

Regrowth Concentration Zero (RC_0) as Complementary Endpoint Parameter to Evaluate Compound Candidates During Preclinical Drug Development for Cancer Treatment

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Abstract

The screening process for potential anticancer drugs involves expensive and time consuming preclinical and clinical trials (CT) before a drug is approved for clinical use (CU). At present, there is a “bottleneck” at the CT/CU transition because many drugs that showed promising results during preclinical research did not pass clinical trials. We speculated that the endpoint parameters (the inhibitory concentration 50 (IC_{50}) or lethal concentration 100 (LC_{100})) commonly used in proliferation assays for short-term periods (24-72 h) are not useful to predict the antiproliferative effect *in vivo*, especially during clinical trials. We propose the use of a parameter, regrowth concentration 0 (RC_0), which will define the concentration and time necessary to kill 100 % of the cells and prevent regrowth when drug is removed. The RC_0 might introduce a new bottleneck at the preclinical stage, “preclinical bottleneck”, that will select for drugs with more chances to pass clinical trials and improve the success rate of anticancer screening programs. Our proposal is supported by experiments done with the DBTRG-05MG human glioma cell lines exposed to short and long-term incubation with three different DNA replication inhibitors (aphidicolin, hydroxyurea and etoposide) and retrospective analysis of clinical trials for these drugs.

Keywords: Drug screening; DNA replication; Glioma; Aphidicolin; Hydroxyurea; Etoposide

Introduction

In general, the standard approach to evaluate novel compounds for cancer treatment after drug synthesis or discovery is based in preclinical testing and clinical trials (Figure 1 top). The pre-clinical phase involves *in vitro* as well as *in vivo* research. The entire process is very expensive and time consuming (DiMasi et al., 2003; Emanuel et al., 2003). Commonly, *in vitro* experiments for determining the antiproliferative effects of a potential antineoplastic drug are done either in cell free systems or in cell lines by means of short term proliferation assays which measure the incorporation of tritiated thymidine or BrDU into DNA or mass cell by colorimetric methods (e.g. MTT assay). In these assays, cell line(s) are tested against a broad range of drug concentrations typically for 48-72 h and the results are usually reported as a plot and the IC_{50} and LC_{100} (See Glossary) are calculated by interpolation (Brown, 1997; Iljin et al., 2009). For instance, in the NCI60 human tumor cell line anticancer drug screen program, the GI50 (50% growth inhibition) and

LC_{50} (50% lethal concentration) are derived from concentration-response curves by linear interpolation while the TGI (total growth inhibition) is read as the x-axis intercept (Shoemaker, 2006). The main pitfall of these assays is that the short term incubation is not enough to determine the minimum concentration of the drug that actually kill 100 % of the cells preventing regrowth when the drug is removed from the culture. In practice, the continuous growth of the untreated cells (control) and cells exposed to low concentration of the drug limits the assay because of loss of linearity over time. Long term survival for screening purposes has been assessed by the tumor colony-forming assay on a moderate scale due to technical limitations (Shoemaker et al., 1985). When used, researchers often report the IC_{50} for the tested drug (Sasaki et al., 2008). After the optimal drug concentration (e.g. IC_{50} or LC_{100}) is determined, the next step involves testing the compound in animal models *in vivo* where a tumour is induced by injecting cancer cells into specific organs. The drug is administered in control and experimental groups and the antineoplastic effect is usually assessed by tumor growth and/or survival rates (Kaplan-Meier plots). Due to ethical considerations, animals are usually sacrificed after few weeks and long term relapses are not evaluated after treatment discontinuation. In case the compound shows promising *in vivo* effect on tumor growth, acceptable side effects and toxicity, the drug is considered a good candidate to be tested in clinical trials. These stages are associated with a significant percentage of the total cost of the entire drug evaluation process (DiMasi et al., 2003; Emanuel et al., 2003). There are several examples of newly developed compounds that might be considered for clinical trials (Roth et al., 2009; Xu et al., 2009; Yakisich et al., 2009) but at present it is difficult to estimate the chances that they will be successful. Unfortunately, when promising drug candidates are tested in clinical trials, the majority of them fail. In most cases, the outcome has been disappointing and sometimes

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the trial terminated early due to evident failure (Robe et al., 2009). This problem was recognized by Brown (Brown, 1997) who reported that the popular NCI's anticancer drug screening program does not select for clinically active compounds, but no potential solution or important changes have been implemented to solve the problem.

Our hypothesis is that the high rate of failure when translating preclinical drug screening into successful clinical trials is due to the use of the IC₅₀ (and less common LC₁₀₀) as guiding concentration for mechanistic studies as well as goal for clinical trials. At present, current drug development programs have a "bottleneck" at the "clinical trial"/"clinical use" transition, where the success rate is low (Figure 1 Top). It is estimated that only 5% of cancer drugs entering clinical trials reach marketing approval (Collins and Workman, 2006). In this paper, success rate (SR) is defined as the ratio between the number of drugs approved for clinical use (n_{CU}) and the number of drugs that enter the clinical trial stage (n_{CT}). Conceptually the SR ($SR=n_{CU}/n_{CT}$) is similar to the "clinical approval success rate" defined as "the probability that a compound that enters the clinical testing pipeline will eventually be approved for marketing" (DiMasi et al., 2003). In some cases (e.g. roscovitine as drug candidate for brain tumours), the concentration that actually reach the target tissue is maintained at levels that are below the IC₅₀ reported *in vitro* and might limit the clinical use (Yakisich et al., 2009). Moreover, even if, in the target tissue, concentrations similar to the IC₅₀ are reached, one can anticipate that a fraction of cells will survive and resume cell proliferation when the drug is removed. Interestingly, in a study measuring *in vitro* chemo sensitivity in patient-derived cell lines by a short-term assay (24-72 h), the ID₅₀ (defined as the dose of the drug that inhibited protein synthesis by

50 %), was used to classified between responder and non-responder patients. The relapse-free interval in patients that showed *in vitro* chemo sensitivity (responders) was modestly increased compared to patients designated as non-responder (Thomas et al., 1985).

Increasing the number of clinical trials by simply screening more drugs, without any additional rational guidance, will create an unproductive "financial bottleneck" at the "preclinical/clinical trial" transition due to the astronomical cost associated with the clinical trials. Due to this "financial bottleneck" some drugs that might be of clinical relevance, will probably never be tested while other that enter the clinical trial stage will eventually fail at the same rate (keeping the 5 % rate success constant).

In this paper we propose that the success rate and cost benefit for drug development could be improved by introducing a "bottleneck" during the preclinical stage ("preclinical bottleneck") using the so called RC₀ (see glossary) as endpoint parameter instead of IC₅₀ or LC₁₀₀ (Figure 1 Bottom). The rationale of our proposal is supported by a series of experiments using prolonged exposure to three classical DNA replication inhibitors with different mechanism of action (aphidicolin, hydroxyurea and etoposide) in the human DBTRG-05MG glioma cell line and a retrospective analysis of clinical trials with these same drugs.

Materials and Methods

Cell lines

Stock cultures of human DBTRG-05MG glioma cell line were obtained from the European Collection of Cell Culture (ECACC). Cells were routinely cultured in RPMI-1640 medium supplemented with 10 % Foetal Bovine Serum (FBS), 2 mM glutamine, 1 % HT and 1 mM sodium pyruvate (complete media) at 37 °C in a humidified atmosphere containing 5% CO₂.

Drugs

Hydroxyurea, Aphidicolin and Etoposide (Sigma, Sweden) were prepared as stock solutions (1 M in H₂O, 2.5 mM in DMSO and 25 mM in DMSO respectively) and stored at -20 °C until use. Fresh dilutions in culture media were prepared just before use.

Antiproliferative assay

DBTRG-05MG cells were plated in 96- well flat bottom plates at 5000 cells/well and were allowed to adhere overnight. Then, the cultures were exposed to a concentration range of the three drugs selected and control cultures where treated with the equivalent concentration of the corresponding vehicle (DMSO or H₂O). After 72 h, cell growth was monitored using the CCK kit (Sigma, Sweden) according to supplier instructions. For long-term antiproliferative assays, drugs were maintained in culture for 2-10 weeks. The media and drugs were changed twice a week and were found to be sufficient to prevent cell growth (see result) indicating that the drugs remain active during *in vitro* prolonged cultures.

The presence of surviving cells during prolonged exposure and the (re)growth after drug removal was evaluated using a routine inverted microscope. Regrowth was defined as the abil-

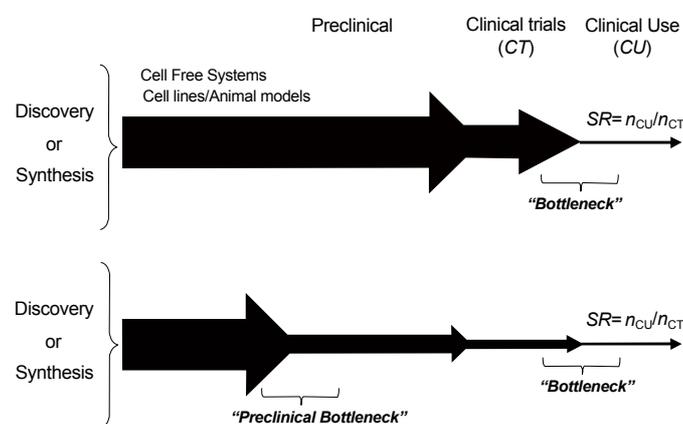


Figure 1: Top) Simplified diagram showing the common steps during drug development from drug discovery to clinical trials. After discovery or synthesis of a new potential antineoplastic drug, the compound undergoes preclinical research where is tested in cell free systems as well as *in vivo* in cell lines and animal models. Promising candidates that pass the preclinical stage are approved for clinical trials and few reach the "clinical use" stage. At the "clinical trial"/"drug use" transition the presence of a "bottleneck" limits the success rate. The success rate (defined as the number of drugs that reach the stage of clinical use divided by the number of drugs that enter the number of drugs clinical trial; n_{CU}/n_{CT}), value between 0-1, is an indication of the cost/benefit of the program.

B) Proposed outcome of drug development by introducing a "preclinical bottleneck" by using the RC₀ as endpoint parameter during preclinical research. The presence of the "preclinical bottleneck" will reduce the number of cells entering the "clinical trial stage" and might increase the success rate improving the cost/benefit.

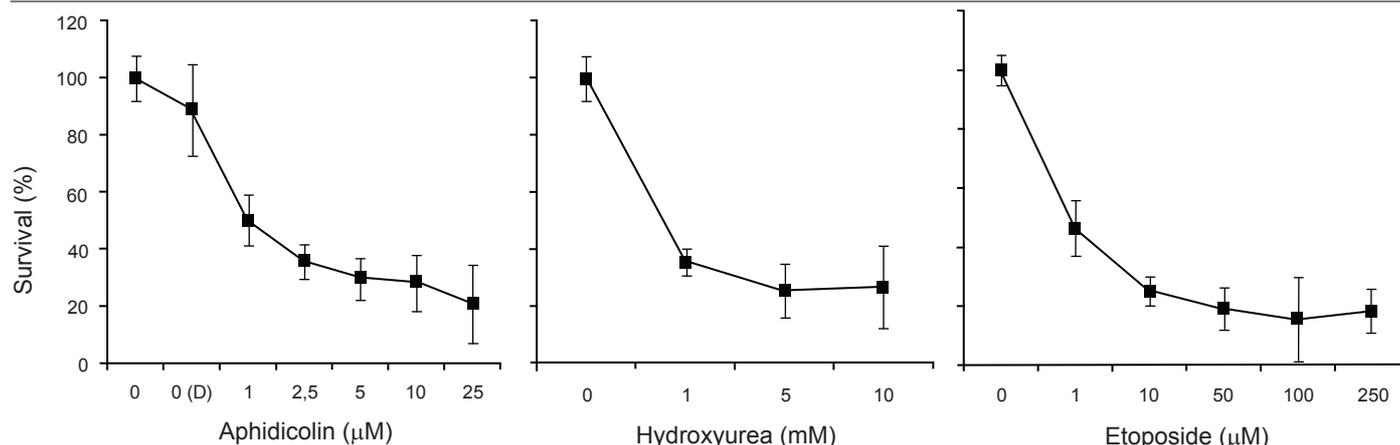


Figure 2: Antiproliferative effect of short term incubation with Hydroxyurea, Aphidicolin or Etoposide. DBTRG-05MG glioma cells were incubated with the indicated concentration of drugs during 72 h. Cell proliferation was measured by the CCK kit. Results are representative of three independent experiments performed by quadruplicates.

ity of surviving cells to form a monolayer after 1-2 weeks of incubation with drug-free media.

Results

We evaluated the short term antiproliferative effect of hydroxyurea (HU), aphidicolin (Aph) and etoposide (Et) on DBTRG-05MG cells in culture. Exponentially growing cells were incubated with different concentrations of each drug and cell proliferation was measured at 72 h using the CCK kit assay. All three drugs tested showed a concentration-dependent inhibitory effect reaching the maximum effect at 2.5 μM, 10 mM and 50 μM for Aph, HU and Et respectively (Figure 2). The IC_{50} was estimated by interpolation as 0.9 μM, 0.75 mM and 0.9 μM for Aph, HU and Et respectively.

Long-term incubation (4 weeks) with concentrations $> IC_{50}$ (Aph, 2.5 μM; HU, 10 M; Et, 5 μM), showed a subpopulation of cells resistant to these drug concentrations. Microscopic examination revealed that a small fraction of cells remained attached to the surface. When the drugs were removed from the culture, the surviving cells were able to resume cell division and, they proliferate reaching a monolayer morphologically indistinguishable from the original culture. To prevent cell regrowth higher concentrations of HU (50 mM) or Et (> 25 μM) for at least one week were required.

Discussion

We used the human glioma cell line DBTRG-05MG as an experimental system to retrospectively analyze the failure of several drugs that inhibit *in vitro* cell proliferation of cancer cell lines during clinical trials. Gliomas are the most common primary brain tumours and remain poorly responsive to multimodality therapeutic interventions, including surgery, radiotherapy, and chemotherapy. The highly proliferative activity of glioma cells compared to normal brain makes DNA replication an interesting target for therapeutic purposes. In this paper, we evaluated the outcome of DBTRG-05MG glioma cells exposed to three different DNA replication inhibitors for short and long-term exposures (Figure 2, Figure 3).

Aphidicolin: The *in vitro* IC_{50} for aphidicolin has been determined in cell free systems as 0.5, 0.9 and 5.8 μM for polymerase α , δ , and ϵ respectively (Wright et al., 1994) without affecting

dNTP pools (Sheaff et al., 1991). Due to the poor solubility and because pharmacologically active levels had not been achieved for aphidicolin, it was decided early (after two phase I studies) to stop further evaluation (Beijnen et al., 1995). Therefore, aphidicolin represents a good example of a potential anticancer drug that reached the stage of clinical trials and its use for cancer treatment was stopped early based in its pharmacokinetic properties.

Hydroxyurea: In our study, the IC_{50} for DBTRG-05MG cells was ≈ 0.5 mM (Figure 2). Pharmacological studies showed that the level of HU after a single dose of 1200 mg can reach only 0.04 mM and 0.26 mM in cerebrospinal fluid and plasma respectively (Gwilt et al., 2003). DBTRG-05MG cells were able to resist prolonged treatments (> 4 weeks) with 10 mM while higher concentrations (50 mM) were required to prevent regrowth. Plasma concentration might be enough for treatment of some types of cancer, but definitely not for brain tumors. Hydroxyurea alone has been proven at early stage to be of no use for glioma treatment and represents an example of a drug that (based only in IC_{50} values) could have been stopped at the “preclinical bottleneck” before engaging in costly and time consuming clinical trials.

Etoposide: Several large numbers of clinical trials and pharmacokinetic data showed that a daily oral etoposide dose of 50 mg/m² produces serum concentrations > 1 mg/L (~ 1.7 μM) lasting several hours each day (Hainsworth, 1999). Potentially cytotoxic concentration in cerebro spinal fluid (CSF) was achieved with doses higher than 300 mg/m² i.v. (levels of 0.175 μM; range, 0.066 to 2.12) in children with acute lymphoblastic leukemia (Relling et al., 1996). In our study, the IC_{50} for DBTRG-05MG cells was < 1 μM but the RC_0 was > 25 μM (Figure 3). Even though at present etoposide alone has been of limited use in brain tumors, our data predict that clinical trials with etoposide will continue to fail unless intracerebral levels of > 25 μM are reached. The use of etoposide at very high doses (800 mg/m²) in combination with autologous bone marrow transplantation increase the CSF levels (similar to IC_{50} but not to RC_0 levels) still, in brain tumor tissue the outcome has been disappointing (Giannone and Wolff, 1987; Leff et al., 1988). Etoposide is an example of a drug that based in IC_{50} values should have been successful for glioma treatment, but clinical trials proved to be of very limited use (Finn et al., 1985; Fulton et al., 1996; Tirelli et

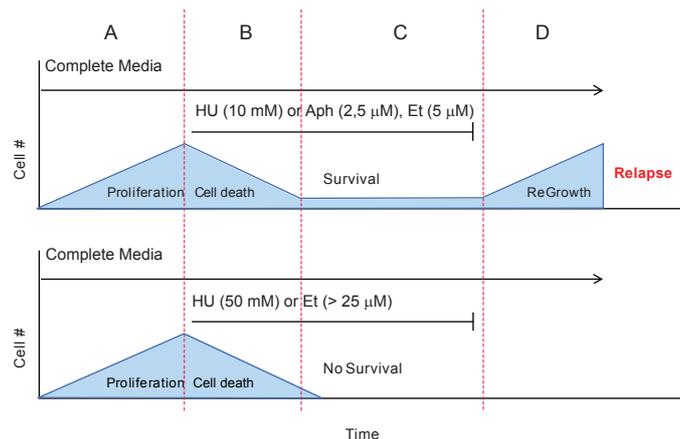


Figure 3: Top) Antiproliferative effect of long-term incubation with Aphidicolin (Aph, 2.5 μM), Hydroxyurea (HU, 10 mM) or Etoposide (Et, 5 μM) on DBTRG-05MG glioma cells. Exponentially growing cells were incubated in complete media for 2-3 days (A), exposed for 4 weeks to the indicated drugs concentrations (B-C). During this period the media and the drugs were changed twice a week. A clear decrease in the cell density was observed by microscopic examination indicating extensive cell death (B) followed by a stable low cell density (C). When the drug was removed, the surviving cells resumed proliferation and formed a monolayer indicated as regrowth in (D). Bottom) same as top but cells were incubated with higher concentrations of HU (50 mM) or Et (25 μM).

al., 1984). If the RC₀ have been used as guide, etoposide as single agents would have never been attempted saving cost and time. Instead, etoposide would have been used from the beginning in combinational therapy with other drugs.

The three drugs we evaluated have RC₀ much higher than IC₅₀ and retrospectively demonstrate that clinical trials have poor outcome when the drug concentration reach similar or just slightly higher values than the IC₅₀. In m-AMSA treated cultures at LD₈₀ concentration, a fraction of stem cells survive prolonged exposure (Sabisz and Skladanowski, 2009). m-AMSA is a topoisomerase II inhibitor that also works as an alkylating agent, suggesting that our study can be extrapolated to other drug types. Thus, classical end-point proliferation parameters used to evaluate drug effects (IC₅₀, LC₁₀₀) when used in short term assays, are insufficient for preclinical testing since they do not predict long term effects. This is due to the fact that a small fraction of cells may survive prolonged exposure to similar or slightly higher concentrations than the IC₅₀. The surviving cells, when the drug is removed from the culture will resume cell division. At the clinical level, when the chemotherapy regime is discontinued, surviving cells will resume cell division leading to relapse of the tumour. We propose the use of a new parameter, RC₀ (Regrowth Concentration 0; defined as the lowest concentration that produce 0% regrowth when the drug is removed from the culture) as a complementary end-point proliferation parameter. Other authors also proposed that assays that monitor surviving cells should be included during drug screening (Sabisz and Skladanowski, 2009). The main disadvantage of the RC₀ is the length of the assay (2-4 weeks) that in turn limits the number of drugs that can be screened. High throughput assays such as the NCI60 (Shoemaker, 2006) or similar cell-based screening methods (Iljin et al., 2009) can in short time, select several potential anticancer drugs (based in IC₅₀ values) from compound

libraries. After that, the RC₀ can further help to identify those drugs with higher chances to succeed in clinical trials from the initial selection.

The RC₀ provides two types of valuable information: 1) The necessary concentration to kill 100 % of tumoral cells, 2) the lapse of time the cells need to be exposed to a certain concentration. The time factor has been demonstrated to be important. For instance extended-schedule oral etoposide shows more efficacy in selected cancers (Hainsworth, 1999). Thus, preclinical *in vitro* testing should include long term proliferation assays to determine the RC₀. This can simply be done by incubating the cells for a prolonged period of time (e.g. 2-4 weeks) after that, remove the drugs and incubate the cells for another 1-2 weeks and determine the concentration of drug that killed 100% of the cells (No regrowth after incubation in drug free media).

To be of clinical use, the CR₀ should fulfil two other essential requirements: a) the drugs should be non toxic (or mild) to normal cells for the period required to kill all tumoral cells and b) It should be possible to reach this concentration in the target tissue. In this context, the data obtained from phase 0 might constitute an essential step before pursuing more advanced research (e.g phase I). A rational use of CR₀ in combination with pharmacokinetic and toxicological studies in healthy individuals (Phase 0) might lead to a more successful rate of antineoplastic drugs with clinical relevance.

Since the RC₀ > LC₁₀₀ > LC₅₀, higher toxicity to normal cells will be expected, and less number of drugs will fulfil the criteria to be approved for clinical trials. At a first glance, this will reduce the number of clinical trials that in turn will reduce the number of drugs that reach the market. However, by avoiding unnecessary highly expensive clinical trials with drugs that have little or no chances to be of clinical use, more drugs can be screened before the “preclinical bottleneck”. Resources can be put into developing more specific drugs with RC₀ levels that can be achieved in target tissue with tolerable toxicity. The RC₀ will eventually move the drug development “bottleneck” from clinical trials to preclinical stages. This displacement will eventually produce a drop in drugs that reach clinical trials but it will select for those with higher rates of success reducing the high expenses and time of clinical trials.

Thus, the use of the RC₀ has the potential to increase the successful rate and maybe, overcome the bottleneck when translating preclinical research into clinical trials. From the economical perspective, predicting which drug will successfully pass clinical trials will have a tremendous impact in the drug industry by lowering cost and time by stopping clinical trials of high number of drugs. On the other hand the use of the RC₀ will help at early stages to decide which drugs have little chance to be useful for monotherapy but might have use in combinational regimes.

Glossary

GI₅₀: drug concentration that causes a 50% reduction in cell number in test plates relative to control plates (equivalent to IC₅₀).

LC₁₀₀: drug concentration that causes a 100% reduction in cell number in test plates relative to control plates (equivalent to

IC_{100}). It is usually determined by interpolation from concentration response curves.

RC_0 : drug concentration that kill 100% of cells preventing re-growth when cells are incubated in drug free media. In contrast to LC_{100} , RC_0 is determined empirically, not by interpolation.

Disclosure of Potential Conflicts of Interest

None

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