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IDENTIFICATION OF NEW CRYOPROTECTIVE AGENTS FOR CULTURED MAMMALIAN CELLS

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SUMMARY

Thirty-one compounds have been identified that act as cryoprotective agents for cultured mammalian cells. Eight compounds were comparable to dimethylsulfoxide (DMSO) in cryoprotective effectiveness. Many of the cryoprotective compounds studied also (a) promote cell fusion and (b) induce cell differentiation in erythroleukemia and other cell systems. Thus, previously unrecognized effects on the differentiated state of cells may occur when cells are treated with cryoprotective agents.

Key words: cryoprotective agents; cell fusion; dimethylsulfoxide; cell differentiation; erythroleukemia.

INTRODUCTION

Several structurally unrelated compounds have been shown to act as cryoprotective agents for mammalian cells. Dimethylsulfoxide (1), glycerol (2), poly(vinylpyrrolidone) (3), and hydroxyethyl starch (4) have demonstrable cryoprotective activity for culture mammalian cells whereas several polar organic compounds (5) have been shown to protect red blood cells from lysis during freezing. Thirty-one compounds, which promote cell fusion (6) and possess cryoprotective properties for cultured mammalian cells are described here. Several cryoprotective agents were identified that are as effective as DMSO.

MATERIALS AND METHODS

Maintenance of cells. CHO Chinese hamster ovary cells (7) were maintained in Dulbecco's medium (Flow Laboratories, McLean, VA) supplemented with 1 m*M* L-proline, 10% newborn bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Hereafter, the above is referred to as culture medium. L-Proline was incorporated in the culture medium because CHO is a proline auxotroph (7).

Cryoprotection assay. A series of compounds were assayed for their ability to protect CHO cells from freezing damage. In brief, a 1.2 ml Nunc cryotube (Vangard International, Inc., Neptune, NJ) containing 10^5 CHO cells in 1 ml was cooled to −80° C. Cells were frozen in complete culture medium containing various concentrations of a given test compound. Each cryotube was inserted at the center of a 512 cm^3 block of styrofoam (density = 0.25 g/cm^2) before being placed in a −80° C freezer for at least 16 h. Cells were frozen at a rate of approximately 2.6° C/min from room temperature to the freezing point and 3.2° C/min in the range from −10° C to −30° C. With the aid of a digital thermometer (Model 2500-C, Extech, Boston, MA), the cooling rates above were measured by inserting a thermistor probe into an insulated cryotube containing 1 ml of water. Cells were thawed at a rate of approximately 38° C/min by rapid agitation in a 37° C water bath. The warming rate was determined, between −80° and 0° C, by measuring the time required for the disappearance of ice after rapid shaking in a 37° C water bath. The cooling and warming rates employed are comparable to recommended rates (8). Thawed cells were inoculated at densities of 10^4, 10^3, and 10^2 cells/culture flask (Corning Glass Works, Corning, NY) in 5 ml of complete culture medium. Inasmuch as the protocol employed results in a 1:5,000 dilution of cryoprotective agent in the 10^2 cells/flask points, thawed cells were not washed before assay to avoid loss of cells during washing. After 7 d,

colonies were stained with Wright's stain and enumerated. Each experiment was carried out with two controls; namely, cells frozen in either 10% DMSO in culture medium or cells frozen without a cryopreservative. The cryoprotective effect elicited by a test compound is presented as a percent of the cryoprotective effect of 10% DMSO (Table 1). The compounds studied are listed in decreasing order of cryoprotective effectiveness (Table 1). Results were calculated from the following ratio:

$$\text{Percent DMSO cryoprotectiveness} = \frac{\text{Number of colonies cryoprotected by a test compound}}{\text{Number of colonies cryoprotected by 1.3 } M \text{ DMSO}} \times 100.$$

TABLE 1

COMPOUNDS WITH CRYOPROTECTIVE PROPERTIES

Compound	Percent DMSO Cryoprotection Effectiveness	Optimum Concentration (Molar)	Concentrations Tested (Molar)	Number of Experiments	Promotion of Poly(ethylene glycol) Mediated Cell Fusion	Induction of Erythroleukemia Cell Differentiation
Ethylene glycol[a]	230%	1.6	5.0-0.16	3	+	
Methyl acetamide[a]	140	1.0	2.0-0.0001	4	+	(16)
PEG-3000[b]	130	20%	20-1%	3	+	
Polyvinyl pyrrolidone[b] (10,000 mw)	115	40%	40-0.01%	2	+	
DMSO[b]	100	1.3	7.5-0.012	25	+	(11)
PEG methyl ester[c]	58	10%	50-0.1%	2	+	
N,N'-dimethylformamide[c]	53	0.1	1.0-0.001	2	+	(16)
Glycerol[d]	49	0.55	3.3-0.03	2	+	
PEG-200[b]	33	1.0	1.0-0.05	3	+	(16)
Dextran-4, 700[b]	28	20%	20-0.1%	2		
PEG-600[b]	28	10%	30-0.1%	2	+	
Sucrose[b]	26	1.0	1.0-0.001	2		
Fructose[b]	23	1.0	1.0-0.001	2		
Methanol[d]	16	1.0	6.6-0.03	3		
N,N'-Dimethylacetamide[a]	15	0.1	1.0-0.001	2	+	(16)
Glucose[b]	13	1.0	1.0-0.001	2		
Tetramethylene sulfone[a]	12	0.01	0.1-0.001	2		
Acetamide[a]	12	0.1	1.0-0.001	2	+	(16)
Potassium bromide[c]	6	0.1	1.0-0.001	2	+	
Decaglycerol	6	3%	30-0.1%	2	+	
Sodium sulfate[b]	5	0.001	0.1-0.0001	2	+	
Methyl sulfone[a]	5	0.1	1.0-0.001	2	+	
N-Methylformamide[a]	5	0.1	1.0-0.001	2	+	(16)
Guanidine HCl[d]	4	0.001	1.0-0.0001	2	+	
Dextran (18,000)[b]	3	10%	10-0.1%	2		
Urea[c]	3	0.3	1.0-0.03	2	+	
Dextran-40,000[b]	2	10%	10-0.1%	2		
Valerolactam[a]	2	0.001	0.1-0.001	2		(16)
Pyridine-*N*-oxide[a]	2	0.01	1.0-0.001	2		(16)
Dextran (10,500)	1	10%	10-0.001%	2		
Hydroxyethyl starch[b]	1	1%	10-0.001%	2		

Compounds are listed in order of their decreasing effectiveness as cryoprotective agents. Results are expressed as a percent of the cryoprotective effect of the reference compound, DMSO. The optimum concentration for the cryoprotective effect of each compound is indicated, as well as the range of concentrations tested. With the exception of high molecular weight polymers, whose concentration is given in percent wt/vol, all other concentrations are given in molar units. The values presented under "percent DMSO cryoprotective effectiveness" (*see* Methods) are averages obtained after at least two separate trials at the optimum concentration for cryoprotective effect. In all but one case (acetamide), cryoprotective effects were observed at one or more concentrations, in addition to the optimum concentration. Literature references provide information concerning the ability of each cryoprotective agent to induce erythroleukemia cell differentiation. The ability of the compounds studied to promote cell fusion is drawn from (15).

[a] Aldrich Chemical Co. (Milwaukee, WI).

[b] Sigma Chemical Co. (St. Louis, MO).

[c] J. T. Baker Chemical Co. (Phillipsburg, NJ).

[d] Fisher Scientific (Houston, TX).

Reagents. The source of compounds tested for their cryoprotective effect is given in Table 1. All other compounds were of reagent grade.

Results and Discussion

Under the conditions employed for the detection of cryoprotective compounds, CHO cells frozen under optimal conditions with a cryopreservative had an average plating efficiency of as high as 95% whereas cells frozen without a cryopreservative had an average plating efficiency of 0.6%. Thus, the assay system employed provides a sensitive method for the identification of new compounds with cryoprotective properties.

The evidence presented documents the relative effectiveness of 31 compounds as cryoprotective agents for cultured mammalian cells (Table 1). Compounds found to possess cryoprotective properties are listed in Table 1 in descending order of their effectiveness with respect to the reference compound, DMSO. Table 1 presents the most effective concentration tested for each entry in the table as well as the range of concentrations tested. The well known cryopreservative agents, DMSO and glycerol, were among the most effective cryoprotective agents studied. Eight other compounds, comparable to DMSO in cryoprotective effectiveness, were identified by the assay system employed.

Synergism between several cryoprotective agents is demonstrated in Table 2. The cryoprotective effects of combinations of cryoprotective agents was greater than one would expect if the action of each agent was simply additive. Synergism between chemical cell fusion agents (6) and inducers of erythroleukemia cell differentiation (9) have been reported previously. The observation of synergistic action between cryoprotective agents indicates that several structurally unrelated compounds may have similar modes of action.

TABLE 2

Synergism Between Cryoprotective Agents[a]

Compound	Percent DMSO Cryoprotective Effectiveness
DMSO (0.13 *M*)	1.4%
N,N'-dimethylformamide (0.03 *M*)	3
PEG — 200 (0.05 *M*)	5
Glucose (0.03 *M*)	0.011
DMSO (0.13 *M*) + *N,N'*-dimethylformamide (0.03 *M*)	18
DMSO (0.13 *M*) + PEG - 200 (0.05 *M*)	12
DMSO (0.13 *M*) + Glucose (0.03 *M*)	3

[a] Suboptimal concentrations of cryoprotective agents were employed in combination to protect CHO cells from freezing injury. When cryoprotective agents were used in combination, their combined effect was greater than one would expect, due to their additive effect. The studies described above were carried out in an identical fashion to the survey presented in Table 1 except that cells were frozen in the presence of two cryoprotective agents at the concentrations indicated. Experimental points were compared to the cryoprotective effect of 1.3 *M* DMSO (100% cryoprotection).

Inasmuch as DMSO has well known effects (a) as a cryopreservative (1,8), (b) as an inducer of erythroleukemia cell differentiation (10,12), and (c) as an enhancer of poly(ethylene glycol) induced cell fusion (13,15), Table 1 also indicates the existence of such biological effects for other cryopreservatives detected in this survey. Of the 31 cryoprotective agents studied, 20 possessed the ability to either promote cell fusion or induce erythroleukemia cell differentiation (Table 1) or both. Because it is clear that agents that promote cell fusion have membrane active properties, it is also evident that many cryoprotective agents have direct effects on cell membranes. It has been demonstrated previously that several cryoprotective agents are inducers of erythroleukemia cell differentiation (14,16) and, hence, previously unrecognized alterations of the differentiated state of cells may occur after freezing in the presence of many cryoprotective agents.

References

1. Lovelock, J. E.; Bishop, M. W. H. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 183: 1394-1395; 1959.
2. Hay, R. J. Preservation of cell-culture stocks in liquid nitrogen. TCA Manual 4: 787-790; 1978.
3. Farrant, J. Is there a common mechanism of protection of living cells by polyvinylpyrrolidone and glycerol during freezing? Nature 222: 1175-1176; 1969.
4. Persidsky, M. D.; Ellett, M. Hydroxyethyl starch as a cryoprotective agent for nucleated mammalian cells. Cryobiology 8: 586-588; 1971.
5. Nash, T. The chemical constitution of compounds which protect erythrocytes against freezing damage. J. Gen. Physiol. 46: 167-175; 1962.
6. Klebe, R. J.; Mancuso, M. G. Chemicals which promote cell hybridization. Somatic Cell Genet. 7: 473-488; 1981.
7. Kao, F. T.; Puck, T. T. Genetics of somatic mammalian cells. IV. Properties of Chinese hamster cell mutants with respect to the requirement for proline. Genetics 55: 513-524; 1967.
8. Akhtar, T.; Pegg, D. E.; Foreman, J. The effect of cooling and warming rates on the survival of cryopreserved L-cells. Cryobiology 16: 424-429; 1979.

9. Priesler, H. D.; Lyman, G. Differentiation of erythroleukemia cells in vitro: Properties of chemical inducers. Cell Differ. 4: 179–185; 1975.

10. Bertolini, L.; Tsuei, D.; Holland, J. G.; Friend, C. Variations in the response of cloned murine Friend erythroleukemia cells to different inducers. In Vitro 17: 284–289; 1981.

11. Friend, C.; Scher, W.; Holland, J. G.; Sato, T. Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: Stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA 68: 378–382; 1971.

12. Reuben, R. A.; Rifkind, R. A.; Marks, P. A. Chemically induced murine erythroleukemic differentiation. Biochim. Biophys. Acta 605: 325–346; 1980.

13. Norwood, T. H.; Zeigler, C. J.; Martin, G. M. Dimethyl sulfoxide enhances polyethylene glycol-mediated somatic cell fusion. Somatic Cell Genet. 2: 263–270; 1976.

14. Priesler, H. D.; Christoff, G.; Taylor, E. Cryoprotective agents as inducers of erythroleukemic cell differentiation in vitro. Blood 47: 363–368; 1976.

15. Klebe, R. J.; Mancuso, M. G. Enhancement of poly(ethylene glycol) mediated cell hybridization by inducers of erythroleukemia cell differentiation. Somatic Cell Genet. 8: 723–730; 1982.

16. Tamaka, M.; Levy, J.; Terada, M.; Breslow, R.; Rifkind, R. A.; Marks, P. A. Induction of erythroid differentiation in murine virus infected erythroleukemia cells by high polar compounds. Proc. Natl. Acad. Sci. USA. 72: 1003–1006; 1975.

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