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IMPROVING THE EFFICACY OF COMBINATION THERAPY IN CANCER

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ПОЛІПШЕННЯ ЕФЕКТИВНОСТІ КОМБІНОВАНОЇ ТЕРАПІЇ РАКУ
БіоМедЕС, Леггат Хаус, Кейтхолл, Інверюр, Абердиншир, AB51 0LX, Велика Британія

Стаття відомого вченого Деніса Н. Уїтлі присвячена найважливішій клінічній проблемі — підвищенню ефективності комбінованого лікування злоякісних новоутворень. Автор підходить до розв'язання проблеми, аналізуючи існуючі уявлення про унікальність патоморфозу злоякісного процесу і, у зв'язку з цим, відсутність єдиного, правильного для всіх типів пухлини підходу до лікування. На думку автора, процес лікування має полягати не в підвищенні ефективності окремих методів лікування, а в пошуку можливості отримання контролю над пухлинним процесом. У статті наведено дані власних багаторічних досліджень автора, серед яких і експериментальні. Застосування аргініну в комбінованій терапії дозволило автору істотно знизити дози застосовуваних токсичних агентів, зокрема променевої терапії, і відповідно поліпшити прогноз лікування різних видів пухлин, у тому числі дисемінованих.
Ключові слова: злоякісні новоутворення, комбінована терапія.

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The article of a famous scientist Denis N. Wheatley is dedicated to an important clinical problem — increasing of efficiency of malignant neoplasms combined treatment. The author touches the problem analysing the present ideas about a unique nature of malignant process pathomorphosis and the absense of the only correct approach to treatment for all the types of tumor. From the author's point of view the treatment process consists not in increase of efficacy of separate treatment modes, but in search for a possibility to take control over the tumor process. The article presents both the own data of long term researches, but experimental ones. Arginin usage in combined therapy allowed the author to decrease significantly the doses of applied toxic agents including X-ray therapy and correspondingly improve the prognosis for different kinds of tumors, disseminated ones as well.
Key words: malignant neoplasms, combined therapy.

1. General Introduction

We have come a long way from seeing carcinogenesis as a single event or mechanism that can occur in all forms of life. Equally, we have come a long way from expecting to find the panacea, "a cure" for all cancers. Every cancer is unique, and it therefore follows that each needs individual attention in its management if current treatments are going to be more effective. The prime aim of the oncologist should be to *gain control* over a cancer rather than necessarily expect to cure it.

Gaining control over anything in life demands as deep an understanding of it as possible. Apart from improving preventive measure to reduce cancer mortality, early detection in diagnosis is the next important means of lowering death rates. But cancer is often not detected at its early stages, and therefore much, if not most, clinic effort is devoted to the therapeutic side in established tumours. This is where greater knowledge of cancer behaviour and the mechanisms of action of different modalities on them are most needed in reducing cancer deaths. With these considerations in mind, improving cancer therapy should come with *more subtle and rational* approaches, and fewer "trial and error" or blunderbuss methods that leave the cancer patient traumatized and truly a victim of both the tumour and its treatment, with loss of quality of life. To gain better control, more insight is needed on the following questions and issues:

1) What causes cancer? This must come with the realisation that we remain relatively ignorant of the causes of many cancers. What is certain is that there are a lot of mechanisms involved and no single cause. There is, in effect, no exclusive hypothesis. However, knowing about causation is not necessarily going to help in the treatment of cancer, but it might give ideas on which biomarkers to explore.

2) What must be done to diagnose cancer at it earliest stages? Here we have to consider two things: (I) is there a hereditary reason for suspecting that a

cancer might arise at a particular age? And (II) have we got sufficient knowledge of biomarkers (e. g. PSA?) which indicate the possibility of the early development of a cancer? The problem of early diagnosis, in the absence of highly effective preventive measures, remains of the highest priority. New technologies should help detect smaller tumour masses (e. g. helical computerised tomography in lung cancer).

3) Assuming most cancers present late, it is worth remembering that a nodule of about 1 cm in diameter will contain many millions of cells, which soon becomes billions of cells. By the time even a small tumour has got to this state, it has become increasingly *heterogeneous*, which means it will have cells that grow ever faster, others will evade cytotoxic drugs, and many acquire the potential to invade other tissues to metastasise and become widely disseminated, a classic example being melanoma (see 4 below).

4) We need to know more out about which biomarkers might distinguish non-metastatic from metastatic cells. Dissemination is the scourge of cancer since it stops it from being containable and more curable by, e. g. surgery and/or radiotherapy. Reliable and highly specific markers of dissemination are therefore extremely important, remembering that they do not have to be qualitative, but can be quantitative.

5) A holistic approach to therapy needs to be more widely adopted and improved; after all a tumour is a part of a suffering human. It is an unwanted development, and whatever is done to it affects the rest of the body. It stands to reason that every effort is required to treat tumour and patient as an entity. Until relatively recently this was not done, but much has been achieved in a short space of time to address this problem, as will be discussed later. On the more scientific and clinical sides, we need to understand much more about the stromal, immunological and other corporeal responses to a tumour, for example, the extent to which immune responses play into the hands of tumours rather than against them needs greater consideration (Prehn, 2010). More difficult matters relate to will, temperament, attitude, and other psychological aspects of both patient and carers. The management team of a cancer patient needs to be familiar with many of the issues raised in (1) to (5).

Although this introduction emphasises many of the issues facing cancer treatment, it is not to say that they are all intractable; some will arise that can be dispatched without trace. The early success with choriocarcinoma is a case in point where chemotherapy is 90–95% effective (Bagshawe et al., 1989). It is noteworthy in this context that the 5–10% of fatalities from choriocarcinoma occurs almost exclusively in cases where it has disseminated to the liver or brain (see 4 above; and Rustin et al., 1989). While the former report indicates that non-metastatic choriocarcinoma responds very well to methotrexate (given with a folinic acid supplement), the finding in the latter reference clearly show the problem of effecting a cure in cases with disseminated disease, the few survivors sometimes having responded to weekly alternating injections of 4 potent cytotoxic agents (an example of a sequential treatment which is not a type of regime which will be discussed herein as combinatorial therapy). But the problem is how to treat the vast majority of cancers responding poorly to apparently well-tested therapeutic measures. Repeated treatment becomes increasingly ineffective (usually through the development of drug resistance), and at the same time the side effects make cancer patients chronically sick with very poor quality of life.

2. Combination therapy

It has become increasingly clear that a single or several courses of treatment with one modality is seldom efficacious; this is why much greater emphasis is being placed on using multiple modalities. In talking about multiple modalities, this does not mean that management of a cancer patient simply goes from one modality of treatment after another as each one fails. Sequential treatments of this kind are probably not the best way of "combining drugs". Equally, there are innumerable reports in the literature where two treatments are given simultaneously, with the hope that some synergy is found, as in the experiments of Noh et al. (2004) relating to the use of arginine deiminase in conjunction with dexamethasone (see also section 4.2). This may be the easiest way to experiment, but it should be seen as the starting point for a much deeper analysis to find the *optimal arrangement* in time and dose to attain the best results. Today, a well-devised treatment plan, protocol, regime, strategy (call it what you will) should combine modalities using critical dose (treatment) levels and timings (intervals) that maximise their effectiveness while keeping side effects to a minimum, i. e. it is an exercise in *optimisation*, and this is what will be considered here as combination (combinatorial) therapy.

One drawback is that research on multiple modalities has not been seriously researched at the *in vitro* and *in vivo* levels, e. g. using relevant animal models, as will be clearly demonstrated in the body of this article. This has to be done meaningfully alongside clinical work if more and effective rational protocols are to emerge. Furthermore more dialogue is needed between bench and bedside, and vice versa, since it rarely reaches a satisfactory level.

Because combination therapy is now more widely used, the management of many cancer patients has moved into the hands of teams of experts. It stands to reason that if several different treatment modalities are being employed, experts from different disciplines are going to have to know a good deal about each others' business. In many countries, medical training has led to increased specialisation. To bring experts together from different specialism requires a strong will to integrate and share their skills, especially for the team leaders. To get the best all round management using a holistic approach also involves pharmacologists, virologists, psychiatrists, nurses and hospital chaplains, which is asking enormous commitment from many different people.

To be more rational in the use of combinatorial therapy, a good understanding of the properties and behaviour of a tumour is needed at the molecular and cellular level, as well as its pathology. The problem is that inadequate information is available, and therefore the forethought that goes into devising a treatment protocol for a cancer patient is more often than not based only of characteristics that might be typical in a particular type of tumour rather than on a truly scientific basis. In truth, it is early days in such a scenario, and what is being discussed here will inevitably be idealistic and forward thinking. Nevertheless, we have to strive towards a rational basis for treatment to avoid the alternative — the "trial and error" approach.

While it is self-evident that the problem with using multiple modalities is how best to arrange them in relation to (I) preparation of the patient for the primary intervention, (II) dose levels to be used, (III) times of treatment and the intervals between them, and (IV) the nature of supplementary support given to ameliorate the side effects of therapy. This list is not exhaustive. From basic mathematics we know that the number of arrangements (the permutations and combinations) for just a few items can be sizeable. Taking, for example, just 3 modalities, they can be arranged in 2s and 3s in 4 different ways. In life sciences, unlike mathematics, the fourth dimension cannot be left out. Hence there will be literally innumerable ways of giving the three drugs when administered at many different times and time intervals in relation to one another. Added to this is a yet further "dimension", which is that each modality can be given over a considerable dose range. One ends up with a number of possible treatment protocols that verge towards the infinite. This looks to be a huge dilemma facing the doctor in charge of the management team of a cancer patient, even though many of vast number of possibilities can be reduced principally by the limitations of the individual agencies. Protocols will improve with time as more experience in handling multifactorial problems is gained, hopefully with a more rational basis emerging from it, for this in essence is what is required.

Assuming we will be moving towards better understanding of how cells proliferate, migrate and die by apoptosis or necrosis, this knowledge provides the basis on which treatment protocols should be built (Wheatley, 2005). The point of much more intense scientific investigation is that it should lead us not only the most logical combination of treatments, but to finding *optimal* conditions in therapy, as mentioned earlier. In addition, an inquisitive scientific approach will also lead to technologies and drugs yet to be discovered that may be useful in therapy.

3. Primary intervention

Surgery can effectively remove a localised, well encapsulated tumour, and may need little more to be done to the patient other than normal follow-up at intervals to check that there is no recurrence. But in more widespread tumours and resection is quickly followed by chemotherapy or radiotherapy, as in breast cancer. Clearly when a case is advanced, the primary intervention is administered with considerable urgency, which leaves little time to plan the best possible course of action. Other patients go straight to radiotherapy (prostate), chemotherapy (choriocarcinoma mentioned above), or perhaps enzyme treatment (L-asparaginase in acute lymphoblastic leukemia) as the primary intervention. One element that seems to be missing is that many patients may not be best prepared to receive treatment, which can start of the day of admission to a hospital or in an out-patient department, as in radiotherapy of the prostate. The reason for including this remark will become apparent in the next section.

The primary intervention to be discussed here is a new modality, akin to L-asparaginase, namely the use of L-arginase. From scientific and clinical approaches, we have learned a great deal about combinatorial procedures that might show the way forward in controlling cancer and giving patients a better quality of life.

4. Argininase as a platform in cancer therapy

The several advantages of L-arginase treatment can quickly be seen. Arginine deprivation as a primary intervention is given by simple i. v. injection, causing little or no trauma to the patient, unlike most of the modalities already intimated. Second, it is human enzyme (arginase-1, predominantly found in the liver), unlike L-asparaginase or the alternative to L-arginase (namely L-arginine deiminase). Next, tumours most likely to respond to arginine deprivation are those which lack the enzymes argininosuccinate synthetase and argininosuccinate lyase (ASS and ASL). Through a channelled pathway, ASS and ASL convert citrulline to arginine. In many tumour types, the ASS/ASL genes are highly repressed, and they can be quickly assessed in this respect and identified as suitable for treatment. In a remarkable analysis of the level of ASS in 92 renal cell carcinomas, Yoon et al. (2006) found that all of them were ASS-negative. [However, it is surprising to find how little work has followed from this on renal carcinomas.] Therefore biomarkers of susceptibility are evident and help in selecting patients that will best respond to treatment. It is of note that some tumours that have become resistant to drugs are nevertheless susceptible to arginase treatment. It follows that a number of cases where more conventional intervention has failed can still be treated by a relatively innocuous agency. Removal of arginine is a treatment that can therefore be applied late in the course of a tumour such as hepatocellular carcinoma, such as in cases that are unresectable (Izzo et al., 2004).

Arginine deprivation can be tolerated for considerable periods of time, as shown both in laboratory animals and man, does not cause any loss of quality of life, and shows no evidence of eliciting an immunogenic reaction. Should it do so, L-arginase, like L-asparaginase used in acute lymphocytic leukemia (ALL), can be introduced into erythrocytes for reinjection into a patient to protect the host (*Yann God-

frin, personal communication, ERYtech SA, Lyon France; encapsulated L-asparaginase is GRASPA®). Indeed, it would seem from the data of Hernandez et al. (2010) that it might be advantageous to encapsulate both enzymes to treat ALL.

Using an arginine platform as a "non-toxic"/non-traumatic primary intervention, sensitive tumours can show growth arrest growth and regression; the first objective of gaining control over a tumour is therefore accomplished. But it is preferable to prepare the patient before the enzyme is administered. In experimental mammals, the effect of arginine deprivation will be mitigated by 3 factors, (a) the citrulline pathway mostly in the kidneys converting this precursor into arginine (not relevant where the ASS/ASL channel is highly repressed), (b) availability of arginine from the diet, which indicates that a small or no protein diet is commenced at least 48 h before treatment, and (c) gut bacteria make arginine beyond their own requirements and these will be taken up into the blood stream; hence antibiotics are given at the start of the dieting. This is a good example of making sure treatment is fully effective by "priming" (preparing) the patient. In the study by Cheng et al. (2004), patients were given high dose insulin before IV treatment with L-arginase to induce a hypoaminoacidemia, which clearly would accentuate the effect of any treatment that was going to deplete serum amino acid levels. Although this kind of priming may not be so relevant in other kinds of primary intervention in cancer, it should carefully considered.

Cell culture and animal work has shown that ASS/ASL repression is usually lost in time and tumour growth eventually may return to its earlier rate (Sugimara et al., 1992). The aim of the arginine deprivation platform was initially to extend for as long as possible this period of control along with tumour reduction, which amounted to several weeks to a month or more in many cases. Not only is controlled gained over tumour growth, but animals are far less traumatised than with other forms of primary intervention. From this platform and during this same period time or after it, subjects can be given other modalities to prevent renewed proliferation, sustain control (ideally indefinitely), and optimistically lead to further regression and possibly remission of the tumour. To go deeper into combinatorial therapy in this context, several examples have been chosen from *in vitro* and animal tumour model work that more rational approaches to treatment can be devised that help in the design and improve protocols for cancer therapy, as well as entirely new protocols. Some of these ideas have been discussed in greater detail in several reviews than will be done here (Wheatley, 2004; 2005; Wheatley et al., 2005; Cheng et al., 2007).

4.1. In vitro experiments with L1210 cells

The first example comes from in vitro cell culture investigation with L1210 murine leukemia cells, which has suddenly become more relevant because of new findings that L-arginase treatment has led to good responses in clinical trials on adult T-ALL (Hernandez et al., 2011). In this L1210 experimental series, combination treatment was with hydroxyurea (HU) and differed in that the dose levels, and the order and the times (time intervals) of treatment were carefully chosen from pilot experiments (Wheatley, 2004; see figures 2 and 3 therein). Also HU was the drug of choice because is quickly washed out without trace from cultured cells, and being an inhibitor of ribonucleotide reductase, it produces no significant damage to DNA at low doses, as would 5-FU, cisplatin or alkylating agents. Treatment with HU given after arginine deprivation was remarkably good in arresting further proliferation (given day 2 or 4 after the start of arginine deprivation), causing a very significant drop in cell number with massive cell death leading to almost complete obliteration of the cells at relatively minimal doses. The results of the crucial experiment are shown in Figure 1, with the treatment details being found in the legend. The experiment was repeated with cisplatin at a dose that was ~50% inhibitory ($1 \cdot 10^{-3}$ M), and similar results were recorded except that less recovery was seen with cisplatin treatment in the controls where arginine was present during the drug treatment. This is probably explained by the reversibility of HU at wash-out being far greater than cisplatin from the start of the recovery phase after day 4, and a higher dose of cisplatin relative to the more critical level of HU used. These experiments inevitably lead to intensive and extensive series of experiment to further optimise the protocols, and especially to explore the effects of giving the drugs in different orders and doses, as well as determining the best intervals between treatments.

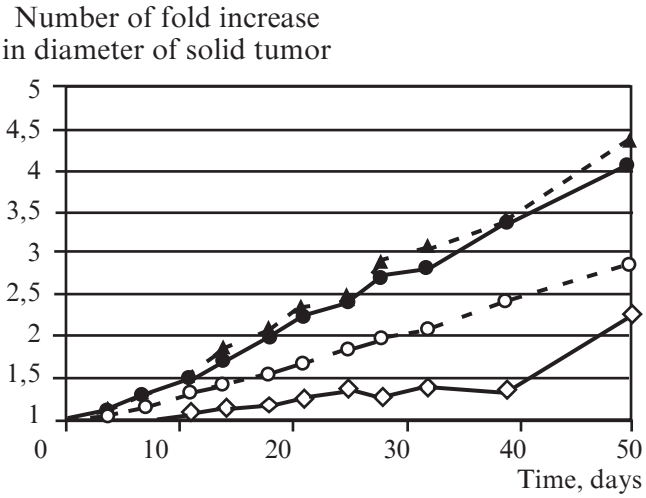

Fig. 1. Effect of rHArg-peg$_{5000mw}$ on the growth of HCC implanted into nuce mice. Mice were implanted with 10^6 Heb3B human HCC cells subcutaneously, and were allowed to grow until tumours had reached 5–10 mm in diameter. The animals were then randomly assigned to four groups (n=10 per group), which then received each week (a) saline (●); (b) 250 IU of arginase (○); (c) 5-FU at 10 mg/kg (▼); (d) both arginase and 5-FU (◇). The tumours were measured every second day and the increase given relative to the time of treatment. (From Cheng et al., 2007; with permission of Cancer Research; AACR journals.)

Despite the apparent fall to an indetectable level of intact cells in the HU-treated cultures that were arginine deprived, when they were washed with fresh medium and "rescued" over a period of 12–28 days, we were surprised to find occasionally some small regrowth occurring from very sparse colonies. Repeating the treatment and taking much greater care during the recovery phase using higher fetal calf serum levels in an enriched medium used conventionally for cloning purposes, this phenomenon was much more clearly seen up tp ~35–40 days. Closer microscopic inspection post-treatment showed that some giant L1210 cells resisted the treatment and persisted (see Philip et al., 2003; their figure 3). The only conclusion that could be drawn is that these cells were probably responsible for "repopulating" the cultures. This notion is in accord with the results of Erenpeisa et al. (2000) and subsequently Rajaraman et al. (2006). The former group showed that giant cells could undergo "restitutive division" and spawn near diploid cells that could proliferate in time as well as the original L1210 cells used in the experiment. The latter group called this process "neosis", which is contrary to the received wisdom in pathology that the frequently seen giant cells in tumour biopsies are non-viable (terminal) cells. Clearly thorough scientific investigation has turned up some unexpected findings that dispel the old dogma (Wheatley, 2008). But this is precisely why we have to consider how best to follow L1210 cells after we thought they had been wiped them out. The logical step now is to add anti-mitotics such as vinblastine to the protocol at the appropriate post-arginase/HU treatment interval, a procedure that urgently needs investigating. The implications of this must resound in all cases were tumour eradication in the clinic has been as thorough as possible, but recurrence is invariably evidence of residual tumour cells, which can occur months or often years after the last treatment.

4.2. HCC animal tumour model responses to combinatorial treatment

In an initial series of experiments on nude mice bearing a xenografted human HCC tumour the first requirements were to find dose levels of L-arginase that would slow down the rate of tumour cell proliferation to an easily discernible (measurable level) of about half that of the controls in order to ensure a clear response had been obtained. The next task was to determine a dose level of a second agent, in this case 5-fluorouracil (5-FU), marginally less than was needed to show a definite effect on tumour growth on its own. With the chosen doses of the 2 agents, animals were treated over a period of nearly 2 months, being given as weekly injections to 4 groups of mice: (I) mice given only saline placebo injections, (II) those given only L-arginase, (III) those given only 5-FU, and finally (IV) mice given both agent. The results of this series in a quest towards minimising dose levels showed quite dramatically that, while 5-FU alone had no effect on tumour growth, a 40–50% reduction was achieved with L-arginase alone, and very much slower growth in the combined treatment, with recovery of tumour growth occurring after about one month (Fig. 2). The health of the animals given L-arginase alone or in combination with 5-FU was very good throughout the treatment, showing that the low dose combination had minimal side effects. The object of this series was not to show regression of tumour, but as a prelude to optimisation of dose levels the combination treatment by further experimentation to find other slightly higher levels that can have a greater impact of the tumour over a longer period of time (in progress).

The combination treatment used so far has been the simplest in that the drugs were administered together. A strategy akin to that seen in the *in vitro* work in section 4.1 needs to be adopted. But experiments using just two agents at different times and different time intervals in relation to each other, in different orders along with adjustments of the dose levels create a very complex and time-consuming programme; however, this is what has to be done to improve protocols that might later translate well to the clinic. Manipulating the variables as experiments proceed from by taking as many interim measurements as possible can eliminate those arms of a

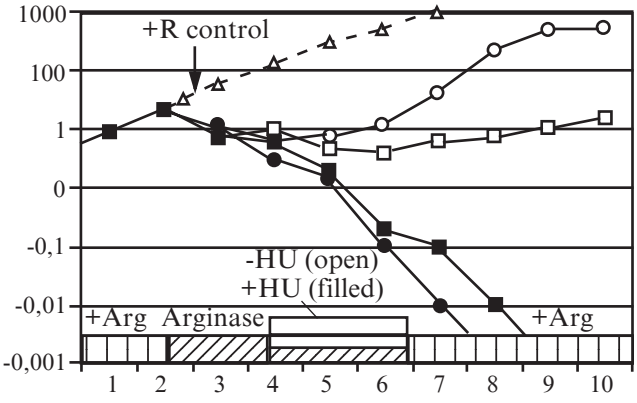

Fig. 2. L1210 cells in culture were grown under arginine deprivation with 1 U/ml arginase for up to 5 days in fresh arginine-containing medium, except for one set of cultures that received no enzyme as the postive control (△). The remaining 4 paralled culture sets (3 at each time-point) received arginase for 2 days (days 2–4) or 4 days (days 2–6) as shown in the lower bars. They were rescued in arginine-containing medium without enzyme at day 4 in 2 cases (●○) and given either $2 \cdot 10^{-4}$ M HU (●) or no HU from days 4–6 (○). In the remaining 2 culture sets, HU was added to one of them with continued arginase treatment (■), while its partner set got no HU (□). These cultures were rescued with fresh arginine-containing medium of day 6. Growth of cells in the cultures after recovery is shown. In the case of the control (△), growth was exponential to ~10^6 cells/well. None was seen for at least 2 days after arginase treatment. In cells exposed to the enzyme for either 2 or 4 days, no growth was seen in the following days and the cultures proved to have nothing more than dead husks of cells (*), some of which still being recorded by the electronic Coulter counter. Cells not receiving HU showed good recovery of growth after the 2-day exposure (○) and a slower recovery after 4 days (□), the latter soon thereafter recovering close to exponential growth (not shown in this experiment) after about day 10. Thus combined treatment of arginase followed by a critically low level of HU was sufficient to prevent recovery of cells from the arginine-deprived state alone. (From Wheatley, 2004; reproduced by permission of Anti-Cancer Drugs.)

strategy that are proving to be ineffective, thereby reducing the seemingly endless possibilities of finding the best combination, while cutting down the use of animals and the costs of expensive resources. This procedure, referred to as "flexible experimentation", gets away from the execution of rigid experimental protocols that have to be pursued without change to the end, no matter what seems to be happening during their time-courses. Flexible experimentation of animals would more closely follow the way cancer patients are treated in the hospital. There is no point in continuing a protocol on experimental animal models if treatments were having no effect on the tumour and/or markedly reduced the quality of life. It stands to reason that faster progress can be made when a more flexible approach is adopted, and combinatorial treatments give excellent opportunities to rethink and redesign an experiment while in progress.

The most important issue in future work on the basic framework of combinatorial treatment in cancer research is that more emphasis should be placed on the timing of treatments relative to one another. The questions emerging from the above experimental example are: what would happen if the 5-FU treatment was given before or half way through treatment with arginase, or perhaps after had been completed, possibly starting a day or a week later? And how much can more changes in dose levels of the agents relative to one another brought about an even better outcome.

4.3. Use of "antimetabolites" in combinatorial therapy

A wide range of therapeutic substances include anti-metabolites. The aim is to block pathways that, in the case of cancer, would nourish and tend to enhance tumour cell growth. Simple starvation has been an approach that sees mixed responses in cancer patients, although there have been some remarkable remissions with this course of action alone. But this is where the arginase platform is particularly useful. Starving a living creature of arginine that cannot make enough itself (it being a semi-essential amino acid) will slow down any sort of growth and over time lead to weight loss, and tumours will also be affected (Anon, 1973). The problem with tumours is that they are sometimes less affected by deprivation than host tissues through their strong ability to scavenge. At one time this was thought to be a prominent feature of many cancers, but many more studies on amino acid deprivation studies in recent years suggests that tumours generally remain vulnerable to their absence, and indeed may be more sensitive than growing host tissues to cell death since they cannot often enter into a quiescent state (Wheatley, 1998). However, removing arginine alone goes further than a response to total amino acid starvation because normal cells can continue because their ASS/ASL activity will allow them to generate arginine from citrulline. Regression of a tumour is commonly seen, but even a good response is unlikely to lead to the complete disappearance of the cancer. But an anti-metabolite can assist this process where only a partial regression is achieved, and again it becomes a matter of which one would be most suitable in any particular circumstance, followed by further decisions on how and when it should be administered in relation to other interventions. In a tumour that is responsive to L-arginase, it follows that an anti-metabolite is probably best given towards a day or two before the end of the period of primary intervention when some arrest or regression has already been recorded. The idea is that the anti-metabolite can inveigle itself in a tumour already weakened by arginine deprivation. The most sensible approach would be to use analogs of amino acids that are known to induce death of tumour cells on a large scale. In tumour cells that still attempt to proliferate, a marked shut-down or disturbance in their synthesis of proteins will affect them more than the same analogs would interfere with normal cells that are becoming increasingly quiescent as arginine deprivation continues. Thus there is a greater selective pressure against cancer cells.

Considerable work has been done on the action of anti-metabolites in cancer. There are a vast array to choose from, but clearly there are some that are much more commonly used than others, While anti-metabolites are mostly substances interfering with purine and and pyrimidine metabolism, in a wider sense it must be a substance that interferes with some (or perhaps many) pathways that constitute the metabolome. With regard to arginine, we need inhibitors of amino acid utilization or analogs that mimic the authentic amino acid. There are now hundreds of analogs of arginine, most prepared as potential inhibitors of the nitric oxide synthetase action on arginine in the production of NO, but we will concentrate of canavanine in arginine-free and arginine-rich medium.

L-canavanine is known to be a toxic amino once it has been incorporated into proteins, with ~6% the efficiency of L-arginine. In the presence of L-arginine, it has almost no effect, but it is devastating to cells in its absence, CHO cultures being destroyed within 24–36 h. The same has been found in at least 3 other cell lines including (Hela, Molt4 and SaOS2). The death of CHO cells is shown in Figure 3 and Molt4 in Figure 4, the latter showing more clearly the demise by using a log-scale on the ordinate axis. As a measure of the severity of interference, tritiated thymidine incorporation into CHO cells was followed for a period of 4 h each day at normal ($4 \cdot 10^{-4}$ M), low (10^{-5} M) or deficient arginine concentrations, with or without ($4 \cdot 10^{-4}$ M) L-canavanine (Table 1). The treatments give the fastest demise seen in arginine deprived cultures, but it can be substantially deferred by adding 10 μg ml^{-1} cycloheximide at the same time as L-canavanine to inhibit protein synthesis by ~90% (not shown), in which case the curve is approximately equivalent to that of arginine deprivation alone. The importance of incorporation of the analog into proteins is confirmed by this finding.

Another effective amino acid analog has been studied in phenylalanine deprived cultures of HeLa

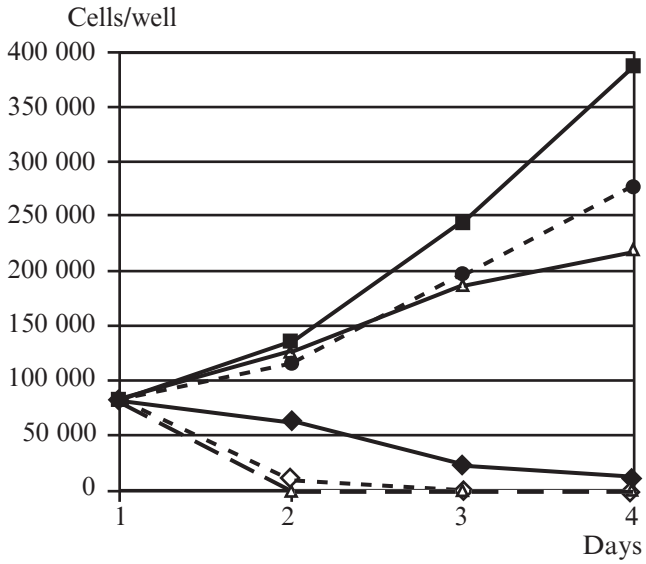

Fig. 3. CHO cells were set up the previous day to reach ~100,000 cells per well on day 1 when treatment commenced. The first pair of culture sets had arginine in the medium at $4 \cdot 10^{-4}$ M alone (■) or $4 \cdot 10^{-4}$ M canavanine present (□). A further pair of culture sets were given low arginine levels ($4 \cdot 10^{-5}$ M) either with (●) or without (▲) canavanine at $2 \cdot 10^{-4}$ M. In a final pair of culture sets, both were arginine-free (open triangles), but again one had canavanine at $2 \cdot 10^{-4}$ M (○). The destructive effect of the addition of canavanine is very pronounced at low arginine or arginine deprived cultures, although it only slowed cultures mildly when at equimolar concentrations with respect to arginine

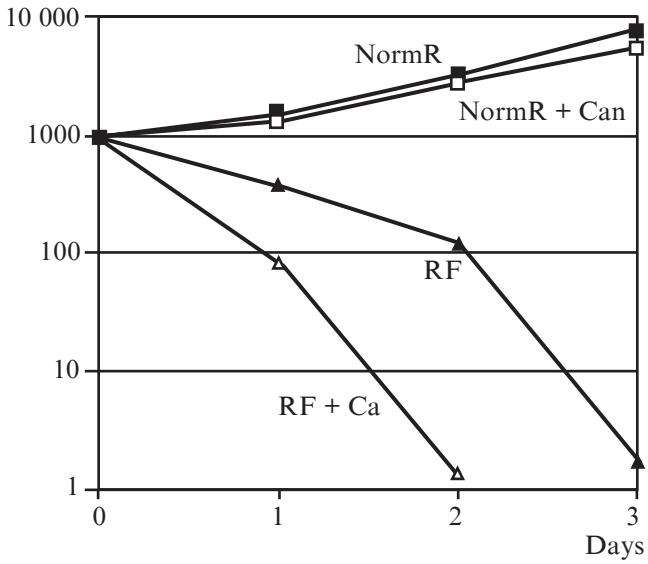

Fig. 4. Molt4 human leukemic cells in normal arginine-containing medium at $4 \cdot 10^{-4}$ M were treated with either no analog (NormR) or $4 \cdot 10^{-4}$ M canavanine (NormR + Can). In the lower curves, both sets of culture were arginine-free from day 0 (RF) with one received canavanine (RF + Can). The ordinate is a long scale showing that cells starting at 100,000 per well (ordinate is × 100) per well were down to an almost uncountable number of cells by days 2 and 3, notably faster in the presence of canavanine. In the presence of arginine, canavanine had no significant effect

and CHO cells, namely p-fluorophenylalanine. Like arginine deprivation, phenylalanine-free medium slows cell growth but seldom induces cancer cell death in vitro on the massive scale see with as arginine deprivation. But when cells in phenylalanine-free medium were given p-fluorophenylalanine that incorporates ~30% as efficiently as phenylalanine into proteins, cell death was several-fold greater but still weak compared with canavanine introduced into arginine-free medium. HeLa cells, however, were arrested from entering mitosis within 15 min of p-fluorophenylalanine administration. We had also shown that cells incorporated the analog into proteins became very heat-sensitive (Henderson and Wheatley, 1974); by raising the temperature of incubation by just 2.5–3 °C led to a very much more significant amount of cell death. This type of combination treatment seems to work well in culture. It is in many ways like other sensitising agents used in cancer treatment that can result in heightened cell death, as for example with photodynamic therapy with agents that need light activation after accumulated in a tumour. However, it does illustrate another general area of intervention that can be usefully applied in a combinatorial manner.

5. Concluding remarks

The experiments discussed herein along with similar reports throughout the literature, give us increasing guidance as to what is needed in vivo as far as cancer therapy is concerned, which has to be based as far as possible on our increasing knowledge of cell cycling of tumour cells and changes post treat-

Table 1

Effect of L-canavanine at $4 \cdot 10^{-4}$ M in medium with $4 \cdot 10^{-4}$ M (normal), 10^{-5} M (low), or no arginine present, as indicated by loss of tritiated thymidine incorporation for 1 h per well (mean plus 1SD of an average of 4 wells per assay in dpm) for CHO cells. (Average background of 33 dpm subtracted from all values.)

Days	Normal medium	Normal medium + canavanine	Low medium	Low medium + canavanine	Deprived medium	Deprived medium + canavanine
0*	11 664±662	10 129±771	6530±1064	6589±1329	2557±145	3346±1046
1	10 407±160	12 800±354	5740±690	820±340	1268±171	427±203
2	18 708±1558	20 421±1323	2441±250	123±67	256±18	28±55
3	24 307±1328	19 700±1249	255±108	27±16	57±14	0±0

Note. * — tritiated pulse given at from 5 to 6 h on Day 0.

ment in their characteristics (through changes in gene expression, invasive potential, metabolomic features, etc.). More animal models, especially of xenografted human tumours, need to be explored with combinatorial therapies, which may well be most appropriate to translate to the clinic, where trials from an arginine basis have begun to increase. Clinical use of combination therapy on a rational basis has to seek for synergies at the *lowest* dose levels of toxic agents, including radiation. This could improve prognosis for many cancer sufferers, notably so in the case of disseminated disease.

The main aim of the oncologist is to gain control over a cancer rather than necessarily strive for complete remission. At the same time, a holistic approach will ensure that a reasonable quality of life is sustained patients under the treatment regimes that have been all too superficially discussed in the space available in this article. The importance of this discussion is that it relates directly to the growing number of clinical trials now in progress with arginine deprivation as it basis (e. g. Izzo et al., 2004; Cheng et al., 2005; Hernandez et al., 2010). Considering the number of sensitive tumour types that are known from their ASS repression status, it is clear that many other trials should soon be initiated; for example, the convincing data from renal cell carcinoma (Yoon et al., 2006) suggests an obvious case in which the arginine platform as a prime intervention can lead on the effective combinatorial therapy.

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