Anti-Cancer Drugs 1994, **5**, pp. 171–176

The influence of storage on cytotoxic drug activity in an ATP-based chemosensitivity assay

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The use of viability assays to assess the effect of antineoplastic agents on cell lines and tumor cells is an important investigative tool and may have clinical relevance. Such assays require very small quantities of drugs and it is the practice of many laboratories to freeze aliquots of drugs for use in these assays as required. We have investigated the stability of 11 different agents in an ATP-based chemosensitivity assay which is being evaluated for clinical use. The results show that most drugs maintain their biological activity well when frozen at −20°C for periods up to 24 months, or occasionally at room temperature. However, 4-hydroperoxycyclophosphamide and mitomycin C are exceptions to this rule, and should not be kept frozen for more than 2–3 months. Cisplatin is unstable when frozen and then thawed, but maintained activity at room temperature for at least 6 months. Since biological activity may not correlate completely with chemical stability, further studies on the effect of storage are required, but it seems unlikely that the appropriate use of frozen aliquots is a major source of error in tumor chemosensitivity assays.

Key words: ATP, chemosensitivity, cytotoxic drug, freezing, luminescence, viability.

Introduction

There is relatively little published information on the long-term stability of cytotoxic drugs frozen in small quantities, although a series of useful reviews has been published over the last few years.[1–3] Since drugs given to patients are usually made up and given immediately, long-term storage of small aliquots has not been important in the past. However, as part of the development of an *in vitro* tumor chemosensitivity assay (TCA),[4–7] we have been using an ATP luminescence method to test the sensitivity of tumor cells to a large number of single agents and drug combinations. The cost and complexity of the assay would be substantially increased if fresh drugs were used for each assay and we have therefore frozen small quantities of most drugs for use in the assay whenever they are required,[7] or kept them at room temperature if the drug was thought to be stable under these conditions.[1–3,8] Intravenous formulations supplied by the manufacturer were used for all drugs.

This approach to drug storage introduces a number of potential problems, such as precipitation, adsorption and altered chemical stability.[1] While there is a need to study the effect of storage on chemical stability as well as biological activity, we have elected to test the effect of storage on the cytotoxic activity of those agents most commonly used in this chemosensitivity assay.

This work was supported by BATLE LE Inc.

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Materials and methods

Drugs

Aliquots (100–1000 µl) of each drug tested were frozen at −20°C in 1.5 ml sterile sealed polystyrene tubes (Bibby Sterilin, Stone, Staffs, UK) on receipt of the vial, which was reconstituted according to the manufacturers' recommendations for clinical use by the hospital pharmacy or one of the authors and not diluted further. Four drugs were thought to be stable at room temperature [mitoxantrone, 5-fluorouracil (5-FU), cisplatin and etoposide]: 1–5 ml aliquots of these drugs were therefore kept at room temperature (20°C). Although 5-FU is known to be stable for up to 4 years at room temperature,[3] it was tested frozen in this study, as this form of storage is generally more secure. At intervals when new drugs were obtained from the hospital pharmacy, stored aliquots were tested against freshly reconstituted drugs to determine whether there was any loss of activity. The drugs tested, their origins, concentrations and the volumes of the aliquots used in these experiments are shown in Table 1. All drugs were kept in the dark.

Table 1. The drugs tested, their concentrations, storage temperatures and the volumes of the aliquots used in these experiments

Drug class	Drug	Origin	Size of aliquot (µl)	Concentration (mg/ml)	Storage temperature (°C)
Alkylating agents and plantinum	4-HC	Nova	100–500	5	–20
	cisplatin	David Bull Labs or Lederle Labs	1000–5000	1	RT
	carboplatin	Bristol-Myers	200	10	–20
Antibiotics	doxorubicin	Farmitalia Carlo Erba	200	2	–20
	epirubicin	Farmitalia Carlo Erba	200	2	–20
	mitomycin C	Kyowa	200	0.5	–20
	mitoxantrone	Lederle Labs	1000–5000		RT
Antimetabolite	5-FU	David Bull Labs	500–1000	25	–20
	methotrexate	Lederle labs	100	25	–20
Spindle agents	vincristine	David Bull Labs	100	1	–20
Topoisomerase inhibitor	etoposide	Bristol-Myers	1000-5000	20	RT
			200	20	–20

RT = room temperature.

Cell lines

Cells from four cell lines (Table 2) were grown in RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamicin, 292 ng/ml L-glutamine and 10% fetal calf serum. All reagents were obtained from Gibco BRL (Paisley, UK). This complete assay medium (CAM) was also used for the short-term cell culture (5–7 days) required as part of the TCA method.

Chemosensitivity assay

TCA-100 assays were performed according to the manufacturer's instructions using reagents from BATLE LE (Fort Lauderdale, FL).[4–7] Briefly, drugs were made up in CAM at concentrations corresponding to eight times a standard test drug concentration (TDC). TDC values were defined by reference to plasma concentrations achievable *in vivo* following a standard dose of each drug tested and adjusted to provide a clear dose response with each cell line. Doubling dilutions were performed in polystyrene (Greiner Labortechnik, Dursley, UK) or polypropylene (Costar, High Wycombe, UK) microplates to give seven dilutions corresponding to 200–3.125% of the TDC per well after addition of cells to a final volume of 200 µl. Six wells were reserved for no-drug controls and six wells for a maximum inhibitor of ATP.

Cells from cultures in logarithmic growth phase were harvested, assessed for viability by Trypan blue exclusion, counted, adjusted to 100 000 or 50 000 cells/ml in CAM and plated at 100 µl per well in the microplates according to a standard plate layout.[7]

The plates were incubated at 37°C in an incubator (Hotpack, Philadelphia, PA) with 96% humidity and 5% CO_2 for 5–7 days. ATP was measured at the end of the culture period by adding 50 µl of tumor cell extraction reagent (TCER; BATLE LE) to each well and pipetting the wells to aid cell lysis. After 20 min at room temperature, 50 µl from each well was transferred into 3.5 ml plastic test tubes (Sarstedt, Numbrecht, Germany). These were loaded into a Berthold LB953 luminometer (EG&G Berthold, Wildbad, Germany), which was set to inject 55 µl of recombinant luciferase/luciferin reagent (LU/LU; BATLE LE) per tube. The luminescence response was counted for 10–20 s.

Data analysis

The luminometer results, in RLU (photons/10), were entered into a spreadsheet for analysis in terms of the percentage inhibition at each drug dose. A summary index[7] was calculated as [700 – sum(%inhibition at each concentration tested)], which decreases as sensitivity increases.

This was used to compare results in terms of percentage change in activity over increasing storage time.

Results

A total of 11 different drugs were tested from six different categories (Table 1) to determine their stability in the chemosensitivity assay. Some aliquots were exposed to two freeze/thaw cycles due to a freezer malfunction. Figure 1 shows the results for four drugs [4-hydroperoxycyclophosphamide (4-HC) cisplatinum, methotrexate, and 5-FU] with a leukemic cell line (CCRF-CEM). Most drugs produce standard sigmoid curves over the range of dilutions tested, although this is not true of methotrexate, vincristine, mitoxantrone and etoposide, which either produced an identical percentage inhibition or showed 100% inhibition, across the range of concentrations tested with some or all of the cell lines used in this study. A sigmoid curve could be achieved as shown in Figure 2 for methotrexate below 3.13% TDC by reducing the concentrations used, suggesting that the TDCs for these drugs were higher than the concentration required to achieve maximum inhibition with the cell lines tested (Figure 2).

All but two of the drugs tested showed less than 10% reduction in activity over time (Table 3), a figure which is comparable to the inter-assay variation obtained with the TCA-100 assay (data on file, BATLE LE). However the reduction observed with 4-HC (Figure 1c) and mitomycin C (Figure 1d) was considerable. The reduction in activity of 4-HC and mitomycin C appeared to be particularly marked after one freeze/thaw cycle or after 7 months, but both drugs can be stored at −20°C for at least 2 months without losing more than 2.1% of their activity.

Discussion

The biological activity of most cytotoxic agents is remarkably stable and few drugs show any significant diminution in activity over varying periods up to 24 months (Table 3). Mitomycin C and 4-HC are the notable exceptions, although even these drugs can be frozen for 2 months without significant loss of biological activity. Both may be more stable at −70°C. All freezers go wrong occasionally: some of the older aliquots used in the assays were thawed accidentally for 24 h at least twice. The results show that even this did not affect their activity substantially.

Biological assays of drug stability are open to criticism as they do not allow chemical degradation to be considered.[2,9] Current evidence suggests that alkylating agents such as 4-HC degrade relatively rapidly and lose their cytotoxicity, even when frozen,[1,10] while anti-metabolites[3,11] and vinca alkaloids are much more stable.[3] Cisplatin (stored at room temperature) and carboplatin (frozen) are also stable,[3] as is melphalan.[12] Doxorubicin is stable when frozen.[2,13] Other agents vary and in general it is probably unwise to keep frozen aliquots of drugs for more than 6 months. While some drugs will withstand freeze/thaw cycles, this should be avoided and stocks replaced after freezer malfunctions. Light exposure is known to affect many drugs: aliquots should therefore be kept in light-tight containers.[1,3] There is also evidence to suggest that the type of container influences the drug storage, particularly as dilute solutions.[14] In this study, we have used drugs in exactly the same formulation as they are administered to patients. The primary reason for this was simplicity—the time and equipment required to reconstitute vials is considerably less than that needed to weigh out small quantities of these hazardous agents.

Conclusion

These results have important implications for tumor chemosensitivity testing, given the expense of making up drugs freshly at low concentrations, or on rare occasions for just a few wells of a microplate. The use of frozen drug aliquots in such assays is practicable and adds much to their overall feasibility.

Acknowledgments

This work was supported by BATLE LE, Inc., who also provided many of the reagents used. We are grateful to the Ninewells Hospital and Medical School pharmacy for their assistance. We wish to thank Mrs N MacElduff, Dr L Newman, Mrs D Wilton and Miss M Hepburn for their assistance.

Table 2. The cell lines used, with details of their derivation and requirement for adherence

Cell line	Derivation	Adherence requirements
CCRF-CEM	T lymphocytic cell line derived from human acute lymphoblastoid leukemia	non-adherent
Daudi	human Burkitt lymphoma cell line	non-adherent
HT29	human colorectal carcinoma cell line	adherent
A431	human vulval epidermoid carcinoma cell line	adherent

Those cells which required adherence were tested in polystyrene microplates, while those which grew in suspension were tested in polypropylene microplates.

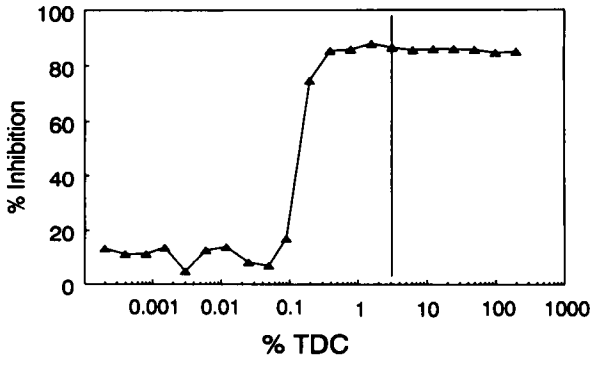

Figure 2. The effect of reducing the concentration of methotrexate in the assay below 3% TDC (bar), showing that there is a sharp transition at 0.1% TDC above which increasing dose does not promote increasing cytotoxicity.

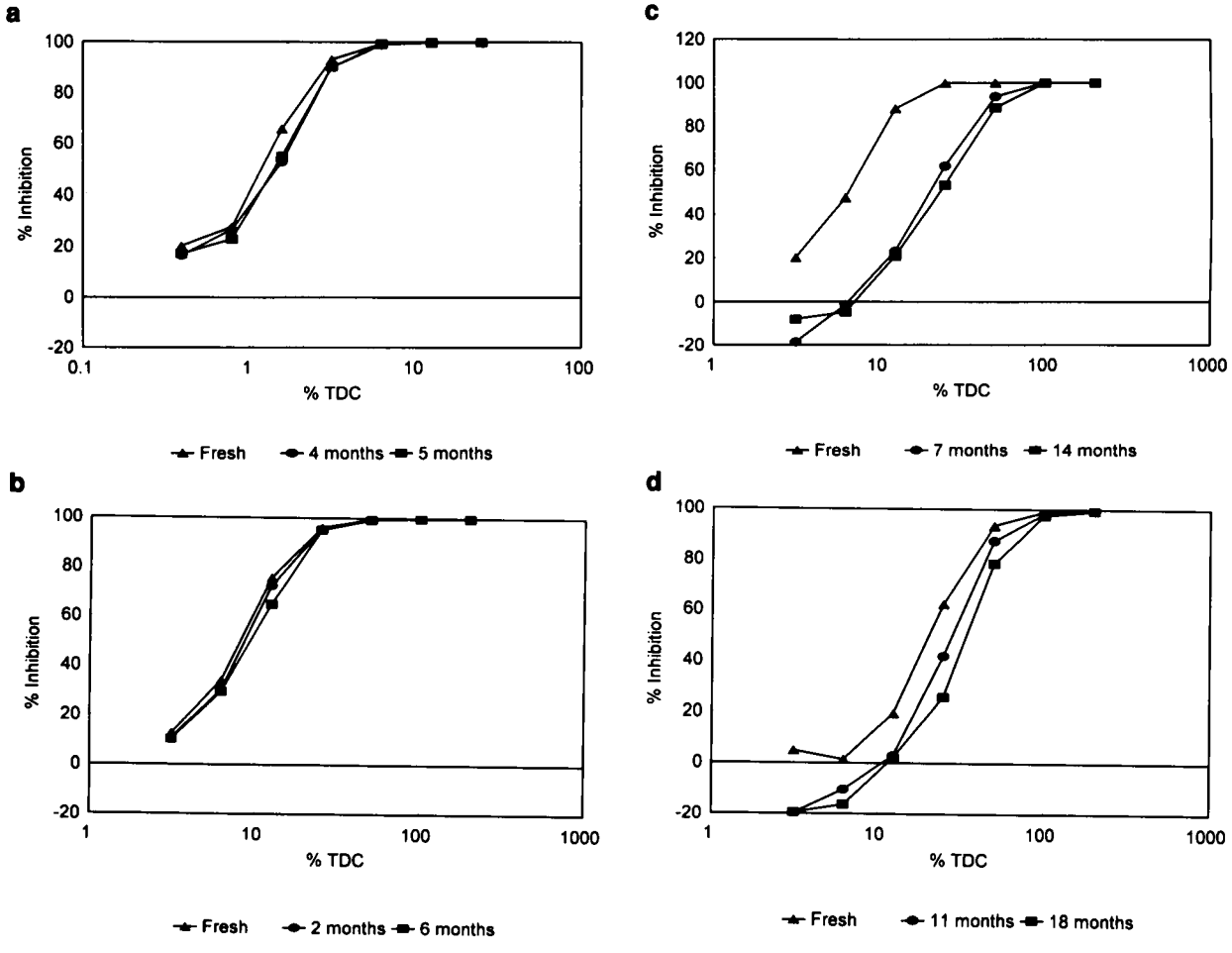

Figure 1. Results for aliquots of four drugs: (**a**) vincristine (−20°C), (**b**) cisplatin (room temperature), (**c**) 4-HC (−20°C) and (**d**) mitomycin C (−20°C). There is considerable reduction in the activity of 4- HC and mitomycin C, but not in vincristine or cisplatin activity. Each point represents the mean inhibition achieved at each concentration for three wells.

Table 3. The variation in TCA index for each drug and cell line assayed after storage of the drugs for the times indicated

Drug	100% TDC (μg/ml)	Cell lines tested	Variation (%)	Storage time (months)	Storage temperature (°C)
4-HC	1.1	CCRF-CEM	−1.9	2	−20
		CCRF-CEM	−2.1	2	−20
		CCRF-CEM	−34.5	7	−20 No F/T
		CCRF-CEM	−92.0	12	−20 No F/T
		CCRF-CEM	−100.0	12	−20
		CCRF-CEM	−36.2	14	−20
	3.0	Daudi	−14.8	7	−20
		Daudi	−33.4	7	−20
		Daudi	−17.7	14	−20
		Daudi	−41.3	14	−20
		HT29	−20.7	7	−20
		HT29	−20.8	14	−20
Cisplatin	3.8	CCRF-CEM	−1.0	2	RT
		CCRF-CEM	−2.8	6	RT
		Daudi	−78.5	1.5	−20
Carboplat	15.8	CCRF-CEM	−3.9	11	−20
		CCRF-CEM	−1.1	13	−20
		CCRF-CEM	+2.8	24	−20
		Daudi	+4.5	11	−20
		Daudi	+0.3	13	−20
		Daudi	+6.8	24	−20
Doxorubicin	0.5	A431	+2.3	1	−20
		A431	+0.6	9	−20
		A431	−0.8	10	−20
		CCRF-CEM	−0.1	9	−20
		CCRF-CEM	−0.3	15	−20
Epirubicin	0.5	CCRF-CEM	0.0	9	−20
		CCRF-CEM	−0.2	15	−20
Mitomycin C	0.12	CCRF-CEM	−9.1	2	−20
		CCRF-CEM	−7.3	2	−20
	0.23	CCRF-CEM	−22.6	11	−20
		CCRF-CEM	−16.4	12	−20
		CCRF-CEM	−31.0	18	+4
		CCRF-CEM	−7.5	18	+4
		Daudi	−10.9	12	−20
		Daudi	−4.7	18	−20
Mitoxantrone	0.04	CCRF-CEM	+0.4	4	RT
		CCRF-CEM	−0.3	9	RT
5-FU	45	HT29	+1.1	3	−20
		HT29	+2.2	4	−20
		HT29	+1.0	4	−20
		HT29	0.0	5	−20
		HT29	+2.2	5	−20
		HT29	+1.6	5	−20
		HT29	+1.2	6	−20
		CCRF-CEM	+0.2	6	−20
		CCRF-CEM	−0.5	9	−20
		CCRF-CEM	−0.5	13	−20
	22.5	Daudi	−0.3	6	−20
		Daudi	−0.3	9	−20
		Daudi	−0.5	13	−20

Continued...

Table 3. (*Continued*)

Drug	100% TDC (μg/ml)	Cell lines tested	Variation (%)	Storage time (months)	Storage temperature (°C)
Methotrexate	2.8	HT29	+2.15	1	−20
		HT29	+2.2	5	−20
		HT29	−2.6	7	−20
		HT29	+9.3	7	−20
		A431	0.0	1	−20
		A431	−2.2	6	−20
		A431	+1.5	8	−20
	0.09	Daudi	−0.3	4	−20
		Daudi	−1.2	11	−20
	0.18	CCRF-CEM	−0.2	2.5	−20
		CCRF-CEM	−1.9	10	−20
Viristine	0.4	HT29	−1.8	2	−20
		HT29	−1.2	2	−20
		HT29	−1.0	5	−20
	0.05	CCRF-CEM	−2.3	4	−20
		CCRF-CEM	−2.4	5	−20
Etoposide	3	CCRF-CEM	−0.1	7	RT
		CCRF-CEM	−0.3	8	−20

The 100% TDC values are given in brackets. Intra-assay error rates <8% are usual: differences greater than this represent significant degradation. F/T = Freeze-thaw cycle, RT = Room temperature.

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(Received 10 January 1994; accepted 24 January 1994)