we instinctively think it to be. Every cell in our body, from the moment of creation until we die, is being told continuously by our body via chemical messengers to die. These so-called *death signals*, if allowed into our cells without restraint, would kill us in a matter of hours. To stop this happening, every cell fights on a continuous basis to resist the death signals. As long as the cell remains healthy and is able to resist, then it is able to stay alive. Should it become damaged, however, it loses the ability to resist the death signals and the cell will die.

Cells self-destruct because of a ‘poison pill’ contained within every cell. This ‘poison pill’ is enzymes known as *caspases* (*cysteine-aspartic proteases*) that are stored in tiny sacs within each cell. These enzymes are highly destructive and literally digest the cell from within if they are released from their sacs. Released caspases will auto-digest a cell within a matter of hours. Releasing the caspases from their protective sacs is what the death signals are trying to achieve. To stay alive, cells need to vigilantly resist these death signals, which they do by putting a protective shield around the caspase sacs. This shield is made up of proteins which the cell needs to make on a continuous basis and which act as decoys, absorbing the death signals and thereby maintaining the integrity of the shield. If the cell becomes damaged to the point of not being able to repair itself, it stops making the shield and the caspases are released. This is the process of apoptosis.

There are a number of fundamental structural features that distinguish a cancer cell from a healthy cell, but one important difference is in the protective shield that surrounds the caspase sacs. Cancer cells make significantly more of the protective shield proteins than normal, healthy cells, meaning that the apoptotic mechanism in cancer cells is essentially turned off because the caspase sacs are surrounded by an impenetrable barrier. The sort of damage that would lead a normal cell to stop resisting the body’s death signals is completely ineffectual in a cancer cell. This ‘resistance’ bar in cancer cells is so high compared to normal cells that they are virtually indestructible. This is why cancer cells are able to resist all attempts by the body to eradicate them, and why cancer cells have the capacity to live forever. Cancer cells

do die, particularly in rapidly growing, large tumors, but that is because the tumour is growing faster than the body can build a blood supply to keep it provided with essential nourishment. In that situation, cancer cells are dying only because they are starving to death. Where they have access to plenty of nutrients, they will live forever.

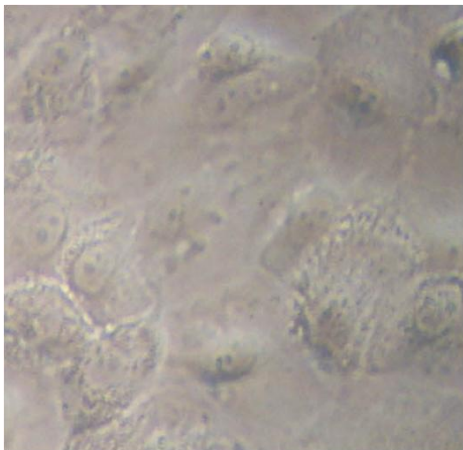
Anti-cancer drugs and radiotherapy are able to kill cancer cells because they inflict such catastrophic damage on the cell that the protective shield surrounding the caspase sacs becomes meaningless. The damage that these agents inflict on the cancer cell's structure, mostly on the DNA, is so severe that the cell is not only incapable of repairing the damage, but is incapable of performing most normal functions, including being able to produce the protective shield that surrounds the caspase sacs. The consequence of this is that without the shield, the body's perpetually-present death signals are able to kill the cell, just in the way that they were intended.

The problem for the rest of the body, however, is that this level of damage is not confined to cancer cells. Healthy cells are just as susceptible to the destructive forces of chemotherapy and radiotherapy, rendering them just as susceptible to apoptosis, perhaps even more so since their level of protective shield surrounding their caspase sacs is nowhere as high as in cancer cells. The fact that **phenoxodiol** was so toxic to all forms of human cancer and was killing cancer cells by reconstituting their self-destruct mechanism in the same way that other potent anticancer drugs and radiotherapy did, begged the obvious question as to whether **phenoxodiol** might have the same blunderbuss, non-specific damaging approach that radiotherapy and drugs such **methotrexate** and **cisplatin** displayed. If this proved to be the case, then there would be every reason to suppose that **phenoxodiol** also would be at risk of inducing serious collateral damage to the body, perhaps even to the point of being too toxic to use. So, the first question that needed to be answered was how toxic **phenoxodiol** was likely to be? This was the first major 'STOP-GO' point in the development process that we needed to address.

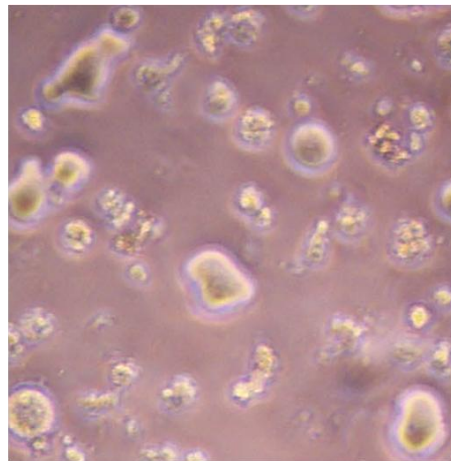
The simplest and quickest way to answer this question was to look at the effect of **phenoxodiol** on non-cancer cells in the test-tube. This was done using the same test-tube test that we had employed in the first place to look at the effect of the drug on cancer cells, only in this case substituting cancer cells with normal animal and human cells. To our relief, **phenoxodiol** proved to have almost no adverse effect on the normal cells. The dose of **phenoxodiol** that killed 100% of cancer cells had to be increased 200 times before it showed even the slightest signs of toxicity (10% of cells dying) in normal cells. Such an outcome was unique in the anticancer therapy field. Every single anticancer drug that kills cancer cells by direct action, as well as radiotherapy, is unable to distinguish between cancer cells and healthy cells. As long as the two types of cells are dividing at the same rate, then drugs such as **methotrexate**, **docetaxol** and **cisplatin** will have exactly the same level of toxicity against both kinds of cells. The only thing that makes such drugs less likely to damage healthy cells is where they are dividing much slower than cancer cells. As we have noted earlier, the damage inflicted by anticancer agents in general terms is proportional to the rate at which cells are dividing...the faster the rate of turnover, the more damage is inflicted. Cells that are resting or dividing at a very slow rate suffer very little damage from chemotherapies. This is why tissues such as bone marrow and the lining of the gut that have a rapid turnover are susceptible to damaging side-effects of chemotherapy and radiotherapy.

The cells that we used in the study to test the effect of **phenoxodiol** on healthy cells were relatively fast-growing....growing roughly at about the same rate as the cancer cells that we

had used earlier. So we were fairly confident that this meant that **phenoxodiol** was unlikely to be associated with the same sort of bad side-effects normally associated with chemotherapy, such as catastrophically low red and white blood cell levels, gastrointestinal problems, nerve damage and hair loss. Of course, that didn't mean that it wouldn't be capable of causing other sorts of side-effects, but we would only find that out once we gave the drug to animals and humans. Nevertheless, the lack of any toxicity in this simple test-tube test gave us considerable confidence that we were going to avoid the usual sorts of life-threatening side-effects associated with most chemotherapies.



Normal human breast cells



Human breast cancer cells

**Human breast cells and breast cancer cells grown in the presence of phenoxodiol. The normal breast cells on the left show adhering, viable cells, while the cancer cells on the right have undergone apoptosis, have detached from the plastic substrate and are fragmenting.**

Ironically, this good news also brought its unfortunate side. For those of us who had been working with **phenoxodiol** and understood its heritage of isoflavones like **genistein** that can kill cancer cells and yet not be toxic in humans, we were entirely comfortable with the fact that we had found a drug that was so highly selective against cancer cells. The fact that this went completely against all previous experience with anticancer drugs did not diminish our faith in what we were seeing. We had tested this enough times to be sure that what we were seeing was real. Over and over again, we had set up normal cells and cancer cells in adjacent test-tubes and added **phenoxodiol** or standard drugs such as **cisplatin** or **docetaxel** to the cells. Each time, the standard drugs killed both normal and cancer cells, while **phenoxodiol** killed only the cancer cells....the normal cells were completely unaffected by **phenoxodiol**. Later animal and human testing would confirm its lack of toxicity, so much so that by 2009 when it had been used in almost 750 patients, not a single incidence of toxicity or intolerance had been reported that could be laid at the feet of the drug. But that was ahead of us....back in the late 1990s, we were struggling to explain its apparent lack of toxicity.

Convincing ourselves was easy. The challenge was convincing others who had been educated to believe that any drug that was directly toxic to cancer cells inevitably was going to cause some collateral damage to healthy cells. This was scientific and medical dogma. All clinical experience since the introduction of **methotrexate** in the mid-1940s had pointed in this

direction. So for us to come along and talk about a drug that was highly toxic to virtually all kinds of human cancer cells, but without any discernible effect on healthy cells, simply flew in the face of accepted dogma.

One group of people in particular who proved a real challenge was the investment banking fraternity. Small biotech companies such as Novogen have a sometimes satisfying and sometimes fractious symbiotic relationship with investment bankers. Biotech companies have an insatiable appetite for capital to fund a growing research and development program, and investment bankers love biotech companies because of that appetite. But before they feed the biotech company, not surprisingly they need to be convinced of the validity of the 'story'. In our case, the overwhelming response ranged from scepticism to outright disbelief. Most major investment banks employ scientific analysts whose task it is to evaluate 'the story'. In the US and Europe where most biotech investment comes from, it is not unusual to find that these analysts are expert in their field to the extent that many are trained oncologists or cancer researchers in a previous life. These people are not easily persuaded, having developed an above-average cynicism based on years of companies such as Novogen knocking on their doors with the begging bowl in hand, telling stories that ultimately prove to be unfounded. The analysts have the luxury of having a smorgasbord of investment opportunities to recommend to their clients, and so their well-developed cynicism is a natural defence against a plethora of opportunities. It is easier for them to run with a story that they can understand... a story that is in sympathy with their understanding of the world and that they can explain to their clients.

You would think, (well, at least I did), that the discovery of a drug that was toxic to all forms of human cancer, but that had no effect on healthy, non-cancer cells would be tremendously exciting to anyone with a background in cancer research. Even in the face of established dogma that held that any drug capable of killing most forms of cancer cells directly must by its very nature have some unfortunate consequences in healthy tissues, I would have anticipated a spark of interest in a drug that ran counter to accepted rules. At least interest to the point of wanting to fully understand what was going on. But this was not the case. Many analysts simply discounted the opportunity of **phenoxodiol** simply because it seemed 'too good to be true'. A drug that killed all forms of human cancer without affecting healthy cells was as close to being the Holy Grail of the medical world that any drug previously had come, that it ultimately proved too difficult a concept for most analysts to grasp.

As frustrating as that early experience with analysts and cancer researchers in general was, in hindsight it perhaps was not all that surprising given that we could provide no rational explanation for how **phenoxodiol** was working. I think if we had had that understanding back then, it might have been completely different. But without any explanation of why **phenoxodiol** was able to be so selective and so broadly active, it was perhaps asking a bit too much for the story to be embraced so enthusiastically.

It took another 6 years to finally get that explanation...to finally understand exactly how **phenoxodiol** was able to target cancer cells and only cancer cells. It is getting ahead of the story to deal with that fact here, but as uncovering the drug's mechanism of action had little or no impact on its pathway of development, and as it touches so much on the drug's credibility and credentials, it is worth breaking the story at this point to deal with this aspect.

The discovery of **phenoxodiol** had not exactly set the scientific and medical worlds alight with interest. Novogen was far too much of a minnow in the biotech world to be generating

much attention, and our energies were more focused in any case on establishing a technology platform than on public relations. But, nevertheless, some interest had been generated within the investment community, and some of this trickled over into the scientific community. The result was approaches from a number of research centres to send them **phenoxodiol** to slot into their research programs. By and large these approaches had to do with understanding a mechanism of action, and so for selected collaborations, we were happy to dispatch off some drug. In other cases, we sought out research groups that we felt were dealing in areas that might be productive in the search for a mechanism of action. But by and large, these collaborations were shots in the dark. We didn't have a starting hypothesis, so there was little or no overriding strategy that we could pursue.

One of the first feedbacks (University of Illinois at Chicago) from these various collaborations was information that **phenoxodiol** was inhibiting the enzyme, *topoisomerase II*. This nuclear enzyme is involved in the restructuring and reassembling of DNA after a cell divides. Blocking its action means that the cell is unable to divide because it can't reassemble new DNA in the daughter cells, leaving it with little choice but to undergo apoptosis. The anti-cancer drugs, **etoposide** and **doxorubicin**, are *topoisomerase II* poisons. This didn't explain why **phenoxodiol** was only killing cancer cells, and its inhibitory effect on *topoisomerase II* was not particularly strong, but it was better than nothing...we at least had something to point to.

Then came the news (National Institutes of Health, US) that **phenoxodiol** was blocking cancer cells from dividing by moderating a number of key enzymes that regulate the progression of the nucleus through the cell cycle during cell division. This collaboration had come about as the result of an interest by the NIH in an experimental anti-cancer drug known as **flavopiridol**. The NIH had discovered this drug some years before and had licensed it to the pharmaceutical company, Aventis. **Flavopiridol** had shown some early promise in the treatment of renal, prostate, colon and gastric carcinomas and non-Hodgkin's lymphoma, and was attracting some attention within the cancer research community in the late 1990s. What had attracted our attention to this work was a remarkable parallel with **phenoxodiol**. **Flavopiridol** was a derivative of a naturally-occurring plant flavonoid known as **rohitukine**, sourced from an Indian plant that was a traditional medicinal plant. With that plant flavonoid heritage in common, it made good sense to explore what similarities there might be in terms of their mechanisms of action.

The anti-cancer effect of **flavopiridol** had been shown to be based on its ability to inhibit the function of enzymes known as cyclin-dependent kinases, and it as well to take some time to look at these enzymes in some detail.

The various phases of a cell's life cycle are represented in the following diagram.



The G0 phase is the resting phase. Most of the body's cells are in this phase. They are still active, but are resting in the sense that they are not dividing. When a cell divides, it first enters the G1 phase which is a time for preparation for division. The cell begins to enlarge and to produce a duplicate set of RNA in preparation for the act of division. The S phase (short for *DNA synthesis* phase) is a particularly active phase during which the cell makes duplicate copies of its DNA, in preparation for the cell splitting ahead. The G2 phase is a time of fine-tuning ahead of the M phase (or *mitosis* phase) when the cell splits and two daughter cells are produced. The two new cells then enter the resting G0 phase until called on themselves to divide.

The progression of the cell through these various phases is marked by a number of checkpoints where the cell ensures that all is in readiness and that the DNA is error-free for it to progress to the next point. The two key checkpoints are G1/S and G2/M.

These various checkpoints are under the control of enzymes known as *cyclin-dependent kinases (cdks)*. This family of kinase enzymes becomes activated by binding to proteins known as cyclins that are produced by extra-cellular signals. The cyclin-dependent kinases permit the passage of the cell through the various checkpoints.

**Flavopiridol** inhibits a range of cdks, resulting in the cancer cell being blocked at both the G1/S and G2/M checkpoints. **Phenoxodiol** also inhibited the progression of the cancer cell through the cell cycle, but its effect was limited to inhibition of the activity of a single cdk (cdk2) resulting in a block at the G1/S checkpoint only. Unlike **flavopiridol** which down-regulated cdk function directly, the effect of **phenoxodiol** was indirect, down-regulating cdk2 activity by up-regulating levels of the endogenous cdk inhibitor, p21<sup>(WAF1)</sup>.

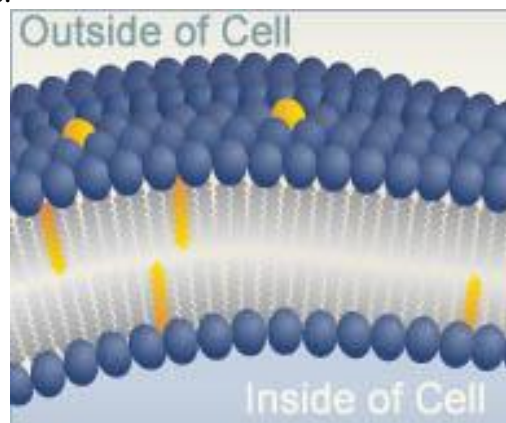
The differences between **flavopiridol** and **phenoxodiol** were relevant because one of the factors holding back general interest in **flavopiridol** was its toxicity. If **phenoxodiol** had shown a similar mechanism of action to **flavopiridol**, then we would have been concerned about its safety in humans (even though all laboratory studies were pointing to a lack of toxicity). **Flavopiridol** was inhibiting a range of cdks and the effect was not limited to cancer cells; **phenoxodiol**, on the other hand, was restricted to inhibiting only cdk2, and only in cancer cells. Toxicity remains an issue for **flavopiridol** to this day, although the drug is undergoing revised interest as a treatment for chronic lymphocytic leukaemia.

The significance of this news was hard to interpret. On the plus side, we had another mechanism of action to point to aside from the topoisomerase 2 story, and we had an explanation for how **phenoxodiol** was arresting cancer cells from dividing by blocking them

at the G1/S checkpoint. But it still didn't explain why it was apparently causing distress to cancer cells within an hour of being exposed to **phenoxodiol**, what was killing the cells eventually, and why it was that only cancer cells were affected. It also was something of a mystery that two entirely unrelated enzyme systems were being inhibited.

The first step on the path to answering some of these key questions came with the news (Flinders University, Australia) that **phenoxodiol** was inhibiting the enzyme, *sphingosine kinase*. [This is such a key part of the story of how **phenoxodiol** works that we will look at it in some detail later, but for now we will keep the story as simple as possible].

The cell membrane of all mammalian cells is a fatty structure (see diagram below) that serves a wide variety of roles.



As the diagram shows, the cell membrane contains what looks like tadpoles (a head + a tail) in two opposed layers. These 'tadpoles' are structures known as sphingomyelin, with the head-to-tail bi-layer forming a hydrophobic membrane.

Traditionally, the cell membrane was just seen as serving a passive role .... acting as a platform for the thousands of different protein receptors awaiting the myriad of regulatory chemical signals reaching the cell, as well as acting as a diffusion membrane for gases and any substance entering and leaving the cell. But in the 1990s, it became increasingly clear that this fatty membrane was far from being a passive structural component, and in fact played a vital, active role in the survival and function of the cell.

A feature of these sphingomyelins is that they are in a constant state of flux, being constantly broken down and reassembled in the following manner:



This flux means that at any one time, the cell membrane contains a mixture of **ceramide**, **sphingosine** and the fully assembled, **sphingomyelin**. **Sphingomyelin** itself appears to be inactive, but **ceramide** and **sphingosine** actively influence a wide range of cellular functions. **Ceramide** is *pro-death* - preventing a cell from growing and dividing and being pro-apoptotic. **Sphingosine** is *pro-life* ... it promotes cell growth and division, promotes cell motility and prevents apoptosis. The balance between **sphingosine** and **ceramide** levels can be seen therefore to be of vital importance to a cell's viability and functionality.

The ratio between **sphingosine** and **ceramide** levels is controlled by a small number of enzymes, the key one being **sphingosine kinase (SK)**. **SK** converts **sphingosine** into **sphingosine-1-phosphate**, and this is the form of **sphingosine** which enters the cell's cytoplasm and exerts its pro-life actions via a range of down-stream signalling pathways. **SK** activity is the single most potent determinant of **sphingosine** and **ceramide** levels in the cell membrane. If **SK** activity falls, **ceramide** levels build up, driving the cell towards death. If **SK** activity increases, **sphingosine-1-phosphate** levels rise, driving the cell towards growth and survival. In cancer cells, **SK** activity is elevated well above normal, and this factor is thought to be a key driving force behind the increased growth and immortality of cancer cells.

**Phenoxodiol** completely inhibited **SK** activity in cancer cells, blocking the formation of the pro-survival factor, **sphingosine-1-phosphate**. In keeping with its cancer specificity, **SK** activity in non-cancer cells was completely unaffected by **phenoxodiol**.

This news was a godsend. It hadn't got us any closer to understanding how and why **phenoxodiol** was selecting cancer cells only, but it went a long way to characterising the drug's mechanism of action. Now we had a *sphingosine kinase inhibitor* that we could talk about, although that turned out to have limited value from an investor relations point of view since no-one else that we were aware of was developing an **SK inhibitor** at the time. But it did explain how **phenoxodiol** was able to kill cancer cells and why it was working across all forms of cancer. **SK** activity was so fundamental to a cell's survival, that to cut it off would be lethal to any cancer cell.

As significant a step forward as this news was, it was clear that the **sphingosine kinase** story represented just the middle part of the riddle. **SK** wasn't the target; it was just another runner in whatever the pathway relay was that was being blocked by the drug. We knew this because **phenoxodiol** had no direct effect on **SK** activity. Mixing **SK** and **phenoxodiol** together didn't it prevent the enzyme from working...it only stopped working when the whole cell was involved. This pointed to an upstream regulator of **SK** function being inhibited by **phenoxodiol**. That raised the intriguing prospect that there were two key book-ends to the **SK** story .... an upstream target that would explain how and why the drug was targeting cancer cells specifically leading to **SK** activity being switched off, and a downstream target that would explain how and why the cancer cells were dying as a result of **SK** activity being switched off.

The answer to the downstream target bookend came first. It came from a research team at Yale University headed by Dr Gil Mor. The team had a long-standing research interest in the biochemical processes involved in apoptosis and the means by which cancer cells circumvent these processes. The Yale team showed that **phenoxodiol** blocked the formation of so-called anti-apoptotic factors. These factors are produced normally by all cells as a means of ensuring that apoptosis is not inadvertently triggered in normal cells. It includes proteins such as c-FLIP and XIAP that are produced in abundance by cells in order to ensure that the self-destructive caspases are not activated. *[More about this later]*. These proteins are over-expressed in cancer cells, thereby raising the bar that cells must overcome in order to induce apoptosis. In the presence of such large amounts of proteins like c-FLIP and XIAP, apoptosis is essentially switched off. It was known that there was a direct link between sphingosine-1-phosphate and the production of anti-apoptotic proteins. Sphingosine-1-phosphate activated a number of different signal transduction pathways (Akt etc) whose end effect was to stimulate the production of anti-apoptotic proteins.

The remaining missing link in all of this was the upstream story bookend. This wasn't revealed until we were well into the **phenoxodiol** clinical program, but it is worth looking at here because of its significance to understanding just how this drug works.

Out of the blue came a telephone call from someone who introduced himself as Jim Morre, from Purdue University in Indiana, USA. He was economical with words and to the point...he said that he was pretty sure that he knew how **phenoxodiol** was working and he would like to get his hands on some of the drug to test out his theory. This stranger from Purdue University would not share with me his theory, but insisted that he was confident of success. Without any great sense of confidence, we sent him some drug, and then promptly put the matter out of our minds. Four months later he rang again with the words: "Morre here. I was right. I know how **phenoxodiol** works." I dropped everything that I was doing and I was on a plane a week later to West Lafayette, Indiana, home of Purdue University and the laboratory of Professor James Morre, Dow Professor of Biochemistry.

What Jim Morre and his team had found was that **phenoxodiol** was targeting a protein that was found only in cancer cells and whose function was critical to the survival of all cancer cells. The significance of this was that it explained why **phenoxodiol** was only killing cancer cells and not normal cells (*because the target protein was only present on cancer cells*), and why **phenoxodiol** was killing all forms of cancer (*because the target protein was present on all cancer cells, irrespective of cancer type*).

In the history of cancer research, nobody had been able to find a single structural factor that distinguished a cancer cell from a normal cell. Cancer cells behave differently from normal cells and they look different in terms of their shape and appearance, but when researchers began to disassemble cells down into their individual building blocks, no-one had been able to identify any structural difference between cancer and normal cells. This is why no-one has been able to develop a single test that can tell whether a person has cancer. There are a small number of cancers such as cancer of the prostate and ovary, where levels of certain proteins in the blood are indicative of the presence or absence of cancer...prostate specific antigen (PSA) in the case of prostate cancer and a protein known as CA125 in the case of ovarian cancer. However, proteins such as these are not specific to cancer cells. They are normal proteins that are found in low levels in normal prostate cells (in the case of PSA) or ovarian cells (in the case of CA125). It simply is a matter of the cell, when it changes from a healthy cell to a cancer cell, making more of that particular protein. For most forms of cancer, however, no such tests exist. So, for Morre and his team purportedly to have discovered a protein that was present only on cancer cells, as well as being present on all forms of cancer cells, was to my mind a monumental discovery that was almost certainly going to change the face of cancer research and cancer therapy.

The background to this finding is that Morre's biochemistry team had been interested for many years in the mechanism of electron transport across cell membranes. All cells have an extensive membranous structure comprising both the plasma membrane that forms the interface between the cell and its surrounding environment, and a complex system of membranes containing all the various internal structures such as mitochondria and the nucleus. One of the functions of this membrane complex is to generate energy. We tend to think of the body deriving energy by burning sugars and fats, which it does, but individual cells generate most (about 95%) of their energy needs from the movement of hydrogen ions ( $H^+$ ) (or *protons*) across cell membranes. Hydrogen ions are generated as by-products of metabolism, and would become a toxic force if allowed to build up inside a cell. A pump

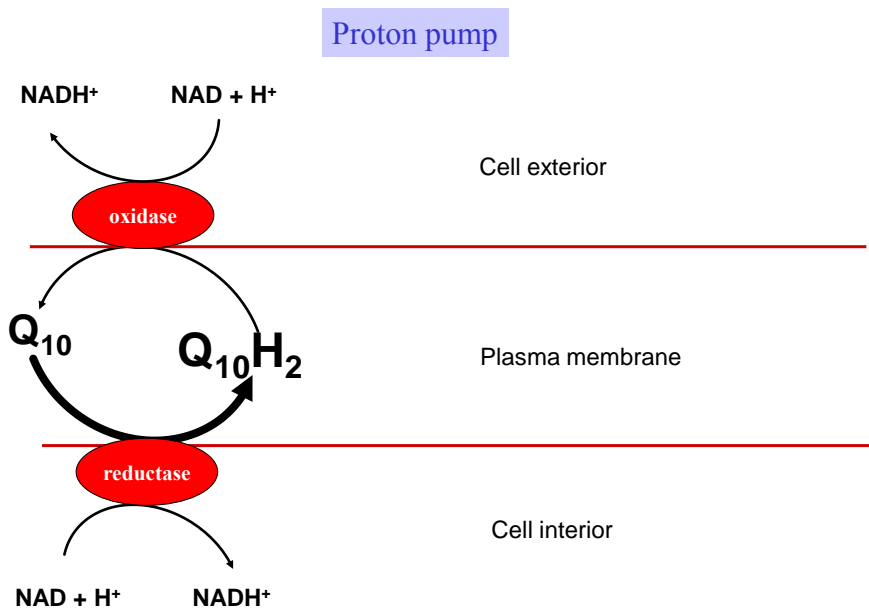
(known as the *proton pump*), moves this excess hydrogen into the space within each membrane and concentrates them there. This concentration of hydrogen ions provides the energy required by a cell's mitochondria to generate the compound, adenosine triphosphate (ATP), which provides the bulk of a cell's energy needs.

The proton pump in the plasma membrane (forming the exterior of the cell), also serves to pump this waste hydrogen out of the cell. Perhaps the most visible function of this pump is the secretion of acid into the stomach as part of our normal digestive processes, and the use of so-called *proton-pump inhibitors* to reduce the amount of acid being produced in cases of esophageal reflux.

Morre's team was particularly interested in the proton pump in the plasma membrane, (as opposed to the rest of the cell's membranes), believing that this particular proton pump served a range of functions beyond that simply of hydrogen excretion. One of those functions was to control the ability of a cell to enlarge after it had divided. Morre's team believed that it had isolated a key protein that regulated the function of this plasma membrane proton pump, and, further, that this protein changed shape and function in cancer, permitting the cancer cell to divide and enlarge more frequently than normal. They further discovered that **phenoxodiol** specifically targets this abnormal protein and disables it. The loss of this pump leads to a build-up of waste hydrogen inside the cell to the point where it becomes lethal.

The Morre hypothesis was contentious when it was first proposed, and elements remain so to this day. Novogen has sought independent verification of the hypothesis and that verification has been obtained, but it has to be acknowledged that only a handful of laboratories worldwide have the means to further verify and extend this hypothesis. Nevertheless, having spent time in Morre's laboratory, speaking with the research staff, and experiencing the data first-hand, I have little doubt about the veracity of the data and the hypothesis.

Regardless of the finer details of the Morre hypothesis, certain basic facts are not in dispute. The following is a general description of the proton pump process based on generally agreed facts.



It starts with the capture of hydrogen ions ( $\text{H}^+$ ) inside the cytoplasm of the cell by the compound, nicotinamide adenosine dinucleotide ( $\text{NAD}$ ), a compound found abundantly within cells and bodily fluids. This results in the formation of  $\text{NADH}$ .

$\text{NADH}$  in turn is coupled to the enzyme,  $\text{NADH reductase}$ , which moves as a complex to the membrane where it passes the hydrogen ion onto the compound *ubiquinone* (also known as *coenzyme  $\text{Q}_{10}$* ). *Ubiquinone* then is transformed by the attached hydrogen ions into *coenzyme  $\text{Q}_{10}\text{H}_2$* .

On the external edge of the membrane sits another enzyme,  $\text{NADH oxidase}$ , which removes the hydrogen ions from the *ubiquinone*. The freed hydrogen ions then pass out of the cell where they are again captured by  $\text{NAD}$ .

The slightly controversial part of this story lies with  $\text{NADH oxidase}$ . Morre and his team identified a difference in the nature of this enzyme between normal cells and cancer cells. The  $\text{NADH oxidase}$  on cancer cells has a different molecular weight and a different functionality compared to normal  $\text{NADH oxidase}$ . *Normal  $\text{NADH oxidase}$*  is referred to as *constitutive  $\text{NADH oxidase}$*  (or cNOX); the  $\text{NADH oxidase}$  on cancer cells is referred to as *tumor-associated  $\text{NADH oxidase}$*  (or tNOX).

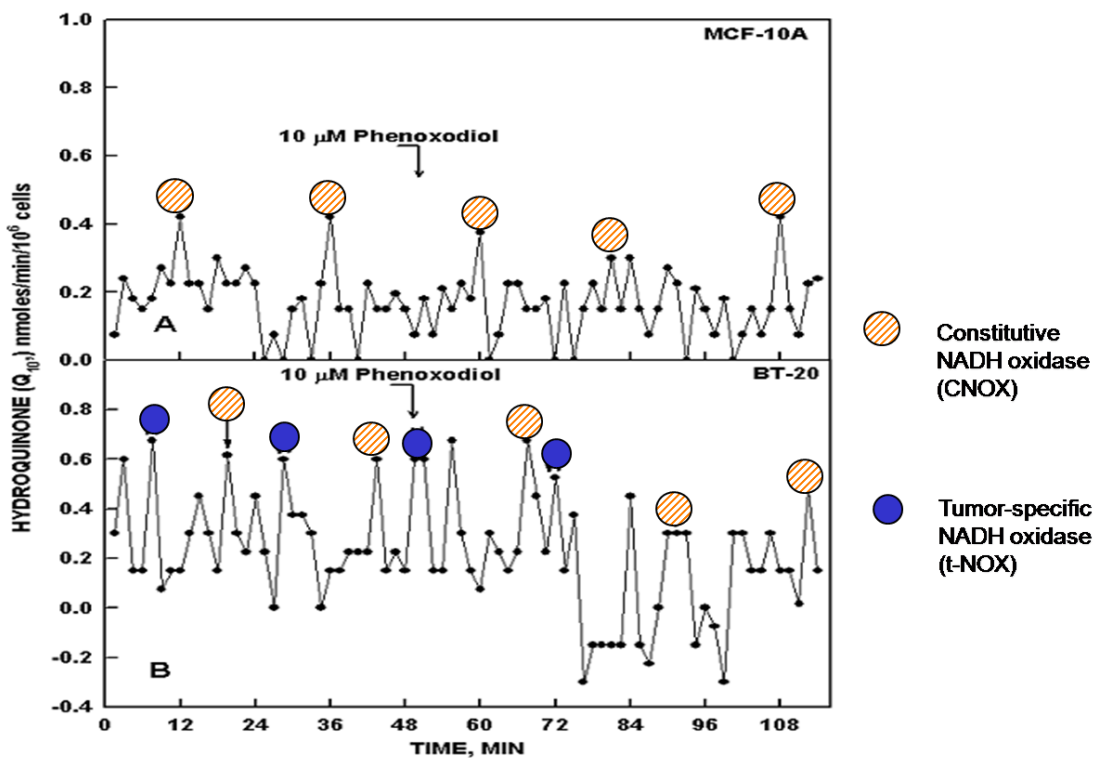
The difference is quite small, and probably comes down to just a slight change in the shape of the NOX protein. It is not clear why it is different and how and when it becomes different, and whether the difference is what makes the cell cancerous or is a consequence of it being turned into a cancer cell. Something that is known about tNOX is that it pumps protons at a slightly faster rate than cNOX, a characteristic that might have to do with the fact that cancer

cells generally have higher metabolic rate than normal cells, and therefore most likely generate waste hydrogen at a faster rate than normal cells. It is not too fanciful to imagine that the NOX protein in cancer cells way back in time, evolved to work at a faster rate than in normal cells to meet this important need. Morre's team speculates that the difference between tNOX and normal NOX lies in the shape of the protein, and it is this spatial difference in shape that determines whether **phenoxodiol** will bind to it or not.

**Phenoxodiol** specifically targets tNOX, and has no effect on cNOX.

When **phenoxodiol** binds to the tNOX protein, it prevents it from performing its normal function, shutting down the pump and so preventing waste hydrogen from being expelled from the cell. The result is that hydrogen quickly accumulates in the cell, poisoning the cell, and interfering with almost every biochemical processes within the cell. The respiratory distress that we had observed within 30 minutes of adding **phenoxodiol** to cancer cells was almost certainly down to this build-up of hydrogen within the cell. The whole process can be likened to the effect of stuffing a tennis ball into a car's exhaust pipe. The inability of the engine to clear waste gases will bring the engine to a sudden stop. **Phenoxodiol** is like that tennis ball – by blocking the cancer cell's waste gas exhaust pipe, the cell's motor eventually stops working.

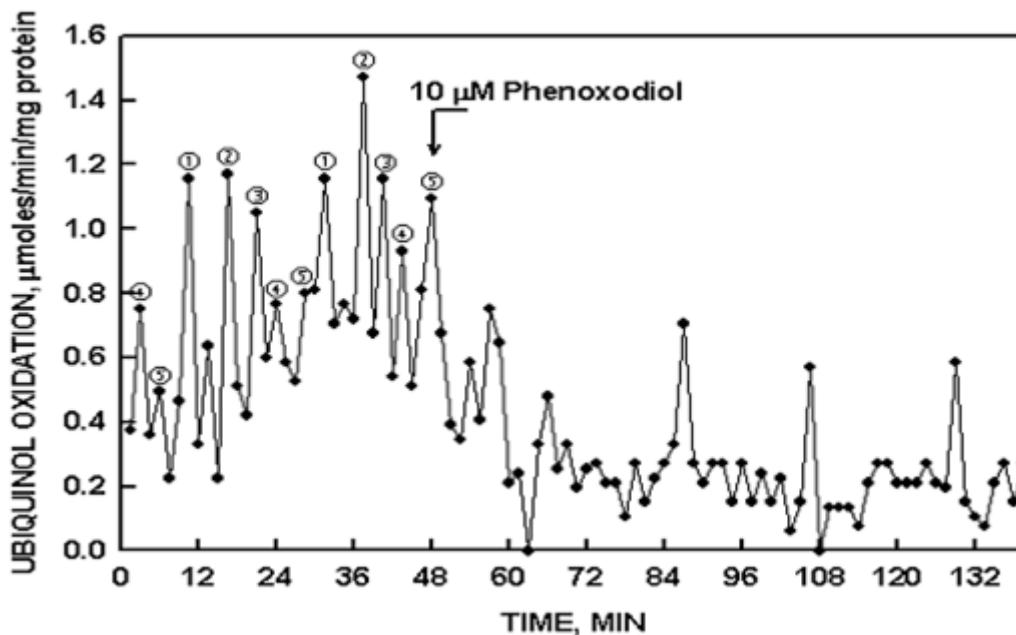
The following figure shows the effect of adding **phenoxodiol** to two types of cells. The first of these (MCF-10A cells) are normal human breast cells. These cells only express cNOX and as the figure shows, the addition of **phenoxodiol** produces no change in the activity of the proton pump in these cells, with their regular cycling of about 24 minutes.



The second type of cell in this study, BT-20, are the same MCF-10A breast cells transfected with tNOX, so that the cells are now expressing both CNOX and tNOX. The addition of **phenoxodiol** to these cells results in an immediate shut-down of the proton pump driven by tNOX, while that driven by cNOX remains unaffected. While cancer cells also express some cNOX activity, it is completely subservient to the more prevalent tNOX activity, so that there is complete shut-down of the proton pump in cancer cells once tNOX is switched off. The absence of tNOX on normal cells accounts for the reason why the effect of **phenoxodiol** is limited to cancer cells.

This shut-down of the plasma membrane proton pump has two immediate consequences. The first is that CoQ<sub>10</sub>H<sub>2</sub> is unable to transfer the hydrogen ions that it is carrying to NAD outside of the cell, leading to a build-up of CoQ<sub>10</sub>H<sub>2</sub> within the plasma membrane. The following figure shows this as measured by the immediate cessation of oxidation (loss of hydrogen ions) by ubiquinol following the addition of **phenoxodiol**.

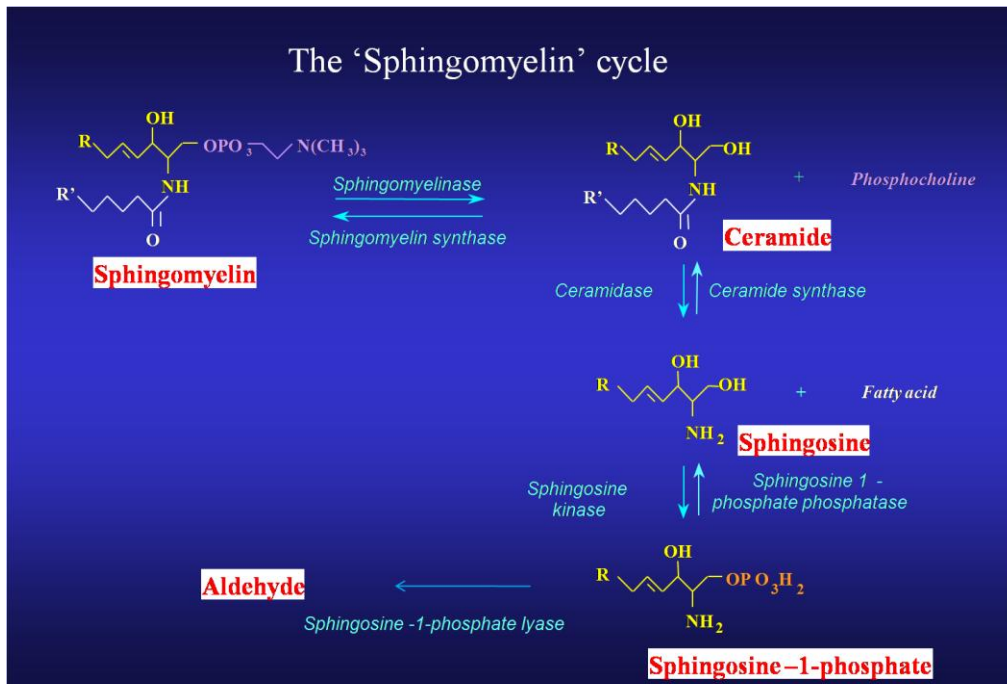
The second consequence of course is that hydrogen ions build up within the cell. Without an active proton pump, the cell has no means of eradicating hydrogen ions being produced as by-products of metabolism. This in itself would be toxic, disrupting all metabolic processes in the short-term, and proving lethal ultimately.



But in terms of how **phenoxodiol** drives the cancer cell towards death, it appears that it is elevated CoQ<sub>10</sub>H<sub>2</sub> levels that is the most relevant factor to the final outcome. The build-up of CoQ<sub>10</sub>H<sub>2</sub> within the plasma membrane has a critical effect on the next part of the sequence of events, the *sphingomyelin pathway*.

We earlier touched on the make-up of the cell membrane with its bi-layer of **sphingomyelin** molecules, and the flux that occurs in the constant assembly and disassembly of the **sphingomyelin** layer. For the purpose of understanding just how the tNOX story interplays

with **sphingomyelin**, we need to go into the cell membrane dynamic in a bit more detail. This flux within the cell membrane is known as the ‘**sphingomyelin cycle**’.



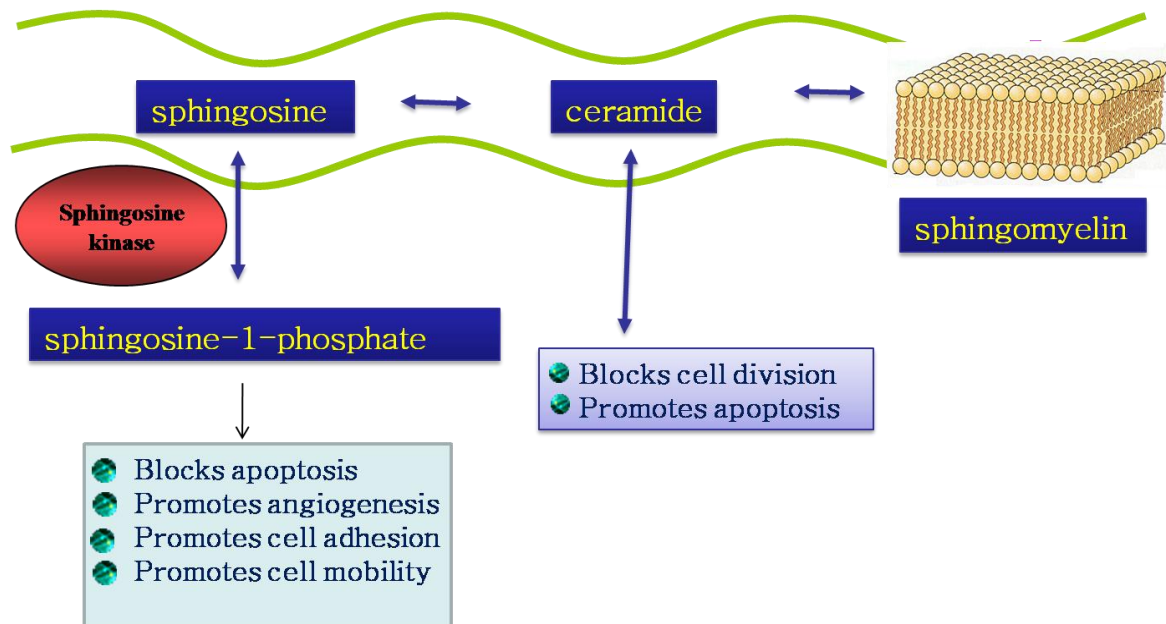
The above diagram shows the flow of the **sphingomyelin cycle**, and the various enzymes involved in the reversible reactions.

At any one time the cell membrane contains variable levels of **sphingomyelin**, and its three principal breakdown products, **ceramide**, **sphingosine** and **sphingosine-1-phosphate**. **Ceramide** is biologically active in its own right, while **sphingosine**, in order to become active, needs to have a phosphate group attached to it (termed *phosphorylation*). This is accomplished by the action of the enzyme, **sphingosine kinase**, yielding **sphingosine-1-phosphate** (or **Sph-1-P**).

We also noted earlier that **ceramide** and **sphingosine** (through its phosphorylated form, **Sph-1-P**) are biologically active messengers that play key roles in cell survival and function, as the diagram below shows.

In normal, healthy cells, an appropriate balance is maintained between levels of **ceramide** and **sphingosine** in the plasma membrane. The various enzymes involved in the **sphingomyelin cycle** are regulated in such a way as to ensure an appropriate balance between the pro-death **ceramide** and the pro-survival **sphingosine**. One of those balancing mechanisms is an enzyme competing with **SK** for **sphingosine**, breaking it down to the biologically inactive compound, **aldehyde**.

Events that tip the scales in favour of **ceramide** production include any stress on the cell such as inflammation and irradiation. The one event known to tip the scales the other way in favor of production of **Sph-1-P** is cancer.



Cancer cells express abnormally high levels of **Sph-1-P**, thought to be the result of increased activity of **SK**, driving the **sphingomyelin cycle** towards the production of **sphingosine**. Over-expression of **Sph-1-P** is a key event in carcinogenesis, preventing apoptosis, and increasing the ability of the cell to migrate and to generate the development of new blood vessels, both important underwriting factors of cancer metastasis.

With these two scenarios in mind, the next step was to see how they were connected. How did switching off the proton pump influence the **sphingomyelin cycle**, if indeed it did at all? At this point we approached a number of experts in the field of cell membrane sphingolipids, one of those being the John Wayne Cancer Institute in Los Angeles. What those collaborations showed was that there was indeed an intimate connection between the activity of the proton pump and the **sphingomyelin cycle**.

CoQ<sub>10</sub>H<sub>2</sub> levels were found to directly regulate the **sphingomyelin cycle**. Increasing CoQ<sub>10</sub>H<sub>2</sub> levels had two effects. The first is that the activity of the enzyme, **sphingosine kinase**, is directly inhibited. The second is that the activity of the enzyme, **sphingomyelinase**, is increased, and this enzyme is responsible for the breakdown of **sphingomyelin** to **ceramide**. The result of this is that **ceramide** levels within the cancer cell rise, while **Sph-1-P** levels fall dramatically.

So here we had a simple but elegant explanation for how **phenoxodiol** was shutting down **SK** activity. By inhibiting the activity of tNOX and switching off the proton pump in the cancer cell, the all-important **ceramide:sphingosine** balance was shifted in favour of **ceramide** production, thus depriving the cell of its pro-survival messenger, **Sph-1-P**.

The final step in this quest for understanding how **phenoxodiol** was working was the second book-end .... what was happening downstream of the **sphingomyelin cycle** that was resulting in the cancer cell failing to divide and then dying. The data coming in on a regular basis from Gil Mor's group at Yale was providing an increasingly clear picture, so much so, that once we had worked out the connection between the proton pump and the sphingomyelin cycle, the downstream consequences were fairly obvious.

Shutting down **SK** activity would undoubtedly deprive the cell of one of the main drivers of important pro-survival signal transduction pathways such as the Akt pathway, and that in turn would clearly affect the ability of the cell to divide and to prevent apoptosis.

The inhibitory effect of **phenoxodiol** on *cdk2* activity (blocking the cell cycle at the G1/S phase), and to a lesser extent, the inhibitory effect on *topoisomerase 2* activity (preventing DNA assembly), were readily explained on the basis of a shutting down of upstream pro-survival pathways. But the main game was even further downstream.....at the level of the ability of the cell to survive.

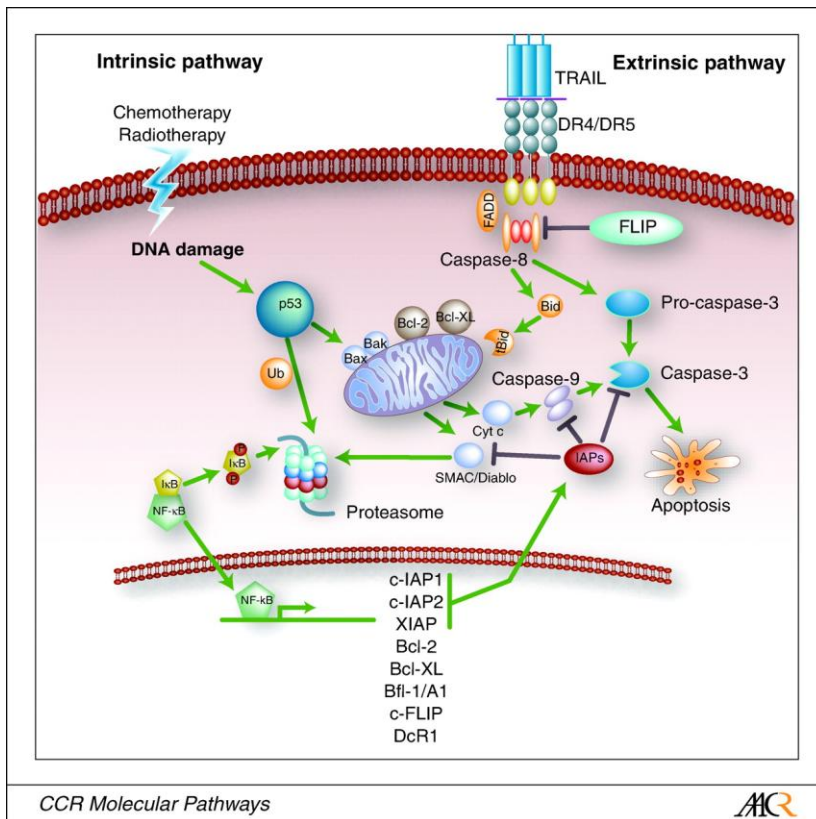
There are two pathways by which a cell can undergo apoptosis. The first is called the ***intrinsic pathway*** which, as the name suggests, comes from within. This pathway is activated when the cell suffers gross injury to its DNA by way of radiotherapy, chemotherapy or heat stress. The second is called the ***extrinsic pathway*** and involves the inability of the cell to reject death signals being received by the cell. Both pathways are immobilized in cancer cells, and both pathways are restored by the action of **phenoxodiol** on the sphingomyelin cycle.

With both pathways, the end result is the release into the cytoplasm of enzymes known as ***caspases*** (*cysteine-aspartic acid proteases*). These are proteolytic enzymes held in sacs within the cytoplasm that when released from the sacs, auto-digest a cell's protein structure. To ensure that ***caspases*** are not triggered either inadvertently or by very minor events, the cell produces a wide range of chemical factors that must be overcome before the various pro-apoptotic signals can activate them. Cancer cells invariably over-express these protective factors, making it that much harder for the cell, no matter how severely damaged, to either self-destruct (intrinsic pathway) or be instructed by the body to die (external pathway).

The figure below from the American Association for Cancer Research nicely summarises the various pathways and their chemical cascades that lead to apoptosis.

Without getting bogged down in the detail of what is a very complex story, there are a couple of key points to note.

The ***intrinsic apoptotic pathway*** is dormant under normal circumstances. It is only when the cell suffers major injury, particularly to its DNA, that the cell attempts to activate it. The ***extrinsic apoptotic pathway***, on the other hand, is continuously on active status. Chemical signals known as ***death signals*** are being received by all of our cells on a second-by-second basis. They are received by protein receptors located on the cell's surface, which once activated, trigger a cascade of chemical events designed to activate the ***caspases*** both directly and via the mitochondria. To stay alive, all cells must maintain the production of ***IAPs*** (*Inhibitor of Apoptosis Proteins*) whose task it is to act as decoys for the pro-apoptotic cascades being triggered by the death receptors.

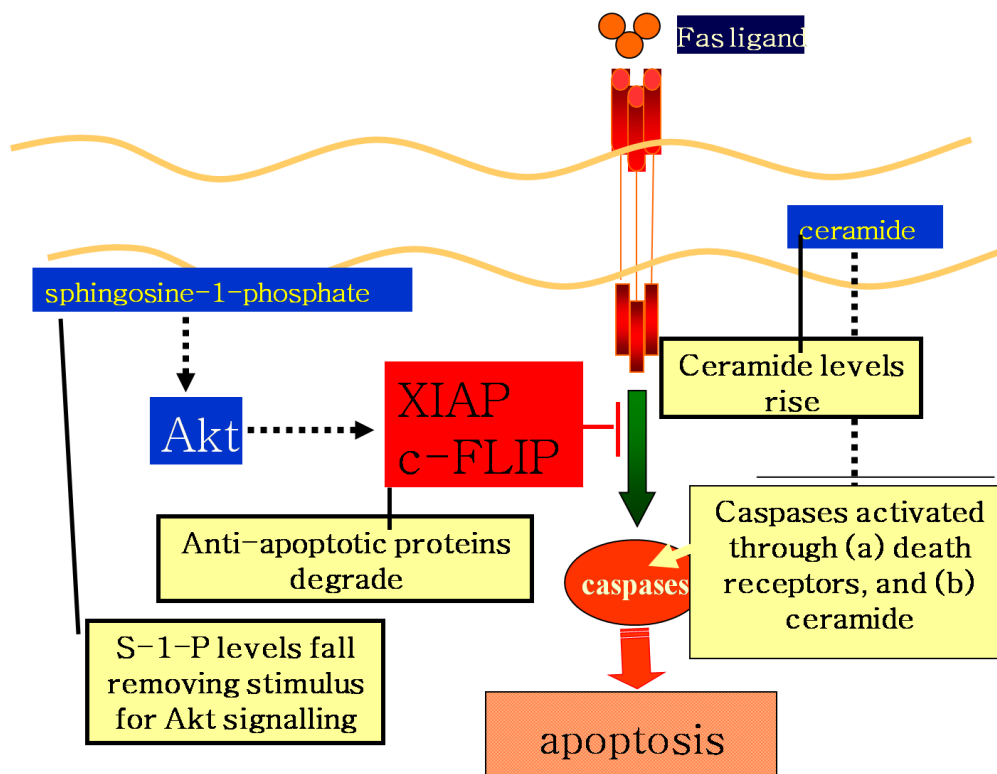


Those anti-apoptotic factors (eg. *C-FLIP* and *XIAP*), and the point at which they block, are denoted in the diagram above by black bars. Cancer cells generally over-express these factors, and reducing the production of these factors is an important step in forcing the cancer cell to die.

The final point to make concerns the type of death receptors. This diagram shows the *TRAIL* death receptor, but the other important one is *Fas*. Both of them initiate essentially the same pro-apoptotic cascades, but different death receptors tend to be expressed on different types of cancer cells. But there must be sufficient difference in their function since **phenoxodiol** appears to be far more effective against cancer cells expressing the *Fas* death receptors than the *TRAIL* death receptors.

The diagram below summarises the effect of **phenoxodiol** on the apoptotic cascades.

Rising CoQ<sub>10</sub>H<sub>2</sub> levels (pursuant to the proton pump being switched off) in the plasma membrane have an immediate knock-on effect on the sphingomyelin cycle. **Ceramide** levels rise dramatically and **sphingosine** and **Sph-1-P** levels fall to negligible levels. The effect of rising **ceramide** levels is to promote the intrinsic apoptotic pathway. The effect of falling **Sph-1-P** levels is to remove the stimulus via the Akt signaling pathway for the production of anti-apoptotic factors.

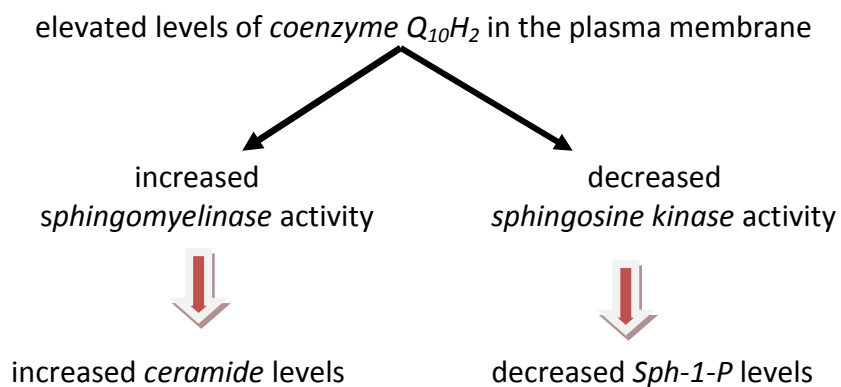


We now had the data to complete an overall flow chart of the mechanism of action of **phenoxodiol**.

The **primary molecular target**

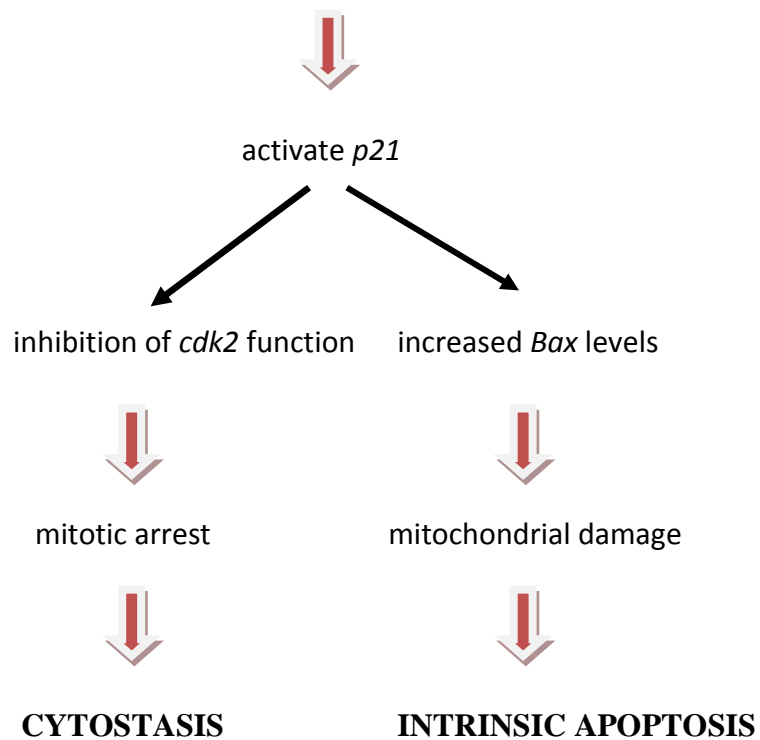
is a protein associated with the proton pump in the plasma membrane regulating the activity of the NADH oxidase component of the pump. The aberrant form of this protein associated with cancer cells, *tumor-associated NADH oxidase (tNOX)*, is inhibited by **phenoxodiol**.

The **primary biochemical consequences** of switching off the proton pump are

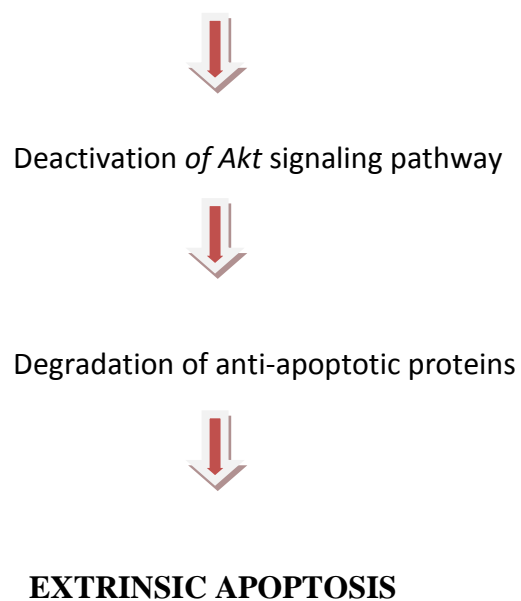


The **primary signalling consequences** of an alteration to the sphingomyelin cycle are

A. *ceramide accumulation*



B. *Sphingosine-1-phosphate* depletion



Of course, none of this answered the key question of why **phenoxodiol** was targeting a protein associated with the proton pump, and only when that protein was behaving abnormally. But a clue to this lies in the plant isoflavone heritage of **phenoxodiol**.

**Rotenone** is a chemical occurring in a range of legumes throughout the world. It displays a number of different functions, most notably being a piscicide, or a fish poison, a property put to good use by a number of native cultures, including Australian aborigines and South American natives, as a means of catching fish. The ground-up roots of various bushes were found to stun fish when added to ponds and rivers. A French botanist and explorer, Emmanuel Geoffrey (1862-1894), became intrigued by this application while travelling in French Guiana, and set about isolating the specific poison. He succeeded in doing so, naming the chemical **nicouline**. Following his death, chemists renamed it **rotenone**.

**Rotenone** was later recognized as having insecticidal and pesticidal properties as well, and for much of the early part of the 20<sup>th</sup> century was used in that way. *Derris dust* became a common garden pesticide and ectoparasite (mites) treatment for animals such as poultry. Its use nowadays is essentially limited to its piscicide activity, being used to eradicate exotic fish from waterways.

The relevance of this to **phenoxodiol** is that **rotenone** is an isoflavone. It also is toxic to insects and fish by its ability to block the transfer of protons to the *ubiquinone* in mitochondria, thus depriving the mitochondria of the ability to generate ATP, their primary energy source. **Rotenone** for this reason also is toxic to humans in sufficient amounts.

**Rotenone** is operating at the initial stage of the proton pump – the initial transfer of protons from NADH to *ubiquinone* – and it does this across all cells. **Phenoxodiol**, on the other hand, is operating on the latter stages of the proton pump – the transfer of protons from *ubiquinone* to NAD – and only then selectively in cancer cells. One action doesn't automatically explain the other, but what it does do is to point to an action against the proton pump as being a prime target for the isoflavone family of chemicals. From that, it is not too much of a stretch to think that one of the natural functions of plant isoflavones commonly consumed by animals is to act as a type of background surveillance mechanism for the detection of abnormality in cellular behavior, and that one of the key abnormalities would appear to be a change in the nature of the proton pump, or more particularly, in the proteins regulating that pump.

This explanation came as a welcome relief to those of who had spent many hours pondering how a drug could be killing cancer cells so selectively and so effectively. In practical terms, this knowledge made little difference to the drug's ensuing development program. Knowing how the drug was working probably did not change in any material way how the next 5 years or so of development proceeded. The real benefit in knowing how it worked lay in making the story far more complete when it was told to clinicians, regulators and investors whose support we were going to need over those 5 years.

## PRE-CLINICAL STUDIES

The understanding of how **phenoxodiol** was working came gradually over a number of years. But back at the time we were ready to move **phenoxodiol** from the test-tube into animals, we had little idea how it was working. The justification for moving forward with animal studies was based on observations that it was working...the how, why and wherefore, we assumed, could come later. With the exception of anticancer drugs developed purposefully by rational drug design, very few of the anticancer drugs in common use today had their mechanism of action understood to any meaningful extent at the time they were tested in humans. To that extent, **phenoxodiol** was simply going to follow a well-worn path.

With the next step being to test the drug in animals, the first decision needing to be made was how we were going to administer the drug, and that basically came down to two choices ... oral or intravenous. For some anticancer drugs, particularly challenging chemical or physical features of the drug can make the process of getting the right dosing formulation a monumentally difficult process. That had been the case with **flavopiridol**, with difficulties in solubilising the drug delaying its introduction to the clinical by some years.

That should not have been the case with **phenoxodiol**, because with the benefit of hindsight it has turned out to be a drug with few formulation challenges. But being the first drug in its class both in terms of chemistry and mechanism of action, back in 1998 we were embarking into a great unknown. That 'unknown' meant that we took some wrong turns before we eventually got onto the right path. But getting there did cost time, probably 2-3 years, and for that reason is a significant part of the story that warrants telling here.

Historically, anticancer drugs have been given to patients predominantly by intravenous injection, mainly because most of them cause nausea and vomiting as a side-effect. An intravenous injection at least ensures that the patient will not lose all or part of the dose where there is uncontrollable vomiting. Choosing an intravenous formulation for **phenoxodiol** upfront therefore would not have been out of the ordinary. But that convention also meant that electing for an oral formulation could become an important point of difference, saving the patients the considerable inconvenience of having to attend an in-patient clinic for intravenous chemotherapy. An oral formulation appeared promising given the total lack of

any obvious toxicity against normal cells that we had seen with **phenoxodiol** in the test-tube. That made it highly unlikely that there would be any gastrointestinal toxicity leading to vomiting.

There was still the question of whether the drug would be absorbed from the gut, but even there we had every reason to be optimistic because of the close chemical relation between **phenoxodiol** and naturally-occurring dietary isoflavones. We knew that dietary isoflavones are readily absorbed from the gut, that they stay in the bloodstream for about 12 hours, and that they leave the body mainly in the urine. If **phenoxodiol** behaved roughly similarly, then oral dosing should be entirely appropriate, with a capsule 2-3 times a day maintaining blood levels at a constantly high level.

Balancing this confidence, however, was one big uncertainty. We already had speculated that **phenoxodiol** was made in the natural course of events when dietary isoflavones were fermented in the bowel, and yet we had never been able to find any **phenoxodiol** in the blood or urine of people eating isoflavones. We therefore had to face the possibility that **phenoxodiol** may be degraded or converted into something else inside the gut. If that turned out to be the case, then that would spell the end of an oral formulation and we would have to revert to the intravenous dosage form.

These questions were readily addressed by conducting a simple pharmacokinetic study in mice. We needed to give the drug to mice by both oral and intravenous routes and compare the outcome. We would measure how much of the administered drug ended up in the bloodstream, and how much of that was present in a useable form. Our prejudice was for an oral dosage form, and the intravenous route would need to be substantially superior to the oral route to justify the extra work and additional cost that would go into manufacturing sterile solutions for injection versus a comparatively lower tech capsule.

This brought us immediately up against a practical problem ..... **phenoxodiol** is totally insoluble in water. It also is insoluble in virtually every other solvent that we looked at. We were well aware of this problem because we had already encountered it during the initial test-tube studies. Cancer cells are grown in the test-tube in a watery medium, and to test a drug's ability to kill the cancer cells means that the drug needs to be dissolved in the medium to ensure that there is direct contact between drug and cell. We found that it was impossible to dissolve **phenoxodiol** in the watery medium, and the only way we could get around this problem was to dissolve the **phenoxodiol** in alcohol, one of the few solvents that it dissolved in that had tolerable toxicity. The amount of alcohol that was used in the test-tube studies was tiny and had no measurable effect on the cancer cells. But that approach was never going to work in the body. The amount of alcohol that we would have needed to use to dissolve the necessary amount of **phenoxodiol** to be given either orally or intravenously would have pickled the mice for days.

This insolubility also made the oral route a significant challenge with an animal as small as a mouse. In a pharmacokinetic study where the general aim is to trace the distribution of a drug in the body, you need to be sure of the amount of drug being put into body, down to the nearest milligram. Such precision is easy when the test drug is injected, and even with an oral formulation when it is delivered in the form of a tablet or capsule and where there is certainty that it has all reached the stomach. The problem with an animal as small as a mouse is that you can't use a capsule or tablet. The normal procedure with mice is to give the drug directly into the stomach by the process of gavage, the same procedure by which patients are fed via a

naso-gastric tube. This means dissolving the test drug in a fluid that could be pushed down into the stomach via a plastic tube. A circular argument that brought us back to the problem that we needed to dissolve the drug in a solvent for the oral route, just as much as we did for the intravenous route.

Close to a year was spent on a search for an appropriate vehicle, until we settled eventually on a material known as *cyclodextrin*. *Cyclodextrin* is a synthetic sugar, constructed to form a large and complex material like starch. *Cyclodextrins* are used widely in the pharmaceutical and cosmetic industries as carriers for water-insoluble materials. The *cyclodextrin* molecule is like a sponge, involving a honeycomb of tunnels and caves where drugs and other chemicals can be stored. *Cyclodextrin* acts like a Trojan horse, delivering into the body thousands of molecules of a water-insoluble chemical, ready to be released under the right conditions. In the stomach, the strong digestive juices break down the sugary *cyclodextrin*, releasing the hidden drug. When given intravenously, the change in pH of the blood compared to the inside of the *cyclodextrin* usually is sufficient to draw out the drug from inside of the *cyclodextrin* into the blood. **Phenoxodiol** proved easy to incorporate into *cyclodextrin*. Mixing **phenoxodiol** with a solution of *cyclodextrin* led to most of the **phenoxodiol** being taken up and residing inside the *cyclodextrin* maze.

For oral administration, a precise amount of **phenoxodiol** was delivered via a naso-gastric tube directly into the stomach of mice; the same amount in a sterile solution of *cyclodextrin* was administered to other mice by intravenous injection. The intravenous dose yielded high blood levels with all of the injected drug being accounted for in the bloodstream. With the oral dose, we were delighted to see that the drug was absorbed readily (starting within about 10 minutes, thus indicating fast release from the *cyclodextrin*), also that a reasonable proportion (about 30%) of the delivered drug was absorbed overall, and that reasonably good blood levels were maintained for about 8 hours. All of which was much as we had anticipated from the experience with dietary isoflavones. Most of the unabsorbed **phenoxodiol** was accounted for in the faeces, indicating that **phenoxodiol** was not being broken down or converted to something else within the gut. A 30% absorption rate might sound relatively inefficient, but that is well within acceptable boundaries for drugs given orally.

The next step proved ultimately to be the fateful step. Having confirmed that we could get the drug into the blood by either route, the next step was to see how the drug was being carried in the bloodstream. As a chemical that is totally insoluble in water, **phenoxodiol** would have to be converted in the body into a soluble form in order to be transported in the blood plasma which is approximately 90% water. For any small molecular weight compound, whether it is a drug or an absorbed nutrient (such as a vitamin) or a chemical made by the body (such as a hormone), it needs to be in a soluble form so that the body can transport it, use it, and then excrete it in the urine.

The classic example of this is our steroid hormones such as estrogen, testosterone and cortisone. Steroids are very poorly soluble in water, not surprising when you consider that they are made from cholesterol, itself a fat and water-insoluble. When steroids are made in the body and released into the blood stream from the ovaries (estrogen) or the testes (testosterone) or the adrenal glands (cortisone), they are attached to special transporter proteins within the blood. The sex hormones have their own transporter protein known as sex hormone-binding globulin; cortisone has its own, known as cortisone binding globulin. These transporter proteins are water-soluble. The steroid hormone is so tiny compared to the large

protein molecule that binding to the protein has no effect on the overall solubility of the protein, thus allowing the steroid to be freely transported around the body.

The liver then takes over, breaking the link between the protein and the steroid, and re-solubilising the steroid by attaching it either to a sugar molecule (known as a *glucuronide*) or to a sulphate ion. Whereas the link between the steroid and the protein is fairly weak, the new link with the sugar or sulphate is strong and can only be broken by enzyme activity. This re-solubilising by the liver has two objectives – first, to make the steroid more available to the various target tissues (the protein-steroid link is not readily broken by tissues other than the liver), and second, to allow the steroids to be excreted in the urine (the kidneys do not filter out protein from blood).

This means that at any one time, the steroids present in our blood are present in 3 forms – attached to transporter proteins, or in a glucuronide form, or in a sulphated form. In all 3 forms, the steroid is biologically inactive, because while it is attached to something else it is unavailable to enter its target cell. To become biologically active, it needs to be released from its attachment. Tissues do this by producing two enzymes – *glucuronidase* and *sulphatase* – both of which snip the bond between the steroid and the sugar or the sulphate ion, releasing the active steroid. All tissues contain these two enzymes.

That above is what happens to steroids made in our bodies. But what happens when we take steroids orally, as pre-menopausal women do with the oral contraceptive pill or menopausal women with hormone replacement therapy? In those cases, the steroid (estrogen) has to pass across the lining of the gut before entering the bloodstream. The mucosal cells lining the gut wall actively transport the steroid, and in order to do so, need to solubilise the steroid. They do so by attaching it to either a *glucuronide* molecule or *sulphate*.

The relevance of all this is that isoflavones behave in a similar manner to steroids, not surprising given the very close chemical relationship between the two structures. Dietary isoflavones, like steroids, are water-insoluble, and when they pass across the gut wall they are treated in exactly the same way as steroids ... by being conjugated to either *glucuronide* or to *sulphate*. The only difference between steroids and isoflavones is that isoflavones show considerably less affinity for transporter proteins in blood than do steroids.

This isoflavone profile was repeated with **phenoxodiol**. When it was administered to mice orally, about 90% of the drug in the blood was present as the *glucuronide* conjugate, with the remaining 10% as the *sulphate* conjugate. The intravenous route produced an almost identical profile with a 90%/10% ratio of *glucuronide* and *sulphate* forms. But with one important difference .... about 10% was present in an unconjugated form. That is, 10% of the recovered **phenoxodiol** in blood following intravenous injection was not conjugated. We assumed that it was bound to protein, but attempts to confirm this were unsuccessful.

The extensive conjugation of **phenoxodiol** came as no surprise. After all, this was how dietary isoflavones and steroids behaved. But where the difference lay was in the fact that dietary isoflavones and steroids were targeting normal tissues, while the **phenoxodiol** was targeting cancer tissue. Normal tissues throughout the body were known to contain plenty of *glucuronidase* and *sulphatase* activity, but there was no such understanding of cancer tissue. Cancerous tissue is considerably different to normal tissue and there was no certainty that it would continue with all normal functions such as the expression of *glucuronidase* and *sulphatase*. An extensive literature search and enquiries to leading authorities in the field of

steroid chemistry failed to answer that question. In fact, the firm advice from several well-renowned authorities in the area of steroid chemistry and function was that it was highly unlikely that conjugated **phenoxodiol** would work as an anticancer agent.

Before proceeding further, Novogen chemists were asked to construct *glucuronide* and *sulphate* conjugates of **phenoxodiol**. When these were tested in the laboratory against cancer cells, it was confirmed that they were totally inactive.

All of this had been done to help decide on the dosage form to take forward, and that decision was needed urgently. A lot of time had already been spent on learning how to solubilise **phenoxodiol**, on top of the pharmacological studies, so a decision was pressing. With no compelling evidence that the oral dosage form would work, and with the vague promise of having approximately 10-15% of **phenoxodiol** in the bloodstream following an intravenous injection in a 'free' (unconjugated) form that had the potential to be biologically active, we decided to proceed with an intravenous dosage form. As time will tell, the basis of that decision proved to be wrong, but that is for the future. At the time, with the limited information of the early part of any program, it was made for what we took to be the best reasons.

The down-side of the intravenous dosage form was the carrier, *cyclodextrin*. We were not aware of any other human drug, anticancer or otherwise, that was being injected intravenously in *cyclodextrin*, so we were going into relatively uncharted waters. On the other hand, *cyclodextrin* was approved in the USA for that purpose even if no-one had yet taken up the opportunity. Having that pre-approval made the task ahead considerably easier.

So it was with a mixture of trepidation and anticipation that we set about testing the injectable dosage form of **phenoxodiol** in mice. This was going to be the first real test that would make or break the program, and, in turn, determine the fate of the company. We chose to test in the first instance one of the most common forms of human cancer – prostate cancer. In the test tube, **phenoxodiol** had shown high anticancer activity against prostate cancer, as it had against a wide range of other cancer types. We were prepared to work our way through the list of different cancers if we needed to in order to find one that responded to **phenoxodiol**, but cancer of the prostate seemed a likely place to start. Human prostate cancer tissue grows in this mouse model at a convenient speed – not so slow that we would need to wait months to see any response, and not so fast that it would overwhelm the mice and prove too much of a challenge for the test drug.

To our delight, daily intravenous injections of **phenoxodiol** for about 7-10 days inhibited the growth of human prostate cancer by at least a 60% compared to untreated mice. This was great news, making us confident at that point that we had an exciting new drug prospect. We still had a long way yet to go before we could declare victory, but this was a key early test that the drug had passed. Over the course of the next year or so, we repeated the study using different prostate cancer cell lines (meaning that they were derived from different individuals originally), which ensured that we were exposing the drug to a range of sub-populations normally present in human prostate cancer. We then extended the studies onto other forms of cancer such as breast cancer and lung cancer cells. In each case, **phenoxodiol** inhibited cancer growth by an order of between 60 and 80%.

With the strong evidence behind us that we had a drug with the potential to deliver a significant anti-cancer effect in humans, we moved onto answering the next key question,

which was, *how safe is the drug?* Up to now we had seen no obvious signs of toxicity in any of the hundreds of mice we had treated with **phenoxodiol** in the studies to date. We hadn't seen any of the obvious signs of toxicity normally associated with commonly used anticancer drugs, such as loss of appetite, weight loss, hair loss, or inactivity. Significantly, we hadn't seen any of these unwanted signs with doses of **phenoxodiol** that were delivering a marked anticancer effect. However, this optimism had to be balanced against the fact that we had not up to this point treated mice for any longer than about 10 days. Once we moved into humans, we knew that treatment almost certainly was going to need to last for weeks, if not months, so a 10-day course of treatment in mice was hardly a rigorous test of safety ahead of human studies. Nevertheless, almost all other potent anticancer drugs such as **cisplatin** and **methotrexate** cause marked toxicity in mice after even 5-7 days of treatment, so the evidence, as preliminary as it was, already was pointing to **phenoxodiol** being far better tolerated than most other commonly-used anticancer drugs.

Government regulatory agencies are justifiably nervous about the safety of drugs that they are required to approve for use in humans. No drug is 100% safe, but the working rule of thumb for most regulators is that the benefits of the drug in question must outweigh the disadvantages. The more serious the disease being treated, the more tolerant they are of side-effects. For a life-threatening disease such as cancer, a new drug can have serious side-effects and still be approved, whereas those same side-effects would be regarded as intolerable and completely unacceptable if the drug were being used to treat a disease such as influenza or arthritis or Alzheimer's disease where death was not normally imminent.

Anticancer drugs are expected to have side-effects. No anticancer therapy in the history of medicine, capable of killing cancer cells, has been without them. Indeed, most anticancer drugs are associated with such serious side-effects that they can be lethal in their own right if not managed carefully. The task of drug developers is to know as much as possible about those side-effects before the drug is given to humans so that the patient's doctors are forewarned, allowing the patient to be monitored and treated appropriately.

The gold standard in the area of drug safety testing has been set by the US Food and Drug Administration (FDA), the regulatory body that approves the sale and use of drugs in the United States. Most new anticancer drugs follow the FDA guidelines when it comes to pre-clinical testing, simply because it leaves fewer questions unanswered when the time comes to approach hospitals and doctors to test the new drug in humans. From the outset, we took the view that we would follow the FDA guidelines when it came to the development of **phenoxodiol**.

The FDA has set a number of key tests of safety. At the most basic level is the test of whether the drug shows any activity against normal cells in the test-tube. We had already passed this test – as mentioned previously, **phenoxodiol** showed no toxicity in the test-tube against various non-cancer cells, including human cells derived freshly from baby's foreskins. Why foreskins? Simply because it is a human tissue that is readily available if you happen to be working near a major hospital with a maternity service, and the cells grow relatively easily and abundantly from the tissue. Doses of **phenoxodiol** that killed 100% of cancer cells in the test-tube had no effect on foreskin cells.

The second level of testing is the question of the agent's carcinogenicity, which means whether the agent has the potential to cause cancer in its own right. Many of the anticancer drugs in common use that work by damaging the DNA of cells are carcinogenic to a greater

or lesser extent. That is just an inevitable consequence of damaging the genetic apparatus of the body. Being carcinogenic wouldn't necessarily stop a drug being used to treat a life-threatening cancer, but it would mean that patients would need to understand the risk that they were facing, and would need to avoid becoming pregnant or fathering children for fear of causing DNA defects in the child. **Phenoxodiol** proved to be completely non-carcinogenic when subjected to the standard laboratory and animal tests.

The third, and final, level of safety testing involves long-term animal testing. This requires testing the drug in at least two different animal species. One species normally comes from the group collectively known as *rodents* (mice, rats, guinea pigs and rabbits). The second species normally comes from a group comprising larger animals (dogs and pigs) or primates (monkeys). The use of at least two substantially different animal species helps to overcome the problem of species-specific side-effects where a drug can cause side-effects in one species but not in another. It is unusual to find a drug that causes the same range of side-effects across different animal species. It is far more usual for a drug to cause one set of side-effects in one species, and another set in another species, with some overlap between the two species. This makes it difficult to predict with any certainty what the safety issues for a new drug might be in humans on the basis of what is seen in animals. Testing in at least two totally different animal species simply helps to lower the risk of missing an important potential side-effect in humans.

The transplant drug (**cyclosporine**) that I had encountered earlier on in my career is a good example of species-specific toxicity. The main side-effects of this drug in humans are damage to the kidneys and liver. These problems are not life-threatening providing that the function of these organs is carefully monitored and the dosage of the drug adjusted appropriately. Rats showed the same toxicity profile as humans. When it was tested in dogs, however, a very different picture emerged. Kidney and liver damage were comparatively mild in the dogs compared to rats and humans. The most significant toxicity in dogs concerned the gastrointestinal tract, with gastric irritation (nausea, vomiting, diarrhoea) and excessive growth of the gums in the mouth, side-effects that almost led to the drug not being developed any further, an outcome that would have been a tragedy given that those side-effects proved to be very rare in humans.

We chose to use rats and dogs as our two test animal species for **phenoxodiol** for no other reason than they are the most commonly used species for this purpose, with dogs representing a significantly less expensive option than pigs or monkeys. A contract research company accredited with the FDA subsequently was retained to perform these studies. The aim was to give **phenoxodiol** by intravenous injection to animals on a daily basis for 4 weeks, with intensive tests being conducted on the animals both during and at the end of the study. Those tests, while never giving a 100% guarantee of picking up subtle side-effects, nevertheless could be relied upon to pick up all serious side-effect likely to be relevant to the safety of human patients.

One of the fundamental rules about testing the safety of a new drug in animals is that it needs to be given to the animals in a way that resembles as closely as possible how you intend to use it in humans. If you think that you are going to have to give it 4 times a day by mouth or twice daily by intravenous injection, then that is how you need to give it to animals. The only room for any variation is in the length of time you think that treatment will need to last in patients. Even if you think that patients will need to be treated repeatedly for a number of months, it would be unusual to have to test the drug in animals for more than about 1 month.

The vast majority of side-effects are going to be evident within a matter of days, so treatment of animals for 4 weeks generally is considered an adequate time to reveal potential side-effects.

Up to this point, we had given the drug to mice by intravenous injection as single daily injections. This is referred to as a *bolus* injection, where the drug is injected quickly over a matter of seconds, resulting in a high level of drug in the bloodstream that gradually falls away over time as the drug is either broken down by the liver or eliminated from the body in the urine. In the case of the mice that we had been treating intravenously with **phenoxodiol** in the studies beforehand, the half-life of the drug was 8 hours, and was largely eliminated from the body by 16 hours. In other words, we were probably only achieving blood levels of **phenoxodiol** capable of delivering an anti-cancer effect for about 10 hours from each injection. For many anti-cancer drugs, the rate at which they disappear from the bloodstream is not overly important. In fact, many of the most commonly used anti-cancer drugs given as a bolus intravenous injection have essentially disappeared from the blood within several hours. For these potent drugs where the anti-cancer effect is essentially a poisoning effect, the damage is inflicted quickly and there is little point in continuing to inflict damage on an already damaged cell. In fact, it becomes counter-productive, with healthy cells being the cells most likely to be damaged with continuing presence of the drug. Because of this, most anti-cancer therapies are based on a *pulsatile* format. This simply means that these drugs are designed to be present in the body for only a limited time each day as a way of maximising damage to cancer cells while minimising damage to healthy cells.

We formed the view early on that this standard *pulsatile* approach probably was not the best way to use **phenoxodiol**. **Phenoxodiol** was not in the same category as most anti-cancer drugs, that is, as a poison delivering a short, sharp, lethal damage. Although **phenoxodiol** killed cancer cells, it appeared to be working through a more gentle, sustained action that suggested that its benefit was likely to be better the longer that it remained in contact with cancer cells. We had some evidence to support this belief. In the test-tube, we had shown that **phenoxodiol** needed to stay in contact with cancer cells for at least 4 hours in order to achieve any significant killing of the cancer cells. Removing the **phenoxodiol** before this time compromised its anti-cancer effect. That observation contrasted with drugs such as **cisplatin** that only needed to be left in contact with cancer cells in the test-tube for as little as 30 minutes in order to achieve a maximum killing effect.

The mice cancer studies we had conducted showed that a single bolus (fast) intravenous injection of **phenoxodiol** each day (providing effective drug levels for about 10 hours of each day) was more than enough to provide a significant anticancer effect. That still begged the question, however, whether we could achieve an even greater anticancer effect if we could keep the drug in the bloodstream at effective levels for 24 hours. There were practical reasons why we weren't able to answer this question in the mice, but our understanding of how **phenoxodiol** was working still led us to believe that an effective 24-hour drug presence would be preferable. The apparent tolerance of the drug by the mice also meant that if safety was not a major barrier, then there was unlikely to be any downside in giving the drug on a continuous basis. That left us with two options to treat patients – either to inject every 12 hours, or to give by continuous intravenous injection. Continuous intravenous drug delivery in humans is achieved by the use of a mini-pump attached to a reservoir, with the drug being drip-fed on a continuous basis via an intravenous line. The whole apparatus is relatively miniaturised and portable and patients are able to go about their daily lives relatively

inconvenienced. Compared to the inconvenience of attending hospital for twice-daily injections, continuous intravenous infusion seemed a far more practical option.

Having decided that this was the method by which we would eventually treat humans, we were obliged to test the same method for safety in animals. We started with rats first. This involved giving **phenoxodiol** to rats by continuous intravenous injection over a 28-day period. For this, rats are trained to tolerate an in-dwelling intravenous catheter that is attached to a mini-pump, and to go about their normal laboratory life. The **phenoxodiol** dissolved in the *cyclodextrin* vehicle then is pumped into the rats on a 24-hour continuous drip. Rats received one of five different treatments over 28 days. The first was saline, just to provide a baseline of the effect of the procedure alone on the health of the rats. The second was the *cyclodextrin* vehicle alone, to determine the toxicity, if any, of this part of the product. The other 3 treatments were increasing dosages of the drug in *cyclodextrin*, with the highest dosage being the practical limit of drug that could be pumped into the body. The rats were subjected to extensive observations and tests both during and following the 28-day test period.

The only significant side-effect found in the rats was mild kidney damage. This occurred both in the rats receiving *cyclodextrin* alone as well as those receiving *cyclodextrin* + **phenoxodiol**, pointing to the fact that the damage was due to the *cyclodextrin*, and not to **phenoxodiol**. This concurred with what we already knew from the manufacturers of *cyclodextrin* about its toxicity, which was that this large sugar tended to cause some damage to kidney cells on its way out of the body in the urine.

We did not regard this kidney damage as anything too serious. First, because the damage in the rats was relatively mild and the animals remained relatively healthy. Second, and more importantly, it was not permanent damage; the damage was repaired within a week of stopping *cyclodextrin* therapy. Third, this was a known side-effect of *cyclodextrin*, and yet the material still had been approved by the FDA for human use, so the same type of damage, if it was to occur in humans, would seem to be acceptable.

So, with this reassurance behind us, we commenced continuous infusion studies in dogs, hopeful that we would see nothing more than the same mild kidney damage that we had seen in the rats. But to our complete dismay, **phenoxodiol** proved to be lethal in dogs, with dogs dying within several days of receiving the drug. The animals were dying from severe internal bleeding problems, which subsequently was traced to the break-down of blood platelets, robbing the animals of the ability of their blood to clot. The effect was identical to a dog being bitten by a highly venomous snake where death comes from massive internal bleeding.

With the initial shock of this dramatic news, came the obvious knee-jerk reaction that this could spell the end of the **phenoxodiol** program. We had seen nothing in our experience of **phenoxodiol** to date to suggest even the remotest hint of adverse effect of the drug on blood platelets, so this news came completely from left field. But on closer examination of the results, the answer became clearer....some dogs had also been given the *cyclodextrin* carrier without **phenoxodiol** and these animals also had severe haemorrhaging, pointing to the problem being with the *cyclodextrin* and not **phenoxodiol**.

Whereas *cyclodextrin* toxicity in rats was mild kidney damage, in dogs it was massive disruption of blood platelets. In preparation for this animal safety study, we had searched the scientific literature and sought as much information from the supplier of *cyclodextrin* as we

could, and no mention had ever been made of this particular side-effect in any animal, including dogs. It was only with considerable further pressure on various parties that we received an acknowledgement that *cyclodextrin* was known to cause platelet disintegration in dogs and that this was considered to be a species-specific effect that would not occur in humans. The relief that came with this news was followed closely by huge annoyance that we had not been informed of this fact. Another valuable lesson learnt ... regulatory authorities see their obligation as only answering questions put to them in the narrowest of terms. Perhaps because of reasons of commercial confidentiality or because they hold dear a notion of non-bias, they do not feel any obligation to offer answers to unasked questions, even when those unasked questions are highly pertinent to the matter under discussion.

Knowing the FDA ground-rule of safety testing of new drugs in at least two animal species, we turned our minds to the alternatives to the dog. The only realistic options were the pig and the monkey, but nobody we contacted was offering a continuous intravenous infusion model with these two species. The most obvious remaining alternative was to drop the continuous intravenous infusion approach and revert to a single daily intravenous injection. That had worked in the mouse cancer model, so we felt that we could always fall back on that approach. That way it would have been practical to use an animal model such as the pig or monkey.

But the attraction of a continuous infusion approach in humans remained strong. We still believed that the concept of 24-hour drug cover was sound and would provide a better response. Moreover it was an entirely practical and accepted method of drug delivery in humans. If we were to stay with this notion, then we were going to have to be creative in order to get around the restrictions of animal testing.

So began the germ of an idea of how we might short-cut the process and to go straight into humans without any further safety testing in animals. This idea was only plausible because of our complete confidence in the safety and tolerance of the product, leaving aside the specific problem encountered in dogs. The irony of this situation was not lost on me. As related previously, my earliest encounter with the challenges of drug development had been with the transplant drug, **cyclosporine**, which had caused significant side-effects in dogs to the extent that its continuing development came under a cloud. It was only the belief of its developers in its safety, to the extent of them using the drug on themselves without permission, that broke through that cloud and resulted in it coming to market to great acclaim. The irony of course being that the drug that I had staked my future on was itself now under a cloud because of dog safety issues.

