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A rapid mechanism-based screen to detect potential anti-cancer agents

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Several mutant Chinese hamster ovary (CHO) cell lines have been adapted to the microtiter tetrazolium assay in order to obtain useful mechanistic information relevant to the cytotoxic activity of marine natural products. The sensitivity of a DNA double-strand break repair deficient CHO line, xrs-6, was compared with that of a DNA repair competent CHO line, BR1, to several known drugs. The deficiency of the xrs-6 cells makes them overly sensitive to compounds [e.g. topoisomerase II (topo II) inhibitors] that produce DNA double-strand breaks. Described here is the validation of this unique cellular screen to detect such compounds. Those drugs thought to produce their effects by the inhibition of topo II, produced the largest differential cytotoxicity against the mutant CHO pair. Other agents that are known to either produce single-strand breaks, cross-links or to inhibit the synthesis of DNA did not possess appreciably enhanced cytotoxicity to the xrs-6 line. The usefulness of the screen was shown by its ability to detect topo II inhibitory activity in several new marine natural products. This activity was confirmed by an *in vitro* enzyme inhibition assay. In contrast, the screen predicted a lack of topo II inhibitory activity in some other structurally related marine natural products and this lack of activity was confirmed by an *in vitro* enzyme inhibition assay.

Key words: Discorhabdin A, DNA double-strand break repair, mutant CHO cells, topoisomerase II, wakayin.

Introduction

In an effort to screen cytotoxic natural products for potential anti-cancer activity and to prioritize these new compounds for further testing, we have adapted several mutant Chinese hamster ovary (CHO) cell lines to the microtiter tetrazolium assay (MTA). The MTA was originally described by Mossmann,[1] modified by other investigators[2,3] and a version of this assay has been used in the National Cancer Institute's (NCI) preclinical antitumor drug discovery screen.[4] We report here some results obtained by comparing the sensitivities of a DNA repair deficient CHO line, xrs-6,[5,6] to a DNA repair competent line, BR1.[7] The xrs-6 cells are one of six mutants of the xrs complementation group isolated from CHO-K1 cells on the basis of sensitivity to X-rays.[8] The BR1 cells were isolated on the basis of their resistance to bis-chloroethylnitrosourea (BCNU)[7] and have elevated levels of O^6-alkylguanine-DNA-alkyltransferase (AT).[9]

The xrs-6 cells are DNA double-strand break repair (DSBR) deficient[6] and, based on the recent reports by Iliakis *et al.*,[10] we estimate that the xrs-6 cells are killed by the production of 15 double-strand breaks (DSBs) in their genome versus approximately 45 DSBs for repair competent CHO lines. The deficiency of the xrs-6 cells makes them exquisitely sensitive to the toxic effects of some important anti-cancer agents. These radiosensitive cells have recently been found to be deficient in V(D)J recombinase activity.[11] The xrs-6 cells are impaired in their ability to form both coding joins and joins of heptamer–spacer–nonamer sequences (RS sequences) flanking V, D or J segments. They apparently can repair single-strand breaks (SSBs).

Recent work by Jeggo *et al.*[5] and Warters *et al.*[12] has shown that the topoisomerase II (topo II) inhibitors, m-AMSA and etoposide, exhibit enhanced toxicity towards the xrs mutants, presumably via the disintegration of cleavable complexes to yield DSBs. Bleomycin, a radiomimetic drug known to fragment DNA,[13] also shows enhanced toxicity towards the xrs cells.[8] The utility of this mechanism-based screen was demonstrated by the detection of wakayin,[14] a marine pyrroloiminoquinone alkaloid isolated from a sea squirt, as a topo II inhibitor. This work has shown the ability of the screen to discriminate between wakayin and two structurally related compounds that are not topo II inhibitors. Also, we have recently reported the isolation of a new

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class of topo II inhibitors (the makaluvamines) isolated from a marine sponge.[15] The activity of the makaluvamines was originally detected by this unique cellular screen. Reported here are the results obtained with 18 different known anti-cancer drugs in this assay.

Materials and methods

Drugs and chemical reagents

Drug standards and MTT (3-(4,5-dimethylthiazol-2 - yl) - 2,5 - diphenyl - tetrazoliumbromide) reagent were purchased from Sigma (St Louis, MO) or were provided by Dr WG Harker or Dr LA Dethlefsen (University of Utah). Methylazoxyprocarbazine (MAPCZ), the active metabolite of procarbazine, was synthesized by Swaffar *et al.*[16] as previously reported. Wakayin was isolated according to the previously published procedure.[14] The marine natural products, discorhabdin A[17] and damirone B,[18] have been previously reported. We have now isolated these compounds from a different marine sponge (*Zyzzya* sp.). All drugs were dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mg/ml and serial dilutions were made from this stock.

Cell culture

The CHO cell lines were grown as monolayers in α-minimal essential medium (α-MEM) (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (Biofluids, Rockville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin and 240 U/ml nystatin (Sigma). The DNA repair competent CHO line, BR1, was developed by Barrows *et al.*[7] by the selection of transfected CHO cells for growth in BCNU. These cells express the hamster gene for AT.[9] The CHO DNA DSBR deficient line, xrs-6,[6] was a generous gift from Dr Penny A Jeggo. The cells were maintained in a humidified 5% CO_2 atmosphere at 37°C.

Cytotoxicity assays using drug-sensitive cell lines

A modification of the MTT-microtiter plate tetrazolium cytotoxicity assay originally described by Mossmann[1] was used to compare the cytotoxicity of a drug against BR1 cells with that of the xrs-6 cells. Briefly, after trypsinization, BR1 cells were counted and adjusted to a concentration of 1.5×10^5 cells/ml and 200 μl of this cell suspension were plated into the wells in the upper four rows of a 96-well microtiter plate (Corning Glass Works, Corning, NY). After adjusting the xrs-6 cells to a concentration of 2×10^5 cells/ml, 200 μl of this suspension were plated to wells in the lower four rows of the plate. After the cells were allowed to attach for 4 h, 2 μl of DMSO-containing drug solutions were added to quadruplicate wells. Initial dose-range finding experiments using order of magnitude drug dilutions were used to determine the range of doses to be examined. Control wells contained a final concentration of 1% DMSO. After incubation of the plates at 37°C in a humidified 5% CO_2 atmosphere for 18 h, medium was carefully aspirated and replaced with fresh α-MEM. Medium was again aspirated from the wells on the third day of the assay and replaced with 100 μl of fresh McCoy's medium (α-MEM medium was found to interfere with the MTT assay). Then, 11 μl of a 5 mg/ml stock solution of MTT in phosphate-buffered saline (PBS) were added to wells of the plate. After incubating the plate for 4 h in 5% CO_2 at 37°C, 100 μl of 0.04 N HCl in isopropanol were added to all wells of the plate and thoroughly mixed in order to solubilize formazan produced by the viable cells. Well absorbances at 540 nm were then measured on a BioRad MP450 plate reader (BioRad, Richmond, CA). The mean absorbance of quadruplicate drug-treated wells was compared with that of control wells and results were expressed as a percentage of control absorbance ± SD. The ratio of the IC_{50} of a drug against BR1 cells to its IC_{50} against the xrs-6 cells was determined. Enhanced cytotoxicity towards the xrs-6 cells was interpreted as evidence for the formation of DNA DSBs by the compounds.

Assay for topo II inhibitory activity

Marine natural product compounds were assayed for possible topo II inhibitory activity by their ability to inhibit the topo II-mediated decatenation of kinetoplast DNA according to modifications of the procedure of Muller *et al.*[19] Each reaction was carried out in a 0.5 ml microcentrifuge tube containing 19.5 μl H_2O, 2.5 μl 10 × buffer (1 × buffer contains 50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol and 30 μg bovine serum albumin/ml), 1 μl kinetoplast DNA (0.2 μg) (TopoGen, Co-

lumbus, OH) and 1 μl DMSO-containing drug. After thorough mixing while on ice, one unit of purified avian topo II (TopoGen) was added immediately before incubation in a water bath at 34°C for 45 min. The reactions were stopped by the addition of 5 μl stop buffer (5% sarkosyl, 0.0025% bromophenol blue, 25% glycerol) and were then placed on ice. DNA electrophoresis was carried out on a 1% agarose gel in TAE (Tris–acetate–EDTA) buffer containing ethidium bromide (0.5 μg/ml). DNA was visualized with a Spectroline Transilluminator (Spectronics, Westbury, NY) at a wavelength of 310 nm and the gels were photographed using a Polaroid Land camera (Polaroid, Cambridge, MA).

Statistics

The IC_{50} values for each drug against the two cell lines were obtained from plots of drug concentration versus percent survival. (These plots are not shown, but all the IC_{50} values are summarized in Table 1.) Points on these graphs represented the mean of the results obtained from two to five combined experiments. The data were smoothed using the method of lowess (locally weighted regression scatter plot smoothing)[20] with a factor of 0.5. Comparative ratios were obtained by dividing the BR1 IC_{50} by the xrs-6 IC_{50} for each drug.

Results

Comparisons of the sensitivities of BR1 cells versus xrs-6 cells to various anti-cancer drugs are shown in Table 1. The xrs-6 cells were much more sensitive than the BR1 cells to drugs known to act by the inhibition of topo II. For instance, the IC_{50} of mitoxantrone against the xrs-6 cell line was 0.0005 μg/ml, while its IC_{50} against the BR1 cells was 0.0027 μg/ml, giving a BR1 IC_{50}/xrs-6 IC_{50} ratio of 5.4. Etoposide, teniposide, m-AMSA, daunomycin and doxorubicin exhibited ratios of 7.0, 6.22, 5.63, 4.12 and 3.77, respectively. With most of these inhibitors, it was generally noted that the IC_{90} against the xrs-6 cells was approached before the IC_{50} towards the BR1 cells was exhibited, e.g. a concentration of 0.033 μg/ml doxorubicin inhibited the growth of 90% of the xrs-6 cells but only about 50% of the BR1 cells.

Also shown in Table 1 is the BR1 IC_{50}/xrs-6 IC_{50} ratio for camptothecin, a drug known to produce its cytotoxic effect through the inhibition of topo I.[21,22] The xrs-6 cells were not more sensitive to this topo I inhibitor than the BR1 cells were (the ratio was 1).

Table 1. Sensitivity of BR1 versus xrs-6 CHO cells to some anti-cancer drugs

Drug	BR1 IC_{50} (μg/ml)	xrs-6 IC_{50} (μg/ml)	BR1 IC_{50}/ xrs-6 IC_{50}
Etoposide	0.56	0.08	7.0
Bleomycin	5.0	0.73	6.84
Teniposide	0.28	0.044	6.22
m-AMSA	0.04	0.0071	5.63
Mitoxantrone	0.0027	0.0005	5.4
Daunomycin	0.028	0.0068	4.12
Doxorubicin	0.029	0.0077	3.77
Vincristine	0.5	0.18	2.78
Colchicine	0.195	0.075	2.6
Vinblastine	1.3	0.62	2.09
Cisplatin	4.0	2.4	1.66
Mitomycin C	0.59	0.52	1.13
MAPCZ	79.0	71.0	1.11
Streptozotocin	100.0	91.0	1.1
Camptothecin	0.055	0.055	1.0
Trimetrexate	10.05	10.1	0.99
Melphalan	1.45	1.5	0.97
5-CdUrd	40.0	>100.0	<0.4
HMM	NA	NA	NA

Several different types of alkylating agents were tested in this screen for their effects on the mutant CHO lines. None of these showed enhanced cytotoxicity to the xrs-6 cells (Table 1). Melphalan, a potent nitrogen mustard alkylating agent, exhibited an almost identical IC_{50} against both CHO lines. Concentrations of drug over 10 μg/ml were equally cytotoxic to both cell lines, essentially inhibiting the growth of 100% of the cells. Mitomycin C becomes a monofunctional or bifunctional (depending on the enzymatic or chemical reducing system) alkylating agent after chemical or enzymatic reduction of the quinone.[23] In addition to inhibiting the synthesis of DNA and forming DNA cross-links, DNA SSBs are also formed. Enhanced cytotoxicity to xrs-6 cells was not exhibited with mitomycin C (Table 1). The IC_{50} ratio was approximately 1; both cells were equally sensitive to all concentrations of mitomycin C. Streptozotocin is a naturally occurring nitrosourea alkylating agent.[24] The xrs-6 cells did not appear to be more sensitive to it, though the IC_{50} against either CHO cell line was just barely reached at concentrations just below 100 μg/ml. MAPCZ is the active cytotoxic metabolite of procarbazine,[16,25] and has recently been shown to be produced

non-enzymatically by autooxidation of procarbazine.[26] MAPCZ may then undergo an intramolecular rearrangement reaction leading to a free radical species that can produce methylation of DNA,[27] ultimately causing DNA strand breakage. As seen in Table 1, its BR1 IC_{50}/xrs-6 IC_{50} ratio was approximately 1. A concentration of 100 μg/ml inhibited the growth of approximately 100% of both CHO lines. Hexamethylmelamine (HMM) is a methylating agent that produces alkylation of DNA after successive enzymatic demethylation and hydroxylation to more active metabolites.[28] It did not exhibit sufficient cytotoxicity to either CHO cell line to effect an IC_{50} (NA = not achieved, in Table 1). This may be due to the fact that microsomal activation of the drug is thought to be required for cytotoxicity.[29]

Cisplatin is a platinum coordination complex, that after displacement of the chloride atoms by water molecules, becomes activated and can rapidly react with two different sites on DNA forming predominantly intrastrand but also interstrand DNA cross-links.[30] A differential cytotoxicity ratio of approximately 1.66 was observed (Table 1) with this cross-linker.

As had been shown before,[13] significant sensitivity of xrs-6 cells to bleomycin was exhibited (Table 1). At all concentrations of drug used, the xrs-6 cells were more sensitive than the BR1 cells. The BR1 IC_{50}/xrs-6 IC_{50} ratio was 6.84.

Interestingly, the nucleotide analog, 5-chloro-2'-deoxyuridine (5-CdUrd), appeared to show enhanced cytotoxicity to the BR1 cells (Table 1). This occurred at every concentration of drug used. The IC_{50} against the BR1 cells was only reached at a concentration of 40 μg/ml while that of the xrs-6 cells was not reached at concentrations up to 100 μg/ml. The comparative ratio of the IC_{50}s thus would be less than 0.4. This may, in part, be due to the more rapid and extensive growth of the BR1 cells and a presumed increase in incorporation of the analog into the cell's DNA.

Trimetrexate is a methotrexate analog that has recently completed phase I studies. It produces its cytotoxic effect by the inhibition of dihydrofolate reductase; however, compared with methotrexate, it has a greater intracellular accumulation due to its higher rate of influx and lower rate of efflux.[31] No differential cytotoxicity to the xrs-6 cells was noted as seen in Table 1, i.e. both CHO cells were equally sensitive to its effects.

Unexpectedly, the Vinca alkaloids, vinblastine and vincristine, exhibited more than 2-fold enhanced cytotoxicity to the xrs-6 cells. These drugs act by binding to tubulin causing dissolution of microtubules in the mitotic spindle, ultimately arresting cell division in metaphase.[32] Vinblastine exhibited a comparative IC_{50} ratio of 2.09 and vincristine had a ratio of 2.78. Due to this unexpected result in our assay, we tested colchicine, a drug which is known to bind to tubulin at a different site than that of the vinca alkaloids[32]. Colchicine also exhibited greater cytotoxicity to the xrs-6 cells, showing an IC_{50} ratio of 2.6.

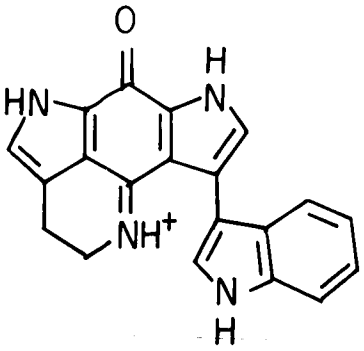
We have recently reported the isolation of wakayin,[14] a pyrroloiminoquinone alkaloid isolated from the ascidian *Clavelina* (a sea squirt). It exhibited potent cytotoxicity against HCT 116 human colon carcinoma cells[14] and our xrs-BR1 screen showed greatly enhanced toxicity toward the xrs-6 cells, as seen in Table 2. Its BR1 IC_{50}/xrs-6 IC_{50} ratio was 9.8. This was the largest differential cytotoxicity that we have seen with this screen so far; etoposide approximating 7. With such a large ratio and because of its planar quinone structure, it was thought that the most likely mechanism for the generation of DSBs would be the inhibition of topo II. Indeed, this was shown by the ability of wakayin to inhibit the decatenation of kinetoplast DNA by topo II (Figure 1). In Lane 1 (C) no topo II or drug were present and it is seen that the highly catenated DNA did not migrate through the gel. Lane 2 (T) contains topo II and catenated DNA, but no inhibitory compound was present. The kinetoplast DNA was effectively decatenated by topo II. Lanes 3–7 contain increasing concentrations of wakayin. (When the enzyme is inhibited, no reaction product monomer circles are released.) A concentration of 40 μg/ml clearly did not inhibit decatenation, but concentrations between 40 and 133 μg/ml showed a disappearance of the monomer circles released from DNA by topo II. The assay thus confirmed the topo II inhibitory activity of wakayin which was dose-dependent.

Also shown in Table 2 is a comparison of BR1 versus xrs-6 sensitivities to two structurally related compounds, discorhabdin A and damirone B. These compounds have been previously isolated from

Table 2. Sensitivity of BR1 versus xrs-6 cells to some marine natural products

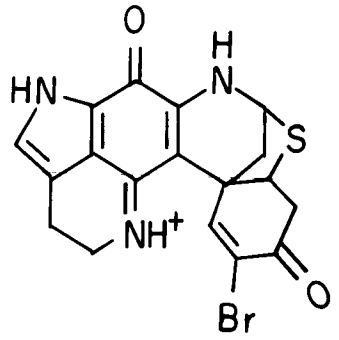
Drug	BR1c IC_{50} (μg/ml)	xrs-6 IC_{50} (μg/ml)	BR1 IC_{50}/ xrs-6 IC_{50}
Wakayin	3.05	0.31	9.8
Discorhabdin A	0.8	0.45	1.77
Damirone B	100.0	76.0	1.31

marine sponges of the genus *Latrunculia*[16] and *Damiria*,[17] respectively. Discorhabdin A is very cytotoxic (HCT 116 IC_{50} of 0.1 μg/ml), while damirone B is not (HCT 116 IC_{50} is greater than 100 μg/ml) and we now have isolated these same compounds from another marine sponge, *Zyzzya* sp. While discorhabdin A was very toxic, its BR1 IC_{50}/xrs-6 IC_{50} ratio was only 1.77 (Table 2). This work has suggested that discorhabdin A probably exerts its potent cytotoxic effects through a mechanism other than by topo II inhibition. This result was confirmed using the topo II decatenation assay and, as seen in Figure 2, concentrations up to



Wakayin

Figure 1. Inhibition of the topo II decatenation of kinetoplast DNA by the marine natural product, wakayin. Wakayin was incubated with kinetoplast DNA and topo II for 45 min at 34°C. Reactions were stopped by the addition of a stop buffer containing sarkosyl. Agarose (1%) gel electrophoresis was used to visualize the release of monomer-length kinetoplast DNA circles from kinetoplast DNA, a highly catenated complex of monomer circles. Gels contained 0.5 μg/ml ethidium bromide. Lane 1 (C) contains kinetoplast DNA but no topo II or drug. Lane 2 (T) contains the enzyme and substrate but no drug. Lanes 3–7 contain increasing concentrations of wakayin and show inhibition of decatenation at concentrations of 133 μg/ml.



Discorhabdin A

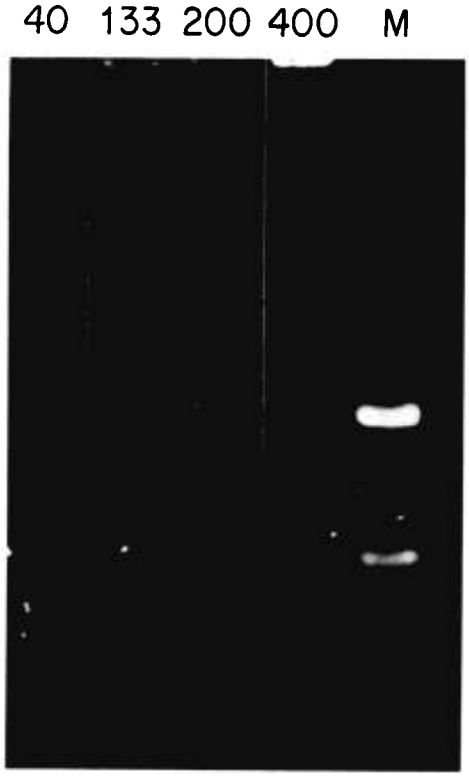


Figure 2. Lack of topo II inhibitory activity by discorhabdin A. The decatenation assay was performed as described in Figure 1. (C and T lanes were the same as those in Figure 1; M lane contains marker decatenated product.) All concentrations of the drug used did not inhibit the topo II decatenation of kinetoplast DNA.

400 μg/ml did *not* inhibit topo II decatenation of kinetoplast DNA (monomer circles were seen at all concentrations of the drug). Similarly, damirone B did not possess enhanced cytotoxicity to the xrs-6 cells (Table 2). The BR1 IC_{50} was barely reached at 100 μg/ml and the xrs-6 IC_{50} was just slightly lower (76 μg/ml). It is obvious that, compared with wakayin, the structural modification seen with damirone B almost completely eliminated topo II inhibitory activity. Damirone B did not prevent the decatenation of kinetoplast DNA by topo II (data not shown).

Discussion

The isolation of natural products with potential anti-cancer activity is often based on the cytotoxicity of the compound. The active (cytotoxic) fractions of a crude extract are usually further fractionated until purified compounds responsible for the cytotoxic action are obtained. To further assess the new isolate's anti-cancer activity, the compounds are often tested against a panel of human tumor cell lines, in hope that screening profiles will suggest some organotropic activity or, alternatively, perhaps resemble that of a known drug and thereby suggest the compound's mechanism of action. One of the objectives of our work is to develop improved screening procedures to aid in the prediction of a novel compound's anti-cancer potential.

It is widely considered desirable to have some knowledge of the cytotoxic mechanism of a potential anti-cancer drug. Novel structures possessing the mechanisms of known, useful drugs might be expected to display unique pharmacokinetics or dose-limiting toxicities while possibly retaining reasonable anti-cancer activity. Consistent with this point of view is the fact that many useful anti-cancer drugs share common mechanisms of action (e.g. topo II inhibition, tubulin disruption or DNA alkylation). This consensus has lead to the recent development of 'mechanism-based assays' for potential anti-cancer agents, such as the recently described set of assays directed toward the discovery of topo I inhibitors.[33] Unfortunately, these screens are often only *in vitro* enzyme assays of one sort or another; or rely on the response of one or another microorganism. Microbial screens include those that use yeast mutants that are completely resistant to topo I inhibitors due to deletion of the topo I gene[34] and the biochemical induction assay in *Escherichia coli*.[35] Draw-backs of enzyme inhibition type screens include their laborious time-consuming nature, the questionable relevance of *in vitro* enzyme inhibitors obtained at super-toxic drug concentrations and specificity that might overlook a potentially useful compound that possesses an alternate mechanism of action. Also, it is possible that such enzyme inhibition screens might identify non-specific enzyme inhibitors which also inhibit many other enzymes, e.g. novobiocin.[36] One obvious short-coming of micro-organism-based screens is the necessary extrapolation of results to the mammalian case.

The approach validated here is similar to an approach for screening environmental toxins, suggested earlier by Thompson *et al.*[37] A recent description of the 'COMPARE' program used by the NCI drug screening program[38] illustrates the power of comparing cell lines of known sensitivities for identifying potential anti-cancer drugs of novel structure. 'COMPARE' is a powerful tool, but it requires the potential drugs to be tested in approximately 60 different cell lines. Similarly, Weinstein *et al.*[39] at the NCI have recently developed a system of neural computing to predict a drug's mechanism of action. This neural network (8% incorrect predictions) appears to be more sophisticated than linear discriminant analysis (14% incorrect predictions) as a tool for predicting a drug's mechanism of action. (Rather than being programmed to get the right answer as usual computer programs do, the network instead 'learns' from a set of examples). A weakness of the 'COMPARE' program and the neural network is that the molecular basis for the sensitivity or resistance of any given cell line is unknown. On a small scale such as ours, screening a compound against 60 different cell lines would be impractical. It would be more desirable to use cell lines that represent a certain mechanism of action. Our screen begins to approximate this goal by using a pair of mutant CHO lines. To provide more precise information regarding a potential drug's mechanism, we chose to compare a series of closely related cell lines (i.e. CHO mutants), with DNA repair deficiencies that are at least partially understood on a molecular level.

We have assembled a small panel of CHO mutants for use in this new approach to anti-cancer drug screening. (We hope to present results obtained with the other lines in the near future.) Perhaps the most informative mutant line in the panel is the xrs-6 line. Work of several investigators[5,11] illustrated the sensitivity of xrs mutants to the topo II inhibitors, m-AMSA and etoposide,

and agents that cleave DNA. We have shown here that the CHO lines are readily adapted to MTA screening and that their differential sensitivities to various anticancer agents may be readily ascertained using the MTA.

The BR1 cells were established by transfection of the normal CHO cell line, AA8, and selection for BCNU resistance and were shown to exhibit approximately 2-fold resistance to BCNU (BR1 IC_{50}/AA8 IC_{50}) in a clonogenic survival assay.[7] Tano *et al.*[9] showed the BR1 cells to contain moderately high levels of the endogenous hamster DNA repair protein, AT. Using the MTA assay described here, the BR1 cells were only marginally more resistant to BCNU and CCNU (chloroethylnitrosourea) (IC_{50} ratios of about 1.3) than AA8 or xrs-6 cells (data not shown). Thus, while giving much more rapid results, the MTA may be slightly less discriminatory than the standard clonogenic survival assay. Likewise, we have not been able to show the previously demonstrated slight resistance of BR1 cells (compared with AA8 cells) to simple methylating agents such as dimethyl sulfate, using the MTA. The reason for selecting the BR1 cells for this screen was to maximize any difference in toxicity between it, as our DNA repair competent-reference cell line, and any of the DNA repair deficient lines. BR1 cells are as proficient in the repair of DSBs as normal CHO (AA8) cells, while xrs-6 cells have difficulty in the repair of such strand breaks. Thus, xrs-6 cells are much more sensitive to killing by agents that induce such damage. When DNA DSBs are introduced into these cells, there are apparently both impaired coding joins and impaired RS joins.[11] In other words, these cells probably attempt to repair such breaks in their double-strand DNA, but a mutation of V(D)J recombination may impair the normal joining process so that only illegitimate (imprecise) joins are produced. Thus, since these breaks cannot be properly repaired, it would take less DSBs to kill these cells. Our screen used a comparison of IC_{50}s obtained from this pair of mutant CHO cells as an indication of the production of DNA DSBs.

Our results have shown that all the clinically available topo II inhibitors exhibit substantial activity in this screen (BR1 IC_{50}/xrs-6 IC_{50} ratios ranging from approximately 4 to 10). Likewise, an agent known to cleave DNA was readily detectable; bleomycin showed a ratio of 6.84. This is the first description of the enhanced sensitivity of the xrs-6 cells to the anthracyclines, doxorubicin and daunorubicin, and the synthetic compound mitoxantrone. These results were not unexpected, however, since doxorubicin, daunorubicin and mitoxantrone are thought to act via topo II inhibition. Other mechanisms have also been observed in the cytotoxic activity of doxorubicin and this may account for its lower (of the topo II inhibitors) BR1 IC_{50}/xrs-6 IC_{50} ratio. Mitoxantrone lacks the ability to generate free radicals as does doxorubicin, therefore this could explain the greater enhanced sensitivity of the xrs-6 cells to mitoxantrone than to doxorubicin. The non-intercalating agents, etoposide and teniposide, are thought to act only through the inhibition of topo II, and these drugs so far have exhibited the largest BR1 IC_{50}/xrs-6 IC_{50} ratios. Other anti-cancer agents, known to damage DNA in other ways (e.g. alkylation, topo I inhibition, etc.), exhibited little or no enhanced toxicity toward the xrs-6 cells. Thus, with regard to topoisomerase inhibition, the assay appears to be specific for putative inhibitors of topo II.

We have not been able to show slight sensitivity of the xrs-6 cells to some alkylating agents, as has been shown by other investigators.[40] This can probably be attributed to differences in cytotoxicity assays, cell lines used for comparison, alkylating agents and methods of determining enhanced sensitivity of the xrs-6 cells. For example, our screen used the MTA instead of a clonogenic assay for cytotoxicity data and BR1 cells instead of CHO-K1 cells as did earlier reported studies of Jeggo and Kemp.[13] The first published report of the isolation of the xrs mutants showed that they were sensitive to the alkylating agents MMS and EMS[13]. This was determined on the basis of D_{10} values and was at most only a 2-fold effect. According to early studies on the repair defect of the xrs-6 cells,[6] these cells are proficient in SSB repair (so our result was not surprising).

An interesting observation made while running anti-cancer drugs through our screen was that the tubulin inhibitors (viblastine, vincristine and colchicine) showed enhanced toxicity to the xrs-6 cells. All of these drugs produced small but reproducible BR1 IC_{50}/xrs-6 IC_{50} ratios (between 2 and 3) that were below the (arbitrary) threshold (ratio of approximately 4) seen for topo II inhibitors. Experiments currently underway are attempting to correlate increased DNA breakage in xrs-6 cells with their enhanced sensitivity to the tubulin inhibitors.

Data obtained with the marine pyrroloiminoquinone wakayin and two other marine compounds of related structure have been presented to illustrate the usefulness of this screen. Wakayin was identified as a potential topo II inhibitor, while the chemically

related discorhabdin A and damirone B were not. Results obtained with an *in vitro* enzyme inhibition assay for topo II were consistent with the results of the screen. Thus, even though discorhabdin A is more toxic than wakayin (IC_{50} in human colon tumor cells, HCT 116, of 0.1 versus 1 μg/ml for wakayin), knowledge of wakayin's mechanism of action would, in our scheme, give wakayin priority in further screening. The relative lack of toxicity and mechanistic information combine to make damirone B much less interesting in this evaluation system.

The screen's usefulness has also been demonstrated by our identification of the makaluvamines, pyrroloiminoquinones isolated from a marine sponge, as topo II inhibitors and this is being reported elsewhere.[15] One of the compounds in this series, makaluvamine A, possessed considerably enhanced cytotoxicity to the xrs-6 cells (BR1 IC_{50}/xrs-6 IC_{50} ratio was approximately 7). In that report we show potent inhibition of the topo II decatenation of kinetoplast DNA by makaluvamine A and also show its dose-dependent ability to generate numerous DNA DSBs by DNA neutral filter elution. Our cellular screen thus correctly predicted the mechanism of makaluvamine A.

Conclusion

We have developed a novel, rapid, high-throughput, inexpensive screen for obtaining initial mechanistic information relevant to potential anticancer compounds. We have shown that this screen can be used to suggest mechanisms of action by comparing ratios of IC_{50}s between a pair of mutant CHO lines. The largest ratios were produced by topo II inhibitors and bleomycin. Ratios approximating 1 were produced by methylating agents and some alkylators. A slightly greater (1.6) ratio was produced by the cross-linker, cisplatin. Higher ratios were consistently produced by tubulin inhibitors and this result is currently the subject of intensive investigation. Antimetabolites exhibited ratios less than 1. This cellular assay can also be used to rapidly screen structural modifications that eliminate or enhance topo II inhibitory activity by a compound, i.e. structure–activity relationship studies of a known class of topo II inhibitors. The future incorporation of other mutant CHO lines should improve the screen by identifying compounds with alternate mechanisms of action, such as DNA alkylation or topoisomerase I inhibition.

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