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Properties of Anticancer Agents Relevant to in vitro Determinations of Human Tumor Cell Sensitivity

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Summary. *The physical properties of 59 anticancer agents have been examined with respect to solubility in tissue culture media, binding to ultrafiltration materials, and molecular absorbance and fluorescence behavior. Methods for dissolving these agents, which are compatible with in vitro sensitivity testing of human tumor cells to anticancer agents, are reported in this paper. The potential for anticancer agent binding to cellulose nitrate/cellulose acetate and teflon membrane ultrafilters was documented, and quantitation of these anticancer agents based upon absorbance and fluorescence spectroscopy was performed. Post-filtration quantitation of anticancer agents was found to be a reliable method for determining the actual drug concentrations available in tumor cell sensitivity testing in vitro. The properties documented herein are pharmacologically relevant parameters related to in vitro determinations of human tumor cell sensitivity to anticancer agents.*

Introduction

Recently, a fundamental new approach in cancer treatment has resulted from the realization that chemotherapy can be individualized on the basis of determinations which assess the sensitivity in cell culture of tumor cells, prepared from individual cancer patients, to various anticancer agents [5, 6, 8–10, 13]. At present, this type of approach has been more successful in identifying those chemotherapeutic agents which will be ineffective in the patient than in predicting those anticancer agents that will be effective *in vivo*. Specifically, this method has been reported to identify drug resistance with a predictive accuracy of 96%–99% and drug sensitivity with a predictive accuracy of 60%–70% [11, 13].

It should be obvious that a drug must be in solution when presented to tumor cells in the test determination; moreover, the means used to achieve drug solvation must not interfere with tumor cell replication. If a drug is not in solution, its precipitation presents a potential mechanism for concentrating drug onto tumor cells, which can lead to false-positive indications of tumor cell sensitivity. Conversely, if drug is allowed to precipitate at some stage prior to introduction to the test determination, the available drug concentration in the test will be much lower than expected and false resistance may be predicted. Often it is necessary to use membrane filtration to remove potential biocontaminants from drug solutions prepared for test determinations. Drug interaction with filtration materials has the potential for moderating the apparent cytotoxic drug activity by reducing the effective drug concentration.

At present there is no comprehensive source of information on anticancer agent solubility in tissue culture media or on drug interaction with filtration materials, although significant information does exist on the clinical formulation of anticancer agents [4, 12]. Moreover, it is conceptually advantageous to operate within a uniform test background so that only drug-related effects are compared, from test to test, rather than some complex permutation of drug together with buffer, extender, preservative, carrier, solvent, etc.

For these reasons, we report here on the compatibility of anticancer drug solvation with the tissue culture environment and document the potential for solvent-mediated effects, as well as anticancer agent binding to filtration materials.

Materials and Methods

All determinations on chemotherapeutic agents were performed on pure compounds in the absence of preservatives, stabilizers or extenders. Actinomycin D, bleomycin, cyclophosphamide, progesterone, retinoic acid, and retinol were obtained from Sigma Chemical, St. Louis, USA. *cis*-Diamminedichloroplatinum II, *cis*-dichloro-ethylenediamineplatinum, cordycepin, 5-fluorouracil, and mitomycin C were obtained from Boehringer Mannheim, Indianapolis, Indiana, USA. Doxorubicin was generously provided by Adria Laboratories, Columbus, OH, USA; an alternative batch was obtained from Aldrich Chemical Company, Milwaukee, Wisc., USA. Methotrexate and Thio-TEPA were provided by Lederle Laboratories, Pearl River, NY, USA. Bisantrene hydrochloride ('Orange Crush' or 9,10-antracenedicarboxaldehyde bis-[(4,5-dihydro-1H-imidazol-1-yl) hydrazone] was provided by American Cyanamid, Pearl River, NY, USA. Cytarabine, medroxyprogesterone acetate, and streptozocin were provided by the Upjohn Company, Kalamazoo, MI, USA. Etoposide ('Vp 16–213'), *cis*-platinum, and prednimustine were generously supplied by Bristol Laboratories, Syracuse, NY, USA. Finally, the Investigational Drug Branch of the National Cancer Institute provided the following: aclacinomycin, amasacrine, anguidine, azapicyl, aziridinylbenzoquinone ('AZQ'), Baker's antifol, bruceatin, camptothecin, carboquone, chlorozotocin, cyclocytidine, daunorubicin, dibromodulcitol, dichloroally lawsone, dichloromethotrexate, dihydroxyanthracenedione, etoposide, gallium nitrate, hexa-

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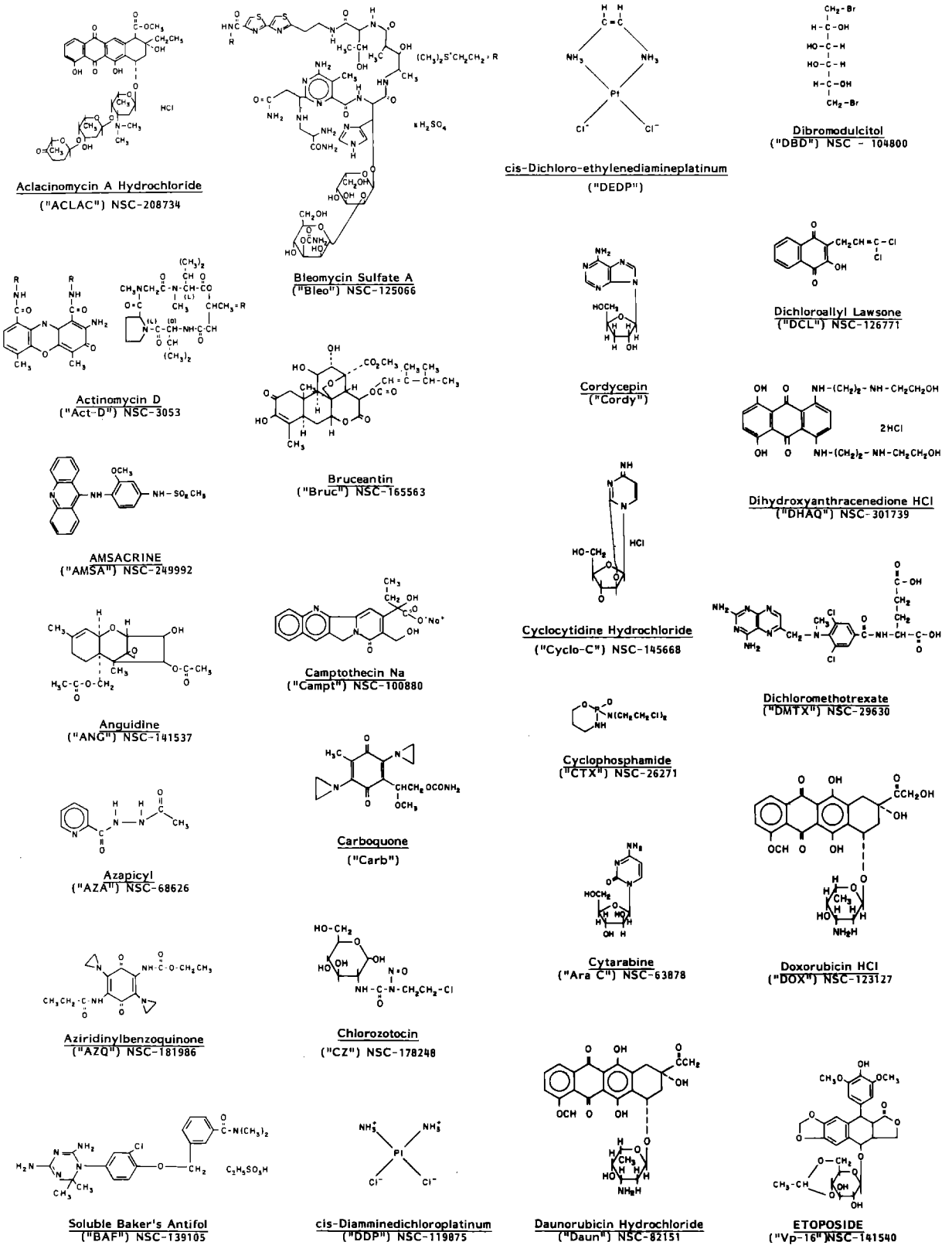

Fig. 1. Chemical structures, National Service Center Identifiers, and abbreviated designations of selected anticancer agents

methylmelamine, ICRF-159 (Razoxane), ICRF-187, ifosfamide, leucovorin, levamisole, lomustine (CCNU), maytansine, melphalan, 5-methyltetrahydrohomofolate, mitoguazone, nafoxidine, PALA, PCNU, pentamethylmelamine, prednisolone, semustine (methyl-CCNU), streptonigrin, tegafur (Ftorafur), and teniposide ('VM-26'). Drugs dissolved in water were used in absorbance and filter-binding determinations, while drugs dissolved in saline were used in fluorescence determinations. A DU-8 spectrophotometer (Beckman Instruments) was used for absorbance determinations, while an Aminco Bowman Spectrophotofluorometer fitted with a high-intensity Xenon lamp was used in fluorescence determi-

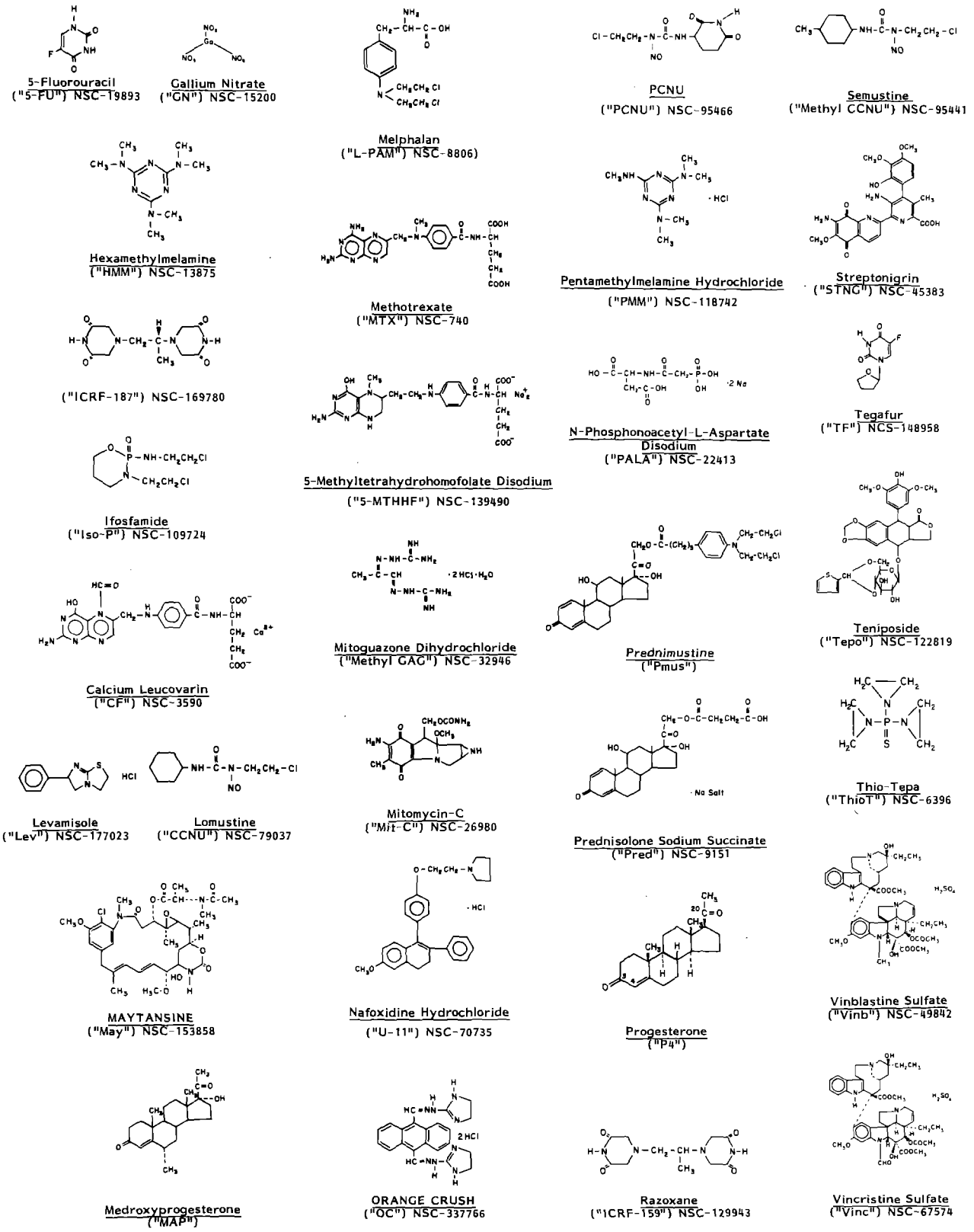

Fig. 1. (continued)

nations. Scans were performed over the range 200–800 nm. Fluorescence determinations were performed in quartz cuvettes (2 ml) with drug fluorescence determined before and after incubation with tumor cells (American Type Culture Collection Cell Line CCL2; 1 h, 37° C). Drugs were dissolved at 1 mg/ml and progressive dilutions were made to identify concentration ranges which remained linear with respect to absorbance or fluorescence. At least eight different concentrations were examined in duplicate over the linear range, and these concentrations were quantitatively reexamined after filtration in 1-ml volumes. Sterile disposable filtration units (27 mm diameter) were used: Millex-OR, 0.22 μm pore and

Millex-FG, 0.2 μm pore (Millepore Corp., Bedford, Mass, USA.

An alphabetic listing of the compounds considered in this study, together with the corresponding National Service Center numbers, chemical structures and abbreviated designations is presented in Fig. 1.

Results

Chemical Solubilities and Filter Binding Characteristics are Properties that can Moderate the Activity of Anticancer Agents in vitro

Agents with Aqueous Solubilities

Two prerequisites which must be accommodated by test determinations for in vitro sensitivity to anticancer agents are that (1) agents remain soluble in the cell culture environment and (2) a uniform test background is employed so that the only variable from test to test is drug alone, rather than some covariant combination of drug together with buffer, extender, preservative, carrier, and solvent. The latter condition precludes the use of sterile pharmaceutical preparations, which rarely contain drug alone; moreover, most investigational drugs are more prudently evaluated by methods that can be dissociated from a preliminary formulation which may not ultimately be clinically optimal. As a consequence, it becomes necessary to remove potential biocontaminations from dissolved anticancer preparations by membrane ultrafiltration.

A large number of anticancer agents are water-soluble and hence soluble in tissue culture media (Dulbecco's MEM, RPMI (1640), McCoy's and CMRL) as identified in Table 1. Vinblastine sulfate differs somewhat from the other agents in Table 1 in that its solubility in tissue culture media requires slight acidification with dilute HCl. We have adopted two criteria for effective solubility in tissue culture media: first, no visible precipitate should form after incubation at 37° C for up to 5 h and second, the spectral profiles, which are unique to each agent, should also persist after incubation. All the agents in Table 1 meet both these criteria, except for anguidine and cyclophosphamide which, while not demonstrating any absorbance properties, did not form visible precipitates at concentrations of up to 500 μg/ml.

The agents in Table 1 have been dissolved in a working stock solution of up to 1 mg/ml. Dilutions of the working stock solution are made and used for in vitro determinations of tumor cell sensitivity to anticancer agents. As a consequence, the working stock solutions for all the agents in Table 1 could be diluted and quantitated by absorbance spectroscopy, except

Table 1. Properties of selected water-soluble anticancer agents

Agent	Molecular weight	Absorbance maxima (nm)*	Linear absorbance range: μg/ml	Millex OR binding	Millex FG binding
Aclacinomycin	884.4	203, 228, 258, 435	10–100	–	–
Actinomycin-D	1255.5	211, 241, 318, 440	4– 50	> 95%	50%–60%
Anguidine	366	no absorbance	–	–	–
Azapicyl	179.17	236, 275	10–100	< 5%	–
(Soluble) Baker's antifol	539	245	10–100	–	–
Bleomycin	1400	249	10–300	–	–
Camptothecin	388.4	217, 252, 294, 367	10–200	< 5%	–
Chlorozotocin	313.7	231, 248, 387	10–300	5%–10%	–
cis-DED-platinum	326.1	224, 292	75–300	–	–
cis-platinum	300.0	223, 301, 356, 362	10–300	–	–
Cyclocytidine	261.5	244	10–100	–	–
Cyclophosphamide	261.1	no absorbance	–	–	–
Cytarabine	243.22	241	10–100	–	–
Daunorubicin	564	239, 251, 289, 481	10–100	5%–10%	10%–15%
Dihydroxyanthracenedione	517.4	241, 275, 319, 608, 661	10–200	50%–60%	10%–20%
Doxorubicin	579.99	233, 252, 299, 453, 494	1– 30	> 95%	30%–50%
5-Fluorouracil	130.1	244	10– 75	< 5%	–
Gallium nitrate	256	229	25–300	–	–
ICRF-187	268	229, 319	10–300	~ 20%	–
Ifosfamide	261	203, 285	Nonlinear to 300 μg/ml		–
Leucovorin	483	242, 313, 325	10–300	5%–10%	–
Levamisole	241	243	10–100	15%	10%–20%
Maytansine	692	210, 232, 252, 279, 296	0.5–5	50%–70%	60%–90%
5-Methyltetrahydro-homofolate	517.5	244, 313, 335	10–300	15%–20%	–
Mitoguazone dihydrochloride	275	243, 262, 313, 337	10–300	–	–
Mitomycin C	334	225, 373, 554	10– 75	< 5%	–
"Orange crush"	471.4	236, 246, 255, 273, 408	10–100	–	–
PALA	299	216	10–300	–	–
Pentamethylmelamine	232.7	244, 249, 256	10– 75	–	–
Streptozocin	265	242, 390	10–200	–	–
Tegafur	200	244	10–300	5%–10%	–
ThioTEPA	189	217	10–300	–	–
Vinblastine	909.1	230, 247, 251, 313, 323	10–300	–	–
Vincristine	923.1	243, 313	10–200	10%–15%	10%–15%

[a] The major absorbance wavelengths are underlined; the concentration curves were determined at the major absorbance wavelength

for the two nonabsorbing agents and ifosfamide. Quantitation by absorbance spectroscopy was used to determine drug concentration before and after membrane filtration, to determine whether any agents would bind to the filtration materials. Assessments of filter binding were run at five different drug concentrations over the linear absorbance range. All agents were evaluated for binding to both the cellulose nitrate/cellulose acetate-based Millex OR filters and the teflon-based Millex FG filters. Drug binding estimates were related to the highest linear concentration tested when post-filtration determinations remained linear with a reduced slope. When post-filtration determinations become asymptotically nonlinear, drug binding estimates related the asymptotic extrapolated value to the highest linear concentration tested. Drug binding to the cellulose nitrate/cellulose acetate filters was very significant with actinomycin D, dihydroxyanthracenedione, doxorubicin and maytansine, while some binding occurred with chlorozotocin, daunorubicin, ICRF-187, leucovorin, levamisole, 5-methyltetrahydro-homofolate, tegafur, and vincristine. In general, binding to the teflon-based filters was equivalent to or less than binding to the cellulose nitrate/cellulose acetate filters; however, maytansine was observed to be bound significantly better by the teflon filters (Table 1). Orange Crush (i.e., OC or bisantrene) presents a solubility problem because the introduction of this agent (dissolved in distilled water) into several media (DME, RPMI, McCoy's, CMRL) and buffers (saline, buffered saline, HBSS) results in immediate precipitation. Spectrophotometric determinations revealed that subsequent filtration clears virtually all the bisantrene from the preparation.

A limited number of agents can be solubilized as salts generated with NaOH (20 m*M*) (Table 2). These agents remain soluble after introduction into media maintained at pH 7.4 for up to 5 h at 37° C, as evidenced by a lack of visible precipitates and the persistence of individualized spectral profiles. Only one of these agents (ICRF-159) was significantly bound by cellulose nitrate/cellulose acetate filters, and this interaction could be reduced by the use of teflon filters.

Agents with Nonaqueous Solubilities. A number of agents cannot be directly solubilized in water or liquid media. These agents are soluble in the organic solvents, ethanol, and *N,N*-dimethylacetamide, both of which must be used at concentrations that do not perturb cellular replication in the in vitro test system. Several primary tumor cell preparations, as well as established tumor cell lines, have been exposed to increasing concentrations of these solvents (data not shown). Some tumor cells have greater sensitivity to these solvents and can be used to establish the lower limits of tolerance for ethanol and *N,N*-dimethylacetamide, as shown in Fig. 2. In contrast, tumor cell preparations, which demonstrate an exceptional tolerance to these solvents, continue unperturbed replication at up to 10–12 µl/ml ethanol or 1–1.5 µl/ml *N,N*-dimethylacetamide, and should be considered more unusual than common. From a solvent toxicity standpoint, ethanol is better tolerated and consequently has greater solvent utility than *N,N*-dimethylacetamide.

As a consequence of solvent-mediated effects on replication, it becomes necessary to prepare very high concentrations of certain agents in either ethanol or *N,N*-dimethylacetamide to deliver modest, nontoxic amounts of solvent to cell culture test determinations. Agents that can be dissolved in ethanol at concentrations high enough to enable the great dilution required to avoid solvent mediated effects are listed in Table 3A, while agents which must be dissolved in *N,N*-dimethylacetamide are listed in Table 3B. It should be noted that AZQ (aziridinylbenzoquinone) is much more highly soluble in *N,N*-dimethylacetamide. (Organization of agents in Table 3A

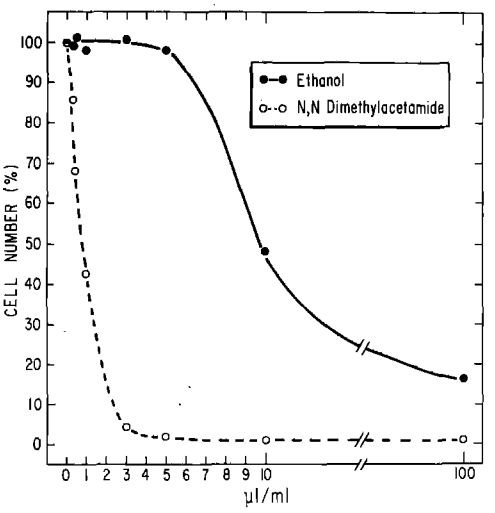

Fig. 2. Solvent-related effects on tumor cell replication. Double glass distilled solvents were obtained from Burdick and Jackson Chemical Company. Tumor cells (ATCC CCL2) were plated in the presence of concentrations of solvent as indicated, and were allowed to replicate for 10 days. Cells were grown in Dulbecco's Modified Eagle's Medium with bicarbonate and Hepes buffering (10 m*M*) at a final supplementation of 10% fetal calf serum and 100 U/ml penicillin/streptomycin. Cells plated in plastic tissue culture vessels (4×10^3 cells/cm^2) were nourished with a single change of medium (0.5 ml/cm^2) and increased to 64×10^3 cells/cm^2 (i.e., 100%) in 10 days. Cells were released to single-cell suspensions by exposure to Hank's balanced salt solution containing EDTA (2 m*M*, pH 7.4) and were counted electronically using a Model ZF Coulter Counter. All determinations were run in triplicate

Table 2. Properties of selected anticancer agents that are soluble as salts generated with NaOH (20 mM)

Agent	Molecular weight	Absorbance maxima (nm)*	Linear absorbance range: µg/ml	Millex OR binding	Millex FG binding
Cordycepin	251.2	215, 260	10–100	< 5%	–
Dichloromethotrexate	559.4	251, 255, 317, 371	10–100	–	–
ICRF-159	268	206, 229	10– 50	30%	–
Methotrexate	454.9	231, 250, 313, 320, 372	10–100	–	–
Streptonigrin	506	244, 313, 389, 554	10–100	–	–

[a] Parameters are as described in Table 1

Table 3. Preparations of select anticancer agents which are solubilized in ethanol (A) or *N,N*-dimethylacetamide (B) and remain soluble after being introduced in liquid media

Agent	Molecular weight	Absorbance Maxima: (nm)*
A. Soluble in ethanol		
Amsacrine	393.5	248, 408
AZQ[a]	364.4	241, 355, 472
Bruceatin	548	232
Carboquone	321.24	243, 313, 373, 541, 554, 565
Dibromodulcitol[c]	308	212
Dichloroallyl lawsone	283.1	236, 276, 313, 355, 377, 555
Hexamethylmelamine	210.3	241
Lomustine	234	241, 383, 398, 415
Medroxyprogesterone	344.48	256, 253
Melphalan[b]	305	215, 255, 258, 301
Nafoxidine[a]	461.5	245, 282, 313, 325
PCNU	262.7	216, 233
Prednimustine[b]	646.6	262, 300
Prednisolone[b]	482.5	248, 354
Progesterone	314.5	248, 250, 260, 308, 316
Retinoic acid	300.42	208, 240, 348
Retinol	286.5	241, 298, 306, 313, 341
Semustine	248	242, 383, 398, 415
B. Soluble in *N,N*-dimethylacetamide		
AZO	364.4	241, 355, 472
Etoposide	588	246, 279, 289, 410
Teniposide	656	285

[a] Must be heated
[b] Acid ethanol: 0.6% HCl in ethanol
[c] Hot, acid ethanol

and 3B is based upon minimizing solvent-related toxicity given by priority to the use of ethanol over *N,N*-dimethylacetamide. Heating of agents is limited to warming the vial under a stream of hot tap water.) The previously discussed criteria for solubility can be satisfied by using these two organic solvents to deliver the agents to aqueous environments; namely, in the absence of visible precipitates unique and individualized absorbance profiles can be obtained even after incubation at 37° C. Finally, it should be noted that both ethanol and *N,N*-dimethylacetamide are microcidal so that ultrafiltration becomes unnecessary.

Except for the agent AZQ, all agents listed in Table 3 can be delivered in soluble form to an aqueous tissue culture environment. AZQ (dissolved in ethanol or *N,N*-dimethylacetamide) was observed to precipitate immediately when introduced into tissue culture media (DME, RPMI, McCoy's, CMRL). Thus, with the present systems for drug solvation, the delivery of dissolved AZQ and Orange Crush to tissue culture determinations for tumor cell sensitivity remains unachieved.

Fluorescent Properties of Selected Anticancer Agents

In general, quantitation based upon fluorescence spectroscopy has a greater sensitivity than absorbance spectroscopy. Hence, whenever dilute drug concentrations must be determined or whenever absorbance quantitation is confounded by multiple co-absorbing components, quantitation based upon fluorescence may be useful. Most of the agents demonstrated some measurable fluorescence properties (52 of 59 agents, Table 4), and quantitation based upon a linear relationship between concentration and fluorescence was observed to be possible for 25 of 59 agents, including anguidine, which had not produced sufficient detectable absorbance. In addition, eight agents that were not fluorescent alone in solution formed fluorescent complexes after incubation with cells; these agents were amsacrine, azapicyl, *cis*-DED-platinum, cyclophosphamide, dischloromethotrexate, ICRF-159, levamisole, and mitoguazone dihydrochloride. This type of fluorescence, which occurs only after interaction with cells, may result from quantum coupling interactions between cellular components and drug. Nevertheless, these observations must be regarded at present as largely phenomenonological. Finally, only daunorubicin and doxorubicin (1–5 mg/ml) could be used to visualize tumor cells under the fluorescent microscope after incubation for 1 or 2 h at 37° C.

Discussion

When the in vitro sensitivities of primary tumor cell preparations to anticancer agents are to be evaluated, it is rather obviously necessary that the anticancer agents remain in solution during the test determinations. In this report we have described how 59 anticancer agents may be solubilized for introduction to tissue culture media; moreover, we have described the limits of solvent concentrations which adversely affect tumor cell replication in culture. The occurrence of stable spectroscopic profiles after incubation at 37° C and the absence of observable precipitation are criteria which most pertinently relate to the *dissolved* status of these compounds; moreover, these criteria do not insure that the absorbing species retains the unaltered active drug form or that the biological activity has not changed. In addition, since it is often necessary to remove potential biocontaminants from preparations of anticancer agents by membrane ultrafiltration, we have examined drug binding to ultramembranes and have observed the potential for interaction between different anticancer agents and filtration materials. This phenomenon involving the binding or adsorption of compounds onto insoluble polymeric materials has long been recognized [1–3, 7]. In general, drugs binding to cellulose nitrate/cellulose acetate filters were found to bind less to teflon filters; however, exceptions can occur (i.e., maytansine) and post-filtration drug quantitation should be performed to unambiguously define the actual drug concentrations in cell culture determinations of sensitivity to anticancer agents. All the drugs considered in this study can be quantitated by either absorbance or fluorescence spectroscopy, except for cyclophosphamide. Nevertheless, it may be possible to quantitate cyclophosphamide on the basis of the fluorescence generated after limited interaction with tumor cells.

Finally, we suggest that the in vitro cytotoxic activity of anticancer agents can be moderated by reduced drug concentrations which result from inadequate solvation or by binding to ultrafiltration materials. For the agents listed in this paper, this moderation can be remedied by the appropriate selection of solvents and filters and the use of post-filtration quantitation.

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Table 4. Fluorescent properties of selected anticancer agents

Agent	Agent alone			Agent and cells		
	Excitation λ (nm)	Emission λ (nm)	Linear fluorescence range: ml × 10^{-6}	Excitation λ (nm)	Emission λ (nm)	Linear fluorescence range: ml × 10^{-6}
Aclacinomycin	340	530	6–60	455	530	3–60
Actinomycin-D	340	525	4–40	340	515	4–30
Amsacrine	300	430	–	360	430	0.2–1.5
Anguidine	340	430	180–600	340	430	300–600
Azapicyl	325	435	–	325	355	1,900–5,600
AZQ	300	340	–	280	340	–
(soluble) Baker's Antifol	300	410	0.6–1.9	350	435	0.9–1.9
Bleomycin	315	375	–	315	390	–
Bruceatin	326	380	–	275	340, 380	–
Camptothecin	346	450	0.003–0.030	372	450	0.005–0.025
Carboquone	300	400	–	300	400	–
Chlorozotocin	325	440	0.4–3.2	358	440	0.4–3.2
cis-DED-platinum	350	405	–	350	405	1,100–3,300
cis-platinum	–	–	–	–	–	–
Cordycepin	350	415	–	350	415	–
Cyclocytidine	300	390	420–3,820	300	390	760–3,820
Cyclophosphamide	300	410	–	360	440	770–3,830
Cytarabine	323	380	370–4,100	323	380	310–4,100
Daunorubicin	300	570	4–50	300	570	4–50
Dibromodulcitol	300	340	–	280	340, 415	–
Dichloroallyl lawsone	–	–	–	–	–	–
Dichloromethotrexate	420	480	–	420	480	0.9–1.8
Dihydroxyanthracenedione	–	–	–	–	–	–
Doxorubicin	300	560	4–50	275	560	4–50
Etoposide	300	–	–	282	405	–
5-Fluorouracil	300	385	–	300	385	–
Gallium nitrate	–	–	–	–	–	–
Hexamethylmelamine	272	340	–	272	340	–
ICRF-159	340	395	–	340	395	6–20
ICRF-187	350	420	8–40	350	420	8–40
Ifosfamide	367	450	–	367	450	–
Leucovorin	327	450	4–20	352	450	4–20
Levamisole	320	375	–	278	360	2,070–4,150
Lomustine	334	390	–	334	390	–
Maytansine	370	440	110–1,450	370	440	110–480
Medroxyprogesterone	293	340	–	275	340	–
Melphalan	353	415, 580	6–16	353	415	6–16
Methotrexate	375	450	7–22	375	450	7–22
5-Methyltetrahydrohomofolate	367	450	10–20	367	450	6–20
Mitoquazone dihydrochloride	375	425	–	375	425	400–730
Mitomycin C	410	505	–	410	505	–
Nafoxidine	350	425	5–10	350	425	5–10
Orange crush	325	520	200–1,060	325	550	200–2,120
PALA	–	–	–	–	–	–
PCNU	310	375	20–40	325	375	20–40
Pentamethylmelamine	442	560	–	442	560	–
Prednomustine	300	355	–	300	355	–
Prednisolone	344	405, 500	4–10	344	405, 500	2–10
Progesterone	280	340, 410	5–16	275	340	8–16
Retinoic acid	–	–	–	–	–	–
Retinol	325	475	0.8–7.0	325	475	1.4–7.0
Semustine	300	340	–	275	340	–
Streptonigrin	300	350	–	270	350	–
Streptozocin	320	395	420–3,800	320	395	750–3,800
Tegafur	450	500	–	450	500	–
Teniposide	300	400	–	300	400	–
Thiotepa	362	430, 505	–	362	430, 505	–
Vinblastine	325	360	6–60	325	360	10–60
Vincristine	318	365	120–1,100	318	365	120–1,100

[a] Agent was incubated with cells in balanced saline for 1 h at 37° C

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