[CANCER RESEARCH 45, 1206–1213, March 1985]

Cytotoxicity of the Alkyl-linked Lipoidal Amine 4-Aminomethyl-1-[2,3-(di-*n*-decyloxy)-*n*-propyl]-4-phenylpiperidine (CP-46,665) in Cells from Human Tumors and Leukemias

Wolfgang E. Berdel,[1] Hans D. Schick,[1] Ulrich Fink, Anneliese Reichert, Kurt Ulm, and Johann Rastetter

Division of Hematology and Oncology, Department of Medicine I [W. E. B., H. D. S., U. F., A. R., J. R.] and Department of Medical Statistics and Epidemiology [K. U.], Technical University, Ismaninger Str. 22, 8000 Munich 80, Federal Republic of Germany

ABSTRACT

The alkyl-linked lipoidal amine 4-aminomethyl-1-[2,3-(di-*n*-decyloxy)-*n*-propyl]-4-phenylpiperidine (CP-46,665) inhibited the *in vitro* incorporation of tritiated thymidine into blasts of eight leukemias and cells of nine different solid tumors of human origin. This activity was well correlated with trypan blue dye exclusion, which was tested to assess cell membrane damage. Scanning electron microscopy revealed loss of cell surface features and severe cell membrane destruction after incubation with CP-46,665. These effects on thymidine uptake and single cell viability were accompanied by a clear loss of the reproductive capacities of human tumor and leukemic cells as measured in a human tumor stem cell assay after incubation with CP-46,665.

The above-mentioned cytostatic and cytotoxic effects of CP-46,665 were dependent on dosage and incubation time. Destruction of leukemic blasts was often completed with ≥5 μg/ml after an incubation of ≥48 hr or ≥10 μg/ml after an incubation of ≥24 hr. Cells from solid tumors usually required a slightly higher drug concentration and longer incubation period for maximum killing. The alkyl-linked lipoidal amine CP-46,665 often showed considerably greater efficacy than did the alkyl-linked phospholipid *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine tested in comparison. In contrast to both drugs, 2-lysophosphatidylcholine showed only minor activity within the same dose range.

INTRODUCTION

Recently, there is increasing interest in the antitumor activities of certain ether-lipids. ALP,[2] *e.g.*, represent a new class of biological response modifiers (28), which inhibit the growth (27, 28) and the metastasis (5) of syngeneic murine tumors, and also have been used successfully in treating experimental rat tumors (10, 11). This activity might be partially mediated by enhancing cytotoxic properties of macrophages (5, 27, 28) and direct effects on neoplastic cells, since some ALP destroy leukemic (3, 26, 35) and tumor cells (6, 31), and others induce differentiation of leukemic blasts (18) or counteract tumor cell invasion (34). Putative molecular mechanisms have been reviewed recently (4). First clinical pilot studies with the analogue ET-18-OCH_3 are in progress (9). However, other groups of ether-lipids have also been shown to possess antitumor activity. They include glyceryl ethers of fatty alcohols (2, 13), haloanalogues of ALP (12), thioether-lysophospholipid derivatives (7), alkylethyleneglycophospholipids (19, 20), and 1-alkyl-2-acetamideglycero-3-phosphocholine, and other analogues of platelet-activating factor (16).

CP-46,665 {4-aminomethyl-1-[2,3-(di-*n*-decyloxy)-*n*-propyl]-4-phenylpiperidine} is an alkyl-linked lipoidal amine (Chart 1), which has been shown to produce clear antimetastatic effects in rodent tumor models, such as the B-16 melanoma in mice and the rat mammary adenocarcinoma line 13762 (38). Although CP-46,665 does not induce interferon production and does not enhance natural killer cell activity (17), its activity was interpreted as being due to biological response modification, since it enhances the tumor-cytolytic properties of peritoneal exudate cells and alveolar macrophages (17, 25, 38). Direct cytotoxic properties of the compound were also noted (17), but these effects have not been explored previously.

Investigating the cytotoxic properties of a broad variety of ether-lipids, CP-46,665 was introduced in our *in vitro* antitumor screening system, consisting of a variety of assays measuring cytotoxic effects of a test compound at different sites of the cell, which not necessarily correlate (30). This report deals with the cytostatic and cytotoxic effects of CP-46,665 in human leukemic and tumor cells *in vitro*. CP-46,665 was tested in direct comparison to ET-18-OCH_3, which thus far is one of the most active ALP, and to 2-LPC, which represents an ester-linked lysophospholipid of low activity.

MATERIALS AND METHODS

Drugs. CP-46,665 was supplied by Dr. K. E. Jensen, Pfizer Central Research (Groton, CT). Details on chemistry, pharmacokinetics, and toxicology have been published before (17, 25, 38). The ALP ET-18-OCH_3 tested in comparison was purchased from Medmark Chemicals (D-8022; Gruenwald bei Muenchen, Federal Republic of Germany). The ester-linked 2-LPC was purchased from F. Roth (D-7500; Karlsruhe, Federal Republic of Germany). Chart 1 depicts the chemical structures of the 3 compounds tested. The substances were dissolved in RPMI 1640 (GIBCO No. 240; Glasgow, Scotland, United Kingdom) supplemented with 10% FCS (GIBCO No. I 76) containing 50 units of penicillin and 50 μg of streptomycin per ml. The solutions were sterilized by micropore filtration (0.22 μm; Millex; Millipore, Molsheim, France) and eventually stored at −20°.

Cells. Cells of 10 solid tumors and 9 leukemias were tested.

Tumor Cells. Cells of 10 solid tumors, histologically identified as neoplastic tissue, were minced into pieces (<1 cu mm) and then sus-

[1] Recipient of Deutsche Forschungsgemeinschaft Grant Be 822/2-3 and a grant from Pfizer Central Research, USA. To whom requests for reprints should be addressed.

[2] The abbreviations used are: ALP, alkyl-lysophospholipid derivative(s); AL, acute leukemia(s); CML/BC, blast crisis of chronic myelogenous leukemia(s); CMRL, Connaught Medical Research Laboratories; ET-18-OCH_3, *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine; FCS, fetal calf serum; GIBCO, Grand Island Biological Co.; HTSCA, human tumor stem cell assay; 2-LPC, 2-lysophosphatidylcholine; PBS, phosphate-buffered saline [43.85 g NaCl, 1.10 g $NaH_2PO_4 \cdot H_2O$, and 11.95 g $Na_2HPO_4 \cdot 12H_2O$ dissolved in 5 liters of water (bidest.) at pH 7.2 (5 N NaOH) and filtered]; SEM, scanning electron microscopy; TLP, thioether-lysophospholipid derivative(s).

Received June 11, 1984; accepted November 29, 1984.

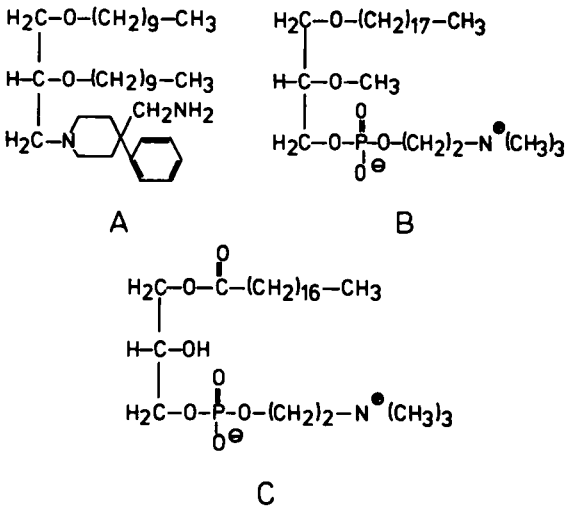

Chart 1. Chemical structures of CP-46,665 (*A*), ET-18-OCH_3 (*B*), and 2-LPC (*C*).

pended in cold PBS. Sediments of this suspension were trypsinized twice as described before (5) and filtered through 4-ply gauze. Trypsinization was stopped by FCS (5). Cells were allowed to grow into dense monolayers by starting cultures in plastic culture flasks (Falcon Plastics, Oxnard, CA) with 1×10^6 cells/ml of "culture medium" [minimal essential medium (GIBCO No. 236) containing 10% FCS, 50 units of penicillin per ml, 50 μg of streptomycin per ml, and 0.3 mg of glutamine per ml] at pH 7.2, 37°, in an atmosphere of 5% CO_2 and high humidity. Culture medium was exchanged daily during the first days. Once a confluent monolayer had developed, cells were subcultured. The monolayer was overlaid with 3 to 4 ml of a 0.9% NaCl:trypsin:EDTA solution, incubated at 37° for 5 min, and then washed from the surface with a jet of culture medium and further dissociated by gentle pipetting. Dilutions of 1:2 were used for at least 2 further successful monolayer cultures before using these continuous cell lines for testing. Monolayer cultures have been characterized as consisting of a malignant cell population by the different criteria of cytological morphology, before cells were added to the test systems.

AL, CML/BC. Venous blood was diluted to RPMI 1640 (1:2), subsequently layered over a density gradient (Lymphoprep; Nyegaard, Oslo, Norway), and spun at 400 × *g* for 30 min. The isolated fractions, morphologically consisting of ≥95% of leukemic blasts with ≤5% of lymphocytes, were washed twice in RPMI 1640 and then diluted to the "test medium" [RPMI 1640 (GIBCO No. 240), supplemented with 10% FCS (GIBCO No. I 76), 50 units of penicillin per ml, and 50 μg of streptomycin per ml]. Cells from HL-60 and K-562 were subcultured as described for tumor cells.

[^{3}H]Thymidine Incorporation. A 100-μl aliquot of the cell suspension in test medium, containing 1 to 2×10^4 tumor cells or 5×10^4 leukemic cells, was placed into each well of a Microtiter plate (Microtest No. 655101; Greiner, D-7440 Nuertingen, Federal Republic of Germany), and then the test agents (100 μl) were added in various concentrations to triplicate cultures. Controls contained 100 μl of pure test medium instead of the test substance. Plates were incubated at pH 7.2 and at 37° in an atmosphere of 5% CO_2 and at high humidity for the times indicated. The cultures were pulsed for 24 hr with 0.5 μCi of [6-^{3}H]thymidine per well (specific activity, 5 Ci/mmol; Amersham and Buchler, D-3300 Braunschweig, Federal Republic of Germany). The samples were harvested (MASH II; Flow Laboratories, D-5300 Bonn, Federal Republic of Germany) after freezing and thawing and radioassayed as described before (6). S.D.s were less than ±20% in continuous cell lines and leukemias.

Trypan Blue Dye Exclusion. The trypan blue dye exclusion test was performed according to the method of Hudson and Hay (21) with minor modifications. The procedure for preincubation of the cells with the drugs was identical to the other test systems (see [^{3}H]thymidine uptake and HTSCA).

SEM. For SEM, cells were grown on glass coverslips and were then incubated with the test substance. The coverslips were washed with PBS and fixed in 1.5% glutaraldehyde in PBS at pH 7.2. They were dehydrated in a graded series of ethanol and subsequently of isoamylacetate, dried by the CO_2 critical-point method (1) in a Bomar SPC-900/Ex apparatus, mounted on stubs, coated with gold, and examined in a Leitz AMR/1200 B scanning electron microscope.

HTSCA. The HTSCA was performed using a method originally described by Hamburger and Salmon (14), Salmon *et al.* (32), and later by Von Hoff *et al.* (36) with the following modifications. Five × 10^6 cells per ml of test medium were incubated (pH 7.2, 37°, in an atmosphere of 5% CO_2 and high humidity) for various times (24 to 72 hr) with the test compounds in the final concentrations of 1, 10, and 20 μg/ml. For incubation of adherent cell populations, *e.g.*, glioblastomas, Petriperm culture dishes (W. C. Heraeus GmbH, D-6450 Hanau, Federal Republic of Germany) with a gas-permeable, hydrophobic Teflon membrane in their bottom were used to prevent adherence. Subsequently, cells were washed twice in McCoy's wash medium (McCoy's Medium 5A:GIBCO No. 041-6600, supplemented with glutamine, antibiotics, and 10% heat-inactivated FCS) and adjusted to 5×10^4 cells per ml in freshly prepared "double enriched CMRL 1066 medium" {40 ml of "enriched CMRL 1066 medium" [500 ml of CMRL 1066 medium:GIBCO No. 041-1535 plus 75 ml of heat-inactivated horse serum plus 20 ml of $CaCl_2$ (100 mM) plus 10 ml of insulin (100 units/ml) plus 5 ml of vitamin C (30 mM) plus 5 ml of penicillin:streptomycin (20.000 units/ml) plus 10 ml of glutamine (200 mM)] plus 0.6 ml asparagine (6.6 mg/ml) plus 0.3 ml of DEAE-dextran (50 mg/ml) plus 0.4 ml of 2-mercaptoethanol (5×10^{-3} M, freshly prepared and light protected)}. For the preparation of the final top layer, 300 μl of 3% agar (Agar Noble No. 0142-01; Difco Laboratories, Detroit, MI) were diluted with 2 ml of the cell suspension. One ml of the resultant mixture was pipetted as top layers on the bottom layers in 35- x 10-mm polystyrene tissue culture dishes (LUX; Miles Laboratories, Inc., Naperville, IL). The final concentration of cells in each top layer was thus approximately 5×10^4/ml. The bottom layers used in this study consisted of 1 ml of agar, which was freshly prepared by mixing 2 ml of 3% agar with 10 ml of plating medium {40 ml of enriched McCoy's medium [500 ml of McCoy's 5A:GIBCO No. 041-6600 plus 25 ml of heat-inactivated horse serum plus 50 ml of heat-inactivated FCS plus 5 ml of sodium pyruvate (2.2%) plus 1 ml of L-serine (21 mg/ml) plus 5 ml of glutamine (200 mM) plus 5 ml of penicillin:streptomycin (20.000 units/ml)] plus 10 ml of tryptic soy broth (3%) plus 0.6 ml of asparagine (6.6 mg/ml) plus 0.3 ml of DEAE-dextran (50 mg/ml)}.

After preparation of both bottom and top layers, the plates were examined under an inverted microscope (Zeiss IM 35; C. Zeiss, D-7082 Oberkochen, Federal Republic of Germany) to ensure the presence of a good single-cell suspension on each of 2 control plates. The plates were then incubated at pH 7.2, 37°, in a 5% CO_2 atmosphere of high humidity. The number of colonies (>50 cells) on control and drug-test plates was determined on an inverted microscope at Day 14.

Statistical Analysis. Due to multiple statistical tests the evaluation was done in the sense of an explorative statistical data analysis. Thus, values in Tables 1 and 2 have been examined in the Wilcoxon matched-pairs signed-ranks test (2-tailed) and in the Friedman 2-way analysis according to their applicability.

RESULTS

Viability of the cells based on trypan blue dye exclusion before testing was >90%. Reliable results in [^{3}H]thymidine uptake tests

were found in 9 of 10 continuously growing tumor cell lines and in 8 of 9 leukemias, accounting for 89% of all tests. Criteria for unreliability were low cpm and high S.D.s, which made it impossible to state positive or negative results and which in leukemic cells seemed to correlate with previous treatment of the patient. Only those reliable results are further evaluated in this paper. All tumors and leukemias tested in [^{3}H]thymidine uptake were assayed for trypan blue dye exclusion at the same time with reliable results found in all tests. HTSCA was performed using cells from 7 different human neoplastic diseases (glioblastoma, hypernephroma, non-small cell lung cancer, small cell lung carcinoma, soft tissue sarcoma, leukemia HL-60, and leukemia K-562) with reliable results in all tests.

CP-46,665 had a marked effect on [^{3}H]thymidine uptake in cells from different AL, CML/BC, and continuously growing leukemic cell lines HL-60 and K-562. A summary is given in Table 1. There was a dependency on exposure time, since at least 24 hr were necessary to reveal full activity, and we found a dose-response relation between 1 and 10 μg/ml. Adding ≥5 μg/ml for ≥48 hr or ≥10 μg/ml for ≥24 hr of incubation, only background counts (<100 cpm) were found in the majority of tests. The rare exceptions from the dose-time-dependency remained in their majority within the S.D. of the test system. In nearly all tests, CP-46,665 showed considerably higher efficacy than did the ALP ET-18-OCH_3, tested in comparison. Furthermore, K-562 cells, which in accordance with the results of other authors (26, 35) revealed comparably low sensitivity to ET-18-OCH_3, could be effectively stopped in their thymidine uptake by CP-46,665. Yet they were also found to be less sensitive to CP-46,665 than were HL-60 cells. In the dose range tested (1 to 20 μg/ml), 2-LPC showed only minor activity. Because of similar molecular weight, all 3 compounds were compared in μg/ml.

Statistical comparison of control values with the values of CP-46,665-incubated specimen of all leukemia cell samples (Friedman analysis; see "Materials and Methods") showed significant effects of the drug at 10 μg/ml after 48 and 72 hr and at 20 μg/ml for all incubation times ($P < 0.05$). Incubation with ET-18-OCH_3 resulted in significant values at 10 μg/ml after 48 and 72 hr and at 20 μg/ml at all incubation times ($P < 0.05$), if resistant K-562 was not taken into consideration. Statistical comparison of CP-46,665 with ET-18-OCH_3 (Wilcoxon test; values expressed as the percentage of control; see "Materials and Methods") showed significant superiority of CP-46,665 over ET-18-OCH_3 at 5 μg/ml after 24 and 48 hr of incubation ($P < 0.05$), at 10 μg/

Table 1

Influence of 2-LPC, ET-18-OCH_3, and CP-46,665 on [^{3}H]thymidine uptake in leukemic blasts

Leukemia type	Analogue	Concentration (μg/ml)	[^{3}H]Thymidine uptake (cpm) at the following incubation times			Maximum % of decrease
			24 hr	48 hr	72 hr	
AMML (1)[a]	Control	0	884[b]	6,646	7,333	0
	2-LPC	20	925	7,855	6,772	7.6
	ET-18-OCH_3	5	540	498	558	92.5
		10	497	34	41	99.5
		20	318	108	49	99.3
	CP-46,665	1	628	625	1,389	90.6
		5	427	312	387	94.7
		10	112	39	50	99.4
		20	52	34	68	99.5
AMML (2)	Control	0	602	598	1,082	0
	2-LPC	20	693	562	805	25.6
	ET-18-OCH_3	5	600	1,062	233	78.5
		10	833	132	132	87.8
		20	371	70	21	98.1
	CP-46,665	5	179	215	386	70.2
		10	202	146	24	97.8
		20	52	19	21	98.1
AML (3)	Control	0	2,163	3,322	4,861	0
	2-LPC	20	1,744	3,101	4,824	19.4
	ET-18-OCH_3	1	1,881	1,799	1,925	60.4
		5	1,443	381	590	88.5
		10	1,499	131	524	96.1
		20	1,286	69	96	98.0
	CP-46,665	1	1,629	2,966	4,299	11.6
		5	418	50	691	98.5
		10	766	115	89	98.2
		20	100	320	41	99.2
ALL (4)	Control	0	2,671	2,088	1,810	0
	2-LPC	20	2,456	2,233	1,311	27.6
	ET-18-OCH_3	1	1,471	361	327	82.7
		5	1,070	159	102	94.4
		10	922	291	89	95.1
		20	778	235	118	93.5
	CP-46,665	1	1,846	769	134	92.6
		5	344	66	95	96.9
		10	264	200	26	98.6
		20	162	227	52	97.1
CML/BC (5)	Control	0	5,688	14,879	13,155	0
	2-LPC	20	5,239	16,547	14,978	7.8
	ET-18-OCH_3	1	2,807	3,739	1,795	86.4
		5	1,078	505	856	96.6
		10	1,004	256	225	98.3
		20	1,171	288	125	99.1
	CP-46,665	1	4,006	7,857	3,202	75.7
		5	364	39	109	99.7
		10	254	26	172	99.8
		20	117	28	34	99.8
CML/BC (6)	Control	0	1,245	2,250		0
	2-LPC	20	669	1,628		46.2
	ET-18-OCH_3	5	418	756		66.4
		10	1,397	198		91.2
		20	524	198		91.2
	CP-46,665	5	246	173		92.3
		10	104	28		98.8
		20	51	18		99.2
HL-60	Control	0	15,899	20,513	28,768	0
	2-LPC	20	18,882	10,691	19,239	47.8
	ET-18-OCH_3	1	15,213	8,127	4,673	83.7
		5	3,849	1,079	384	98.7
		10	1,980	264	212	99.3
		20	1,339	306	86	99.7
	CP-46,665	1	11,834	12,688	19,396	38.1
		5	929	26	143	99.9
		10	444	45	62	99.8
		20	530	26	90	99.8
K-562	Control	0	1,684	2,142	5,178	0
	2-LPC	20	1,499	1,656	4,313	22.7
	ET-18-OCH_3	5	1,577	2,356	3,230	37.6
		10	1,691	1,544	1,708	67.0
		20	1,592	8,658	1,785	65.5
	CP-46,665	5	964	3,577	5,867	42.8
		10	2,343	76	297	96.5
		20	1,140	30	21	99.6

[a] AMML, acute myelomonocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia.
[b] Mean of triplicate cultures (cpm).

Table 2

Influence of 2-LPC, ET-18-OCH_3, and CP-46,665 on [^{3}H]thymidine incorporation in continuous cell lines of solid tumors

Histology	Analogue	Concentration (μg/ml)	[^{3}H]Thymidine incorporation (%) at the following incubation times		
			24 hr	48 hr	72 hr
Hypernephroma (1)	2-LPC	20	100.2[a]	97.0	89.6
	ET-18-OCH_3	10	54.1	30.0	27.4
		20	51.5	25.3	25.2
	CP-46,665	5	77.3	47.6	44.5
		10	33.5	7.6	15.7
		20	15.6	5.9	9.5
Hypernephroma (2)	2-LPC	20	93.9	83.1	100.8
	ET-18-OCH_3	5	29.2	6.9	36.3
		10	12.3	1.3	3.8
		20	4.7	0.6	0.8
	CP-46,665	1	40.3	45.7	58.1
		5	9.4	2.9	12.0
		10	21.9	1.2	6.9
		20	5.4	2.0	5.5
Hypernephroma (3)	2-LPC	20	27.6	47.6	173.7
	ET-18-OCH_3	1	75.6	ND[b]	65.0
		5	77.6	ND	9.4
		10	44.4	ND	10.6
		20	36.6	30.6	8.8
	CP-46,665	1	84.5	ND	32.8
		5	80.4	ND	3.7
		10	23.8	30.7	2.8
		20	14.9	7.2	1.8
Hypernephroma (4)	2-LPC	10	120.5	73.2	72.1
	ET-18-OCH_3	5	34.7	36.3	31.1
		10	33.5	14.6	16.0
		20	26.8	8.1	23.6
	CP-46,665	5	23.6	16.7	30.9
		10	9.7	64.9	9.0
		20	7.4	4.5	2.7
Hypernephroma (5)	2-LPC	20	63.3	62.7	
	ET-18-OCH_3	5	100.5	51.9	
		10	80.7	50.4	
		20	44.5	40.2	
	CP-46,665	5	101.2	74.5	
		10	41.3	193.0	
		20	31.9	0.8	
Ovarian carcinoma (ascites) (6)	2-LPC	20	93.3	143.3	9.2
	ET-18-OCH_3	5	42.8	52.4	33.1
		10	51.3	19.0	2.4
		20	35.2	18.4	6.0
	CP-46,665	5	161.1	55.9	3.1
		10	41.0	183.9	1.0
		20	11.7	16.1	1.5
Glioblastoma (7)	2-LPC	20	97.2	82.8	
	ET-18-OCH_3	5	67.6	56.2	
		10	51.5	34.3	
		20	35.3	17.9	
	CP-46,665	5	50.6	68.6	
		10	29.0	6.9	
		20	3.9	0.4	
Small cell lung carcinoma (8)	2-LPC	20	71.7	50.3	
	ET-18-OCH_3	5	80.5	41.7	
		10	53.7	29.4	
		20	43.2	23.7	
	CP-46,665	5	66.4	189.8	
		10	59.4	20.5	
Squamous cell lung carcinoma (9)	2-LPC	20	93.4	133.9	85.4
	ET-18-OCH_3	5	77.1	71.6	87.7
		10	66.8	58.6	75.2
		20	50.2	50.1	52.5
	CP-46,665	5	97.3	110.9	91.9
		10	47.9	1.5	5.0
		20	11.1	0.9	2.0

[a] Mean of triplicate cultures expressed as percentage of controls.
[b] ND, not determined.

ml for all incubation times ($P \leq 0.05$), and at 20 μg/ml after 24 hr of incubation ($P < 0.05$). The effects of both drugs at the higher dose levels and after longer incubation times proved to become comparable. Differences between 2-LPC and CP-46,665 were significant (Friedman analysis) at 20 μg/ml ($P < 0.05$).

Comparable results were obtained with the continuously growing tumor cell lines (see Table 2). All lines tested were sensitive to CP-46,665 in a dose- and time-dependent fashion. Rare exceptions from the dose and time relationship might be due to the accidental occurrence of tumor cell clusters in the Microtiter plates. However, doses as high as 10 μg CP-46,665 per ml and more and incubation times of ≥48 hr were necessary to produce incorporation rates around 10% of the controls. 2-LPC showed only minor effects in the same dose range. For some of these tumors, CP-46,665 had considerably higher efficacy than did ET-18-OCH_3. Statistical comparison between 2-LPC and CP-46,665 and between 2-LPC and ET-18-OCH_3 proved significant superiority of both ether-lipids over 2-LPC after 24 and 48 hr of incubation at the dose level of 20 μg/ml ($P \leq 0.05$, Friedman analysis). Values after 72 hr of incubation were not explored, because of the small numbers of samples tested. This also holds true in the comparison between CP-46,665 and ET-18-OCH_3 (Wilcoxon test), where significant superiority of CP-46,665 over ET-18-OCH_3 could be established for 10 μg/ml after 24 hr of incubation and 20 μg/ml after 24 and 48 hr ($P < 0.05$). The 72-hr values could not be compared.

Trypan blue dye exclusion, expressed as the percentage of controls, showed a close correlation with the corresponding values obtained for [^{3}H]thymidine incorporation in leukemic blasts and also a good correlation with the values of [^{3}H]thymidine uptake obtained in the continuous cell lines of solid tumors. Examples are summarized in Table 3. Loss of the ability of trypan blue dye exclusion of the cells treated with CP-46,665 and ET-18-OCH_3 also proved to be dependent on dosage and incubation time in contrast to minor effects of 2-LPC in the same dose range. Interestingly, in this assay system, CP-46,665 was again the more active compound and, in some tests, showed even earlier effects on trypan blue dye exclusion than on [^{3}H]thymidine uptake, which was generally the opposite for ET-18-OCH_3 (see Table 3). In both [^{3}H]thymidine uptake and trypan blue dye exclusion, at least 12 hr of drug exposure were necessary to produce visible effects.

As a morphological correlation of these effects, severe membrane damage was observed in SEM. Cells of an astrocytoma, a glioblastoma, and a leukemia were tested in SEM. Cytoplasmic processes with numerous blebs, ruffles, and filopodia have been observed without drug incubation as typical signs of the high membrane activity in malignant astroglial cells (Refs. 15, 33, and 37; Fig. 1*A*). Furthermore, the cell membranes seemed to be intact (see Fig. 1*A*). After incubation with 5 μg CP-46,665 per ml for 24 hr, the described surface structures vanished in many cells, and cell membranes showed numerous round defects resembling punched holes (see Fig. 1*B*). After an incubation with 10 μg CP-46,665 per ml for 24 hr, these morphological disintegrations of membrane structures were even more evident (see Fig. 1*C*). At both concentrations, cells lost adherence properties, another sign of vital membrane function, and many were lost from the surface of the culture dish. This, however, made it impossible to evaluate SEM results quantitatively.

Tested in the HTSCA, both ET-18-OCH_3 and CP-46,665

Table 3

Correlation between [^{3}H]thymidine incorporation and trypan blue dye exclusion of neoplastic cells under the influence of 2-LPC, ET-18-OCH$_3$, and CP-46,665

			[^{3}H]Thymidine uptake and trypan blue dye exclusion at the following incubation times					
			24 hr		48 hr		72 hr	
Histology	Analogue	Concentration (μg/ml)	[^{3}H]thymidine uptake	Trypan blue dye exclusion	[^{3}H]Thymidine uptake	Trypan blue dye exclusion	[^{3}H]Thymidine uptake	Trypan blue dye exclusion
HL-60[a]	2-LPC	10	139[b]	103[c]	57	112	84	111
	ET-18-OCH$_3$	1	96	91	40	55	16	35
		5	24	41	5	6	1	1
		10	12	18	1	0	1	0
	CP-46,665	1	74	102	62	109	67	112
		5	6	26	0	1	1	4
		10	3	0	0	0	0	0
AML	2-LPC	10	99	104	91	92	104	106
	ET-18-OCH$_3$	1	87	101	54	83	40	72
		5	67	87	12	77	12	54
		10	69	93	4	69	11	33
	CP-46,665	1	75	101	89	89	88	89
		5	19	40	2	28	14	16
		10	35	30	12	0	2	1
ALL	2-LPC	10	89	65	114	96	90	79
	ET-18-OCH$_3$	1	55	63	17	66	18	35
		5	40	54	8	55	6	23
		10	35	44	14	35	5	25
	CP-46,665	1	69	53	37	40	7	13
		5	13	8	3	6	5	12
		10	10	8	10	13	1	0
Hypernephroma	2-LPC	20	94	100	83	106	101	94
	ET-18-OCH$_3$	1	43	100	20	98	7	89
		10	12	68	1	66	4	62
		20	5	59	1	59	1	46
	CP-46,665	1	40	87	46	89	58	80
		10	22	0	1	0	7	0
		20	5	0	2	0	5	0
SCLC	2-LPC	20	72	109	50	106	60	121
	ET-18-OCH$_3$	1	110	91	53	106	120	123
		10	54	64	29	57	40	30
		20	43	61	24	29	22	16
	CP-46,665	1	78	102	98	80	96	93
		10	59	4	20	0	24	0
		20	301	0	6	0	11	0

[a] HL-60, HL-60 leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; SCLC, small cell lung carcinoma.
[b] Mean value of triplicate cultures expressed as percentage of the controls.
[c] Values expressed as percentage of the controls.

showed clear suppression of the colony formation of human tumor and leukemic cells when tested after a coincubation of various times in a dose range between 1 and 20 μg/ml. Examples are summarized in Chart 2. Also, in this assay system, the drugs exerted their effects with a dose and time dependency, but CP-46,665 was not always the more active compound.

Furthermore, in both [^{3}H]thymidine uptake and trypan blue dye exclusion, the activity of CP-46,665 was correlated with the concentration of serum (*e.g.*, FCS) in the incubation system. With lower FCS concentrations than 10%, toxicity was even more pronounced, but toxic effects could be nearly totally blocked by adding 50% FCS or more to the culture. This was comparable with the influence of different serum concentrations on the toxicity of ET-18-OCH$_3$ (data obtained with a hypernephroma and a glioblastoma, details not shown).

DISCUSSION

The ether-linked lipoidal amine CP-46,665 (see Chart 1) was tested in our *in vitro* antitumor screening system, consisting of a variety of assays measuring cytostatic and cytotoxic effects of possible anticancer drugs at different cell sites. Results indicate that CP-46,665 inhibited the incorporation of [^{3}H]thymidine into blasts of various types of leukemias and cells of different solid tumors of human origin (see Tables 1 to 3). Since cells were tested in both the stationary and proliferative phases of culture, these effects might reflect antiproliferative activity as well as toxic action on vital steps of cell metabolism. However, testing CP-46,665, the [^{3}H]thymidine uptake of the cells was well correlated with trypan blue dye exclusion, which is widely accepted as a viability test and, in this study, was performed to assess destruction of cell membranes under drug influence (see Table 3). Furthermore, loss of cell surface activity and severe cell membrane destructions caused by CP-46,665 could be directly observed in SEM (see Fig. 1). Similar vacuole-like defects of the outer cell membrane as observed in SEM also could be seen in light microscopy of cytocentrifuge smears of the cells, which has been performed to control the tested cell population cytologically for signs of malignancy (data not shown). The actual cell death might be correlated with a loss of internal material, since the drug has been also found active in release assays (data not shown). However, these different toxic effects in single cells

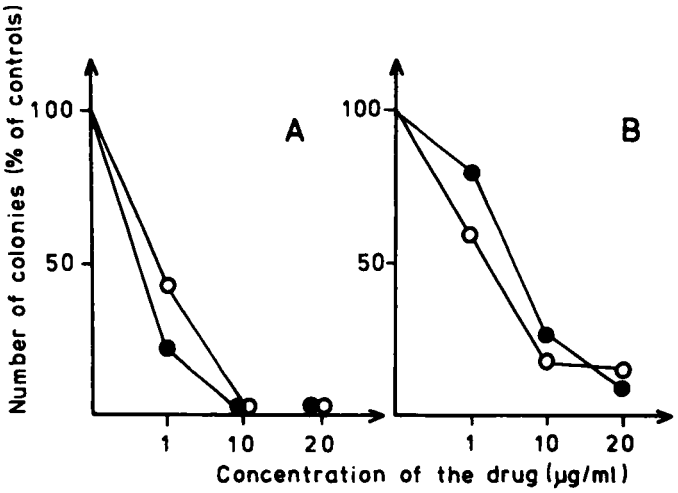

Chart 2. Effects of ET-18-OCH_3 (○) and CP-46,665 (●) on colony formation of cells from a human glioblastoma cell line (*A*) and HL-60 leukemia (*B*). Cells were incubated for 48 hr with various concentrations of the drugs, then washed, and cultured in the agar system (HTSCA) described.

were accompanied by clear suppression of colony formation as tested in the HTSCA (see Chart 2), which was performed to observe the reproductive capacities of the cells under drug influence. The cytostatic and cytotoxic effects of CP-46,665 were dependent on dose and incubation time in all assay systems. Destruction of leukemic blasts was often completed with doses of ≥5 μg/ml after an incubation of ≥48 hr or ≥10 μg/ml after ≥24 hr. In cells from solid tumors, ≥10 μg/ml and incubation times of ≥48 hr were often necessary. In the majority of tests and assay systems, CP-46,665 showed considerably higher efficacy than the ALP ET-18-OCH_3, which was always tested in comparison. This holds also true in 3 different rat brain tumor cell lines tested (data not shown). Thus, the high activity of CP-46,665 is not restricted to one species. 2-LPC remained without comparable activity in the same dose range.

From these data, we conclude that CP-46,665 causes the death of neoplastic cells during coincubation, and in this activity, it is superior to ET-18-OCH_3. This direct cytotoxicity might partially explain *in vivo* antitumor effects of CP-46,665, which can prevent metastasis of various rodent tumors (17, 25, 38). In its *in vivo* activity, CP-46,665 is unique among a series of other lipoidal amines (17, 22–25, 29), since it is the most potent compound and does neither induce interferon production nor enhance natural killer cell activity (17). On the other hand, CP-46,665 activates macrophages to cytotoxic effector cells (38), and the following macrophage tumoristatic activity is associated with the ability of these cells to secrete prostaglandins (17). The lack of a clear dose-response relation and the relatively low dose in which CP-46,665 was found effective *in vivo* support the view of macrophage activation being partially responsible for the therapeutic activity observed so far (22). However, both direct destruction of neoplastic cells and biological response modification might be active. In this respect, it would be of interest to perform further therapeutic studies within a larger dose and time range to reach plasma levels and cell exposure times of the drug, which equal cytotoxic parameters *in vitro*. Whereas toxicological data in different animals (50% lethal dose = 60 to 70 mg/kg in mice and rats) have been published (38), further pharmacokinetic studies remain to be done to determine achievable plasma and tissue levels *in vivo* and metabolism data of the drug.

The pattern of biological response modification by CP-46,665, as well as its phenomenological mode of direct cytotoxicity, is reminiscent of the action seen with other ether-lipids, such as ALP (4).

In particular, the interactions of this compound with structures of the cell membranes, as far as they are visible in SEM, are similar to those of ALP or thioether-lysophospholipid derivatives (4, 7). Thus, it is a tempting speculation, that the activity of these compounds might be based on similar molecular mechanisms. Some ALP among other possible mechanisms exert their direct cytotoxicity via accumulation in and subsequent destruction of lipid structures in the membranes of neoplastic cells, due to their high metabolic stability (for further discussion, see Refs. 4 and 27). However, since other, yet unknown molecular mechanisms might cause its comparably high activity, the molecular basis of the CP-46,665 cytotoxicity still remains to be characterized by further biochemical and pharmacokinetic studies. In addition, further experiments are necessary to characterize the therapeutic index of CP-46,665 with regard to cytotoxicity.

In conclusion, the comparatively high antineoplastic activity of CP-46,665 *in vitro* strongly commends this compound among other ether-lipids for further investigation as an experimental drug for the treatment of cancer.

ACKNOWLEDGMENTS

The authors thank Professor Dr. G. Blümel, Dr. A. Lehmer, and Dr. K. S. Zänker for their helpful cooperation and C. Heinze, I. Eberl, and K. Ziehrer for their excellent assistance.

REFERENCES

1. Anderson, T. F. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. Trans. NY Acad. Sci., *13:* 130–134, 1951.
2. Ando, K., Kodama, K., Kato, A., Tamura, G., and Arima, K. Antitumor activity of glyceryl ethers. Cancer Res., *32:* 125–129, 1972.
3. Andreesen, R., Modolell, M., Weltzien, H. U., Eibl, H., Common, H. H., Loehr, G. W., and Munder, P. G. Selective destruction of human leukemic cells by alkyl-lysophospholipids. Cancer Res., *38:* 3894–3899, 1978.
4. Berdel, W. E., Andreesen, R., and Munder, P. G. Synthetic alkyl-phospholipid analogues: a new class of antitumor agents. *in:* J. F. Kuo (ed.), Phospholipids and Cellular Regulation, Vol. 2. Boca Raton: CRC Press, in press, 1985.
5. Berdel, W. E., Bausert, W. R., Weltzien, H. U., Modolell, M. L., Widmann, K. H., and Munder, P. G. The influence of alkyl-lysophospholipids and lysophospholipid-activated macrophages on the development of metastasis of 3 Lewis lung carcinoma. Eur. J. Cancer, *16:* 1199–1204, 1980.
6. Berdel, W. E., Fink, U., Egger, B., Reichert, A., Munder, P. G., and Rastetter, J. Inhibition by alkyl-lysophospholipids of tritiated thymidine uptake in cells of human malignant urologic tumors. J. Natl. Cancer Inst., *66:* 813–817, 1981.
7. Berdel, W. E., Fromm, M., Fink, U., Pahlke, W., Bicker, U., Reichert, A., and Rastetter, J. Cytotoxicity of thioetherlysophospholipids in leukemias and tumors of human origin. Cancer Res., *43:* 5538–5543, 1983.
8. Berdel, W. E., Greiner, E., Fink, U., Stavrou, D., Reichert, A., Rastetter, J., Hoffman, D. R., and Snyder, F. Cytotoxicity of alkyl-lysophospholipid derivatives and low-alkyl-cleavage enzyme activities in rat brain tumor cells. Cancer Res., *43:* 541–545, 1983.
9. Berdel, W. E., Schlehe, H., Fink, U., Emmerich, B., Maubach, P. A., Emslander, H. P., Daum, S., and Rastetter, J. Early tumor and leukemia response to alkyl-lysophospholipids in a Phase I study. Cancer (Phila.), *50:* 2011–2015, 1982.
10. Berger, M. R., Munder, P. G., Schmähl, D., and Westphal, O. Influence of the alkyl-lysophospholipid ET-18-OCH_3 on methyl-nitrosourea-induced rat mammary carcinomas. Oncology (Basel), *41:* 109–113, 1984.
11. Bertermann, O., and Andreesen, R. Experimental studies with alkyl-lysophospholipids in soft tissue sarcomas. *in:* K. H. Spitzy and K. Karrer (eds.), Proceedings of the 13th International Congress of Chemotherapy (Vienna, August 28 to September 2, 1983), Abstracts SE 12.4.7 A, Part 257, pp. 2–5. 1983.
12. Brachwitz, H., Langen, P., Hintsche, R., and Schildt, J. Halo lipids. V. Synthesis, nuclear magnetic resonance spectra, and cytostatic properties of Halo ana-

logues of alkyl-lysophospholipids. Chem. Phys. Lipids, *31:* 33–52, 1982.
13. Hallgren, B. Therapeutic effects of ether lipids. *In:* H. K. Mangold and F. Paltauf (eds.), Ether Lipids, Biochemical and Biomedical Aspects, pp. 261–275. Academic Press, Inc.; New York: 1983.
14. Hamburger, A. W., and Salmon, S. E. Primary bioassay for human tumor stem cells. Science (Wash. DC), *197:* 461–463, 1977.
15. Haugen, Å., and Laerum, O. D. Scanning electron microscopy of neoplastic neurogenic rat cell lines in culture. Acta Pathol. Microbiol. Scand. (Sect. A), *86:* 101–110, 1978.
16. Hoffman, D. R., Hajdu, J., and Snyder, F. Cytotoxicity of platelet activating factor and related alkyl-phospholipid analogs in human leukemia cells, polymorphonuclear neutrophils, and skin fibroblasts. Blood, *63:* 545–552, 1984.
17. Hoffman, W. W. Lipoidal amines. *In:* R. L. Fenichel *et al.* (eds.), Immunomodulating Agents. M. Derker, in press, 1985.
18. Honma, Y., Kasukabe, T., Hozumi, M., Tsushima, S., and Nomura, H. Induction of differentiation of cultured human and mouse myeloid leukemia cells by alkyl-lysophospholipids. Cancer Res., *41:* 3211–3216, 1981.
19. Honma, Y., Kasukabe, T., Okabe-Kado, J., Hozumi, M., Tshushima, S., and Nomura, H. Antileukemic effect of alkyl-phospholipids. I. Inhibition of proliferation and induction of differentiation of cultured myeloid leukemia cells by alkyl-ethyleneglycophospholipids. Cancer Chemother. Pharmacol., *11:* 73–76, 1983.
20. Honma, Y., Kasukabe, T., Okabe-Kado, J., Hozumi, M., Tsushima, S., and Nomura, H. Antileukemic effect of alkyl-phospholipids. II. Prolongation of survival times of leukemic mice by alkyl-ethyleneglycophospholipids. Cancer Chemother. Pharmacol., *11:* 77–79, 1983.
21. Hudson, L., and Hay, F. C. Practical Immunology, pp. 29–32. Oxford, England: Blackwell Scientific Publications, 1976.
22. Jensen, K. E., Niblack, J. F., Wolff, J. S. Kraska, A. R., Otterness, I. G., Hoffman, W. W., and Hemsworth, G. R. Exploring the immunotherapeutic potential of two lipoidal amines. *In:* Human Cancer Immunology, Vol. 3, pp. 55–63. Amsterdam: Elsevier, 1982.
23. Kraska, A. R., Hemsworth, G. R., Hoffman, W. W., and Wolff, J. S. Antitumor activity of CP-20,961 and CP-28,888. *In:* Current Chemotherapy and Infectious Disease, Proceedings of the 11th ICC and the 19th ICAAC, pp. 1605–1607. American Society of Microbiology, 1980.
24. Kraska, A. R., Pollack, V. A., Hoffman, W. W., Wolff, J. S., and Hemsworth, G. R. Immunomodulatory, antitumor, and antiviral effects of CP-46,665 and its analogs. *In:* The Twenty-first Interscience Conference on Antimicrobic Agents of Chemotherapy, Chicago, 1981, Abstract 329, 1981.
25. LeMarbre, P. J., Neff, R. K., and McIntyre, O. R. A Phase I trial of CP 46,665—a potential new immunostimulant. Proc. Am. Soc. Hematol., *60* (Suppl. 5): 189, 1982.
26. Long, R. C., Small, W. C., Brynes, R. K., Tidwell, T., Goldstein, J. H., and Vogler, W. R. Effects of alkyl-lysophospholipids on human leukemic cell lines measured by nuclear magnetic resonance. Cancer Res., *43:* 770–775, 1983.
27. Munder, P. G., Modolell, M., Bausert, W., Oettgen, H. F., and Westphal, O. Alkyl-lysophospholipids in cancer therapy. *In:* E. M. Hersh *et al.* (eds.), Augmenting Agents in Cancer Therapy, pp. 441–458. New York: Raven Press, 1981.
28. Munder, P. G., Weltzien, H. U., and Modolell, M. Lysolecithin analogs: a new class of immunopotentiators. *In:* P. A. Miescher (ed.), VII International Symposium of Immunopathology, Bad Schachen, Germany, 1976, pp. 411–424. Basel: Schwabe & Co., 1977.
29. Niblack, J. F., Otterness, I. G., Hemsworth, G. R., Wolff, J. S., Hoffman, W. W., and Kraska, A. R. CP-20,961: a structurally novel, synthetic adjuvant. J. Reticuloendothel. Soc., *26:* 655–666, 1979.
30. Roper, P. R., and Drewinko, B. Comparison of *in vitro* methods to determine drug-induced cell lethality. Cancer Res., *36:* 2182–2188, 1976.
31. Runge, M. H., Andreesen, R., Pfleiderer, A., and Munder, P. G. Destruction of human solid tumors by alkyl-lysophospholipids. J. Natl. Cancer Inst., *64:* 1301–1306, 1980.
32. Salmon, S. E., Hamburger, A. W., Soehnlein, B., Durie, B. G. M., Alberts, D. S., and Moon, T. E. Quantitation of differential sensitivity of human-tumor stem cells to anticancer drugs. N. Engl. J. Med., *298:* 1321–1327, 1978.
33. Spence, A. M., and Coates, P. W. Scanning electron microscopy of cloned astrocytic lines derived from ethylnitrosurea-induced rat gliomas. Virchows Arch. B Cell Pathol., *28:* 77–85, 1978.
34. Storme, G., Berdel, W. E., Bruyneel, R., De Bruyne, G., and Mareel, M. Antiinvasive effect of an alkyl-lysophospholipid ET-18-OCH_3 (racemic-1-octadecyl-2-methyl-glycero-3-phosphocholine) which has no microtubule inhibitor activity. Proc. Am. Assoc. Cancer Res., *24:* 31, 1983.
35. Tidwell, T., Guzman, G., and Vogler, W. R. The effects of alkyl-lysophospholipids on leukemic cell lines. I. Differential action on two human leukemic cell lines, HL 60 and K 562. Blood, *57:* 794–797, 1981.
36. Von Hoff, D. D., Clark, G. M., Stogdill, B. J., Sarosdy, M. F., O'Brien, M. T., Casper, J. T., Mattox, D. E., Page, C. P., Cruz, A. B., and Sandbach, J. F. Prospective clinical trial of a human tumor cloning system. Cancer Res., *43:* 1926–1931, 1983.
37. Winslow, D. P., Roscoe, J. P., and Rowles, P. M. Changes in surface morphology associated with ethylnitrosurea-induced malignant transformation of cultured rat brain cells studied by scanning electron microscopy. Br. J. Exp. Pathol., *59:* 530–539, 1978.
38. Wolff, J. S., Hemsworth, G. R., Kraska, A. R., Hoffman, W. W., Figdor, S. K., Fisher, D. O., Jakowski, R. M., Niblack, J. F., and Jensen, K. E. CP-46,665-1: a novel lipoidal amine with antimetastatic and immunomodulatory properties. Cancer Immunol. Immunother., *12:* 97–103, 1982.

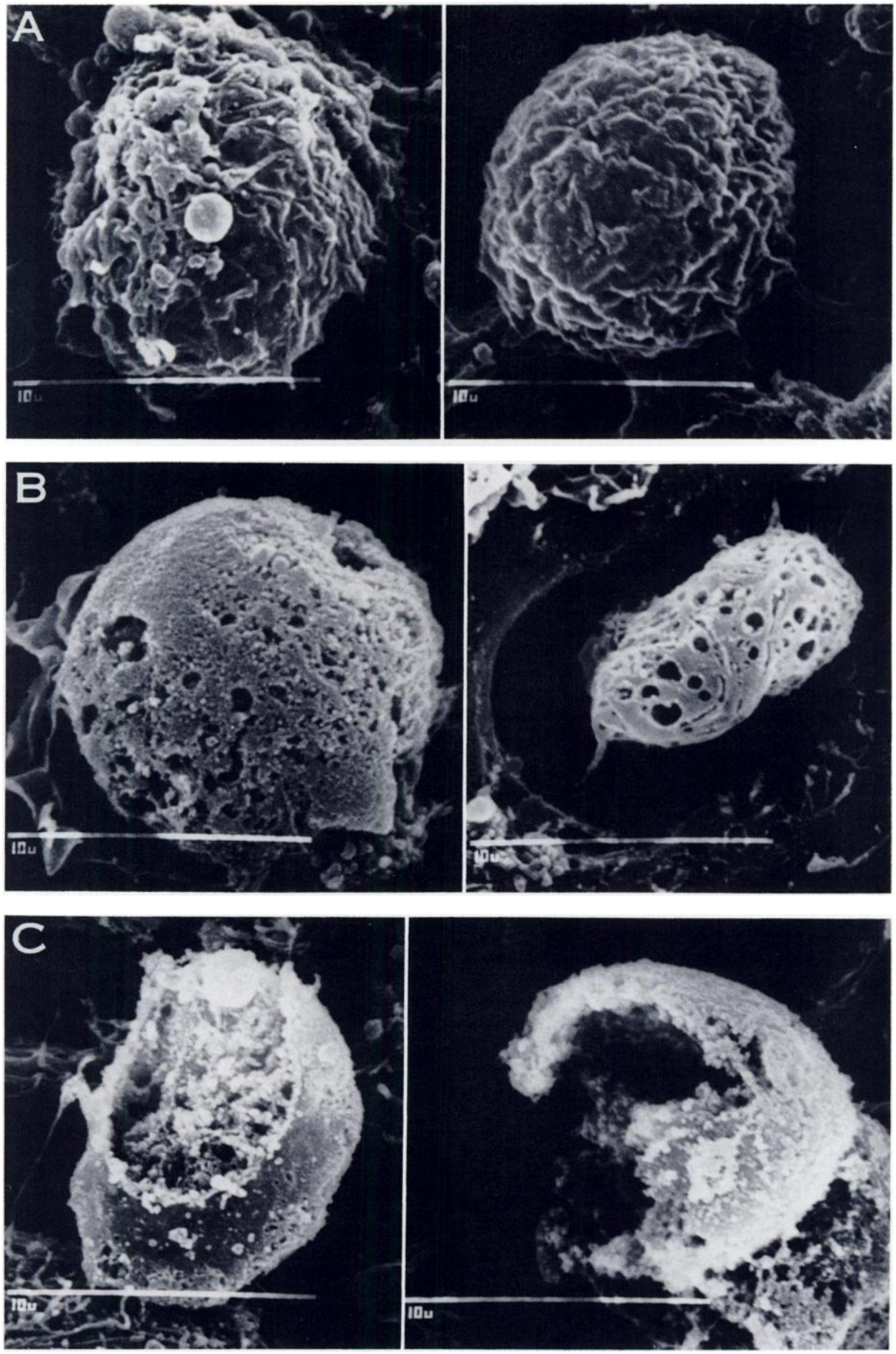

Fig. 1. SEM of cells of a human glioblastoma continuous cell line after 24 hr of incubation without (*A*) and with CP-46,665 in a concentration of 5 μg/ml (*B*) and 10 μg/ml (*C*). *Left* and *right*, 2 different examples of each incubation situation. *Marker*, μm.