Eur J Cancer Clin Oncol, Vol. 24, No. 4, pp. 591–596, 1988.
Printed in Great Britain

0277–5379/88$3.00+0.00

'Petite' Mutagenesis by Anticancer Drugs in *Saccharomyces cerevisiae*

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Abstract—*The mitochondria of cancer cells are potential targets for chemotherapy. Drugs which primarily affect mitochondrial DNA can be screened using a 'petite' mutagenesis assay in* Saccharomyces cerevisiae. *We have used this approach to estimate the antimitochondrial effects of a range of current clinical and experimental antitumour drugs with varying modes of action. Of agents currently in the clinic, the antimetabolites 5-fluorouracil and methotrexate were extremely effective in inducing this respiratory defect, providing cells were growing during treatment. Adriamycin, BCNU, bleomycin, methyl CCNU, cis-platinum, chlorambucil, daunomycin, nitracine, nitrogen mustard and hycanthone were also weakly effective 'petite' mutagens, in either growing or non-growing conditions. None of the currently used agents but some experimental drugs induced high numbers of 'petite' mutants during growing or non-growing conditions. To date, where such agents have been tested clinically, they have proved either ineffective or very toxic. It is possible that antimitochondrial effects on non-proliferating cellular tissues such as the heart might cause unacceptable toxicity and preclude the clinical use of such agents. For those agents effective against proliferating cells, the mitochondria could be an important target for chemotherapy in some cell types. This type of drug appears relatively uncommon in the clinic at present. The 'petite' mutagenesis assay could be more widely used as a screen to optimize this property in development of analogues of current clinical agents, or in developing new types of anticancer drug.*

INTRODUCTION

ENERGY metabolism of tumour cells is known to differ from that of normal cells [1], and a considerable body of work has shown both quantitative and qualitative abnormalities of tumour mitochondria [2, 3]. In the development of antitumour drugs it is important to target agents at some parameter which differs between normal and tumour cells, and mitochondria have been considered as a potential target for antitumour drugs. For example, Wilkie [4] demonstrated selective inhibition of transformed hamster cells *in vitro* and inhibitory effects on growth and development of tumours in mice by two different types of antimitochondrial agent. Kroon *et al.* [5] showed that tetracyclines, which preferentially block mitochondrial protein synthesis, inhibited the growth of both carcinogen-induced animal tumours and also a transplantable tumour of human origin. Fearon [6] suggested that an effective way to attack the mitochondria in cancer cells is to use a combination of two drugs. One of these would inhibit the working of mitochondria while the other would stop new mitochondria being made to compensate for the inhibition. Such a strategy has apparently reduced the size of tumours in rats by 85% within 6 days [6]. It is possible that some of the drugs already in the clinic may have some of the necessary properties for such an approach. Although the mitochondrion has been suggested as a primary target for some drugs currently in the clinic [7], the number of drugs for which this might be true has not been defined.

There are various ways in which compounds can affect mitochondria [4] and it is relatively easy to demonstrate drug effects on oxygen uptake in mammalian cells. However, it is more difficult to show effects at the level of mitochondrial biogenesis in mammalian cells. Screening potential antitumour agents for effects on mitochondrial DNA can be very cheaply and simply achieved using *Saccharomyces cerevisiae* [8, 9]. This yeast is a facultative anaerobe, which can grow in the absence of an intact respiratory chain. Respiratory-deficient cells grow to form a small colony on plates containing fermentable

Accepted 23 September 1987.
This study was supported by a grant from the Auckland Division, Cancer Society of New Zealand.
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media, and are referred to as 'petites'. Chemical or physical agents which preferentially damage mitochondrial DNA can generally be recognized by their ability to induce 'petite' mutants in *Saccharomyces cerevisiae*. This assay provides a useful prescreen which can then be followed by other techniques such as those involving mitochondrial dyes in intact mammalian cells [7] or assays on respiration in isolated mitochondria.

In this study, we have identified antitumour agents which might have some antimitochondrial effects by surveying 31 drugs for 'petite' mutagenesis activity in the diploid strain D5 [10] of *S. cerevisiae*. Agents were selected to cover the range of types which are most commonly used in the clinic, as well as some experimental agents.

MATERIALS AND METHODS

Drugs

These were obtained from various sources, as follows: BCNU and *cis*-platinum, Bristol-Myers; thiotepa and sodium methotrexate, Lederle Laboratories; mitomycin C, Kyowa Hakko Kogyo C Ltd, Japan; dacarbazine, Bayer Laboratories; hydroxyurea, E.R. Squibb and Sons, Inc., New Brunswick; cytosine arabinoside, Upjohn Co., Kalamazoo; vincristine sulphate, David Bull Laboratories Ltd, Victoria, Australia; vinblastine sulphate, Eli Lilly; adriamycin, Farmitalia Carlo Erba. Amsacrine and quinolium bromide were made in the Cancer Research Laboratory and we thank Drs B.C. Baguley and W.A. Denny for their availability. All other drugs were kindly provided by Warner Lambert-Park Davis (Detroit, U.S.A.). Drugs were diluted, stored and used within the time specified on the manufacturers' instructions.

Saccharomyces cerevisiae *strain*

The strain D5 [10] was kindly supplied by Dr B.S. Cox, Botany School, Oxford, U.K. Upon receipt, it was plated, a single-colony isolate inoculated into 100 ml of liquid yeast complete medium (YEPD; 1% yeast extract, 1% peptone (Difco) and 2% glucose), and grown to stationary phase (24 h). DMSO (10%) was added, 1 ml aliquots were frozen to −70°C and stored at this temperature until use.

Experimental method

'Petite' mutagenesis ability of the drugs was measured using the 'microtitre' assay previously described [9]. Yeast cells were incubated with drug for 2 h, in YEPD medium containing 2% glucose and also in 0.87% saline, in order to estimate effects in both growing and non-growing conditions. Cells were washed by dilution and 100 μl aliquots of the diluted culture plated in triplicate onto YEPD plates. After 3 days' incubation at 28°C, colonies were counted and 'petite' colonies distinguished using the tetrazolium overlay method of Nagai [11]. From these data, survival and 'petite' mutagenesis were calculated in relation to dose for each drug, in both growing and non-growing conditions. Linear regression methods were used to estimate the drug concentration which reduced colony numbers to 37% of the value for the untreated control (D37), and the drug concentration necessary to increase the background number of petites more than threefold (PD3) where this was appropriate. We also used the data to determine the maximum percentage of 'petite' colonies which can result from a 2 h exposure to the drug (Px). In order to determine whether effects seen were a result of 'petite' induction or merely selection of pre-existing cells, we have scrutinized the actual numbers of 'petite' colonies on treated vs. control plates. Compounds were only classed as 'petite' inducers if both total number of 'petite' colonies, as well as the frequency of 'petites' among the survivors of drug treatments, was increased. Using this test, all agents which we have scored as 'petite' are inducing rather than selecting agents.

RESULTS

Comparison of 'petite' forming ability in different classes of antitumour drug

Of 31 drugs tested, 4 were non-evaluable because they did not reach toxic concentrations in the cell. Of 27 anticancer drugs which could be evaluated in these assays, 15 (56%) caused some degree of 'petite' mutagenesis in *Saccharomyces cerevisiae*.

Table 1 lists biological data on 'petite' mutagenesis in growing or non-growing cells for various classes of antitumour drug. Also included in the table is the clinical status of the drug, and a classification of the class of 'petite' mutagen [12, 13, 14].

Alkylating agents

cis-Platinum, BCNU, methyl-CCNU, nitrogen mustard and chlorambucil all significantly increased the proportion of 'petite' colonies in either growing or non-growing conditions. In some experiments, a very slight increase was seen with mitomycin C, but this was not consistent and the compound has been scored negative.

2,5-Diaziridinyl-3,6-bis-(carbocthoxyamino)-1,4-benzoquinone (AZQ), busulphan and thiotepa did not induce any 'petite' mutants in these experiments, although they were toxic to the yeast. Although we have tentatively scored cyclophosphamide, dacarbazine and hexamethylmelamine as non-inducers, they did not cause major toxicity and it is possible that other protocols may have revealed antimitochondrial effects. It is known that cyclophosphamide can be metabolically activated by *S. cerevisiae* [15].

Table 1. *Petite mutagenesis activity of antitumour drugs after* 2 *h incubation with yeast strain* D5, *using a microtitre assay* (*see* '*Materials and Methods*'). *Data are compiled from at least two experiments.*

No.	Compound	Growing cells			Non-growing cells				Clinical
		PX*	D37†	PD3‡	Px	D37	PD3	Class§	status‖
Alkylating agents									
1	AZQ	—¶	>5000	—	—	>5000	—	ne	c
2	BCNU	5.3	143	130	6.2	116	93	e	c
3	Busulfan	—	1382	—	—	>2000	—	n	c
4	Chlorambucil	4.8	261	206	3.6	32	25	e	c
5	Cyclophosphamide	—	>5000	—	—	>5000	—	ne	c
6	Cisplatin	17.8	227	287	9.8	353	175	e	c
7	Dacarbazine	—	>4000	—	—	>4000	—	ne	c
8	Hexamethylmelamine	—	1965	—	0.9	4601	—	n	c
9	Methyl-CCNU	17.2	1090	1200	34.8	421	400	e	c
10	Mitomycin C	—	126	—	2.7	60	—	n	c
11	Nitrogen mustard	12.9	23	50	17.1	29	25	e	c
12	Thiotepa	0.9	3820	—	—	>4000	—	n	c
Antimetabolites									
13	Azathioprine	—	4252	—	—	>5000	—	n	c
14	Cytosine arabinoside	—	>10000	—	—	>10000	—	ne	c
15	5-Fluorouracil	68.5	1974	500	—	>8000	—	m	c
16	Hydroxyurea	—	4305	—	—	>10000	—	n	c
17	Methotrexate**	11.7	>40000	284	—	>40000	—	m	c
18	6-Thioguanine	—	3084	—	—	>5000	—	n	c
19	Tiazofurin	—	11345	—	—	>18000	—	n	lc
Mitotic inhibitors									
20	Vincristine	—	1051	—	—	>2000	—	n	c
21	Vinblastine	—	1862	—	—	>2000	—	n	c
Topoisomerase inhibitors									
22	Adriamycin	6.6	113	125	11.8	73	71	d	c
23	Amsacrine	—	1550	—	—	>2000	—	n	c
24	Etoposide	—	3037	—	—	>2000	—	n	c
25	Daunorubicin	12.4	1816	1401	8.6	3830	2651	d	c
Other DNA binding drugs									
26	Bleomycin	8.6	24	25	3.8	222	43	d	c
27	Mitoguazone	—	928	—	—	>4217	—	n	lc
28	DDUG	—	199	—	—	338	—	n	lc
29	Quinolinium bromide	100	1.3	0.005	900	0.8	0.003	b	p
30	Hycanthone	3.9	219	250	7.4	133	185	d	lc
31	Nitracrine	6.1	4.2	4	12.4	3.5	2	d	lc

*Px, the maximum frequency of induction of 'petite' mutants expressed as a percentage of total cells.

†D37, the concentration of drug (μg/ml) required to reduce to 37% the total number of viable yeast cells after drug exposure for 2 h.

‡PD3, the concentration of drug (μg/ml) required to increase the frequency of 'petite' mutants at least three times that of the untreated control.

§Class, the type of 'petite' inducer, as defined in the discussion. ne, Non-evaluable; n, non-inducer; b, berenil-like; d, DNA binding-type; e, ethyl methane sulphonate-like; m, methotrexate-like.

‖Clinical status: c, clinically used drug; lc, tested clinically, but non-active or with limited usefulness; p, tested in preclinical toxicology.

¶—, Negative data. Where the 'petite' frequency in treated cultures does not increase more than three times the value for the untreated control, at any dose, data have been scored as negative.

**The value given is for the highest dose tested (40 000 μg/ml) which resulted in 47% survival.

Antimetabolites

Cytosine arabinoside was not toxic in this yeast with any treatment protocol, while the other compounds were toxic providing that treatment was in growing conditions. 5-Fluorouracil was the most effective 'petite' inducer of all drugs tested, converting around 80% of the cells within the 2 h period, providing cells were growing. Although methotrexate was also very effective, longer treatment periods were necessary to mutagenize most of the culture in this way (unpublished data). Azathioprine, hydroxyurea, 6-thioguanine or tiazofuran, although toxic to the yeast, showed no antimitochondrial effects in these assays.

Mitotic inhibitors

Both vincristine and vinblastine were toxic in this assay, but neither caused any increase in the frequency of 'petite' mutants.

Topoisomerase inhibitors

In general, this class of compounds was preferentially toxic towards growing cells. Amsacrine and etoposide failed to induce 'petites', while adriamycin and daunomycin were weak inducers.

Other DNA cutting agents

Bleomycin was quite toxic and a weak inducer of 'petite' mutants, especially in growing cells.

The bisguanyl hydrazones comprise a series of planar molecules which bind to DNA but do not intercalate [16]. Two representatives in this series, methylglyoxylbisguanylhydrazone (MeGAG), and 4,4′-diacetyl-diphenylurea-bis(guanyl-hydrazone) (DDUG) were not 'petite' inducers in these studies. The related experimental agent, quinolinium bromide, was a very effective 'petite' inducer in both growing and non-growing cells.

Hycanthone and nitracine induced a small increase in the frequency of 'petite' mutants in both growing and non-growing cells.

DISCUSSION

In interpreting the present data it is important to ask how well the 'petite' mutagenesis assay predicts for events in mammalian cells. Wilkie and Evans [17] have pointed to basic similarities between yeast and mammalian mitochondria, and Locker *et al.* [18] found that yeast had eukaryote features of mitochondrial transcription and gene structure. Although extensive correlation studies using both yeast and mammalian systems with a wide range of compounds have not been performed to date, there is reason to believe that yeast has good predictive powers for mammalian cells [17]. In most cases which have been carefully studied, agents which are known to affect mammalian mitochondria either induce or select 'petites' in yeast, and vice versa. For example, the most widely used petite inducer, ethidium bromide, is known to have antimitochondrial effects in mammalian cells [19], and rhodamine 123, known as a probe for mitochondria in mammalian cells [7], induces 'petites' in the present experimental system (unpublished results, this laboratory). It is interesting to note that both ethidium and rhodamine 123 [20] show selective antitumour activity towards carcinoma cells in culture as well as some *in vivo* antitumour effects [21], and both may be considered as potential antitumour agents. We suggest that antimitochondrial effects could constitute a significant part of tumour cell selectively for the 'petite' inducing agents in the present study. It is possible that use of different yeast strains might increase the list of compounds causing antimitochondrial effects [22].

Although there are various types of 'petite' inducer in yeast [12, 13, 14], only three classes were represented by currently used clinical agents. These are:

1. *Enzyme inhibitors such as methotrexate and 5-fluorouracil* [13]. Some of these compounds are 'petite' mutagens but only if cells are growing during treatment, and only if drug concentrations are sufficiently high to reduce growth rate and survival of cells. Long exposures (more than one generation time) are generally necessary for detection of the mutagenic event. In general, these compounds cause 'petite' mutagenesis in a similar fashion to acridines [12] but do not exert their effects through binding to DNA. The present study confirms previous reports of 'petite' induction by methotrexate and 5-fluorouracil [13]. Antimitochondrial effects at the DNA level have been previously proposed to be an important part of the antitumour activity of methotrexate and 5-fluorouracil [7].

2. *Ethylmethanesulphonate-like* [14]. These are alkylating agents which are moderately effective in inducing 'petites' in growing or non-growing conditions. Such agents do not have highly selective antimitochondrial effects, and will also induce nuclear mutations. This study extends the range of such compounds to a number of other alkylating agents.

3. *DNA binding type.* This study indicates that DNA binding drugs may produce anywhere between 1 and 100% of 'petites' in these assays. The most common type of petite inducer seen in this class of agents was only weakly effective in inducing petites. This study confirms previous reports on antimitochondrial effects of daunomycin and bleomycin [4] and also shows these effects for adriamycin, hycanthone and nitracine. Wilkie [4] has previously suggested that such effects may be part of the antitumour activity of these drugs.

The most effective antimitochondrial agents induce 'petites' in either growing or non-growing conditions. The most widely used and effective 'petite' inducing agent is ethidium bromide, representative of a class with a triphasic dose or time-response curve. The related compound ethidium chloride proceeded to clinical trial, but was unsuccessful [3]. The second class of most effective 'petite' inducers is represented by berenil [24] and again is not used clinically at present. However, one experimental agent, quinolium bromide (compound no. 29 in Table 1) falls into this class and shows good tumour cell selectivity. Similarly, a large range of related experimental antitumour agents, including some phthalanilides, have previously shown similar properties [25]. Quinolinium bromide [26] proved rather toxic in preclinical toxicology, and

did not proceed to the clinical level [27, 28]. Similarly, the phthalanilides have proved rather toxic in clinical use [29]. It is possible that potent antimitochondrial effects in non-growing cells may mean that some susceptible tissue, such as the heart muscle, will always be damaged to unacceptable levels if an antimitochondrial agent which can act on non-growing cells is used clinically. Of the present compounds, there are five (methyl CCNU, nitrogen mustard, adriamycin, nitracrine and hycanthone) which appear to be more effective 'petite' mutagens in non-growing cells. With adriamycin, for example, there is some evidence that cardiac arrest results from a depression of mitochondrial biogenesis leading to structural aberrations [30]. The possibility that similar effects would inevitably be true for these classes of 'petite' inducer could be readily studied in animal tumour systems, using experimental antitumour agents which are also effective 'petite' inducers.

If toxic effects towards non-dividing cells are always going to lead to unacceptable toxicity *in vivo*, then it may be more appropriate to concentrate efforts on drugs which act only on dividing cells. 5-Fluorouracil and methotrexate both fall into this category and both are very useful clinical drugs. It has been suggested that mitochondrial effects may be involved in their clinical activity [7]. There are already a number of experimental drugs, including some 9-anilinoacridines [1, 2, 8] and ellipticines [33], which also have these properties, but whose use has been relatively unexplored in the clinic.

If the mitochondrion is to be seriously considered as a target for chemotherapy of cancer cells, then it may be worth considering whether some other types of 'petite' inducer should receive priority for clinical trial. Of eight classes of 'petite' inducing agents which have been recognized, representatives of only three are currently in the clinic. It would appear that the potential of the mitochondria as a target for chemotherapy has been relatively unexploited to date.

Acknowledgements—We thank Dr B.C. Baguley for helpful discussions. This work was financially supported by the Auckland Division Cancer Society of New Zealand.

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