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(54) Title: A NOVEL ANTI-NEOPLASTIC CYTOKINE

(57) Abrégé/Abstract:
The present invention provides a novel human cytokine termed human Oncoinhibin. The human Oncoinhibin protein is secreted by human erythroblastoid cells, has a molecular weight of about 28 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits diverse neoplastic activity.

ABSTRACT

The present invention provides a novel human cytokine termed human Oncoinhibin. The human Oncoinhibin protein is secreted by human erythroblastoid cells, has a molecular weight of about 28 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits diverse neoplastic activity.

A NOVEL ANTI-NEOPLASTIC CYTOKINE

TECHNICAL FIELD

The present invention relates generally to cytokines. More specifically, the present invention relates to a novel cytokine with broad anti-neoplastic activity.

BACKGROUND ART

Cell growth appears to be regulated by a balance between growth stimulatory and growth inhibitory molecules. An imbalance in these growth regulatory cytokines has been proposed as one of the mechanisms of tumor growth.

Several cytokines which stimulate the growth of tumor and normal cells have been described. These include, e.g., epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), insulin-like growth factors (IGF), interleukins (IL), colony stimulating factors (CSF) and transforming growth factors (TGF-α and TGF-β).

In contrast, other cytokines selectively inhibit the growth of certain tumor cells. These include, e.g., interferons (IFN), lymphotoxin (LT), tumor necrosis factor (TNF), oncostatin M, amphiregulin, interleukin-1 (IL-1), interleukin-6 (IL-6) and TGF-β.

These growth stimulatory and growth inhibitory cytokines can be differentiated from each other based on their source, their specificity against tumor targets, their physio-chemical properties and their primary structure. Thus, the identification and

characterization of growth regulatory cytokines is of critical importance in the understanding of cellular growth, including growth of neoplasms.

DESCRIPTION OF THE INVENTION

An object of a broad aspect of the present invention is to provide a human protein which exhibits anti-neoplastic activity.

A first broad aspect of the present invention provides purified and isolated human Oncoinhibin protein which is secreted by human erythroblastoid cells. That protein has a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity.

By a first variant of this first broad aspect of the present invention, the human Oncoinhibin protein is substantially-free of impurities.

By a second variant of this first broad aspect of the present invention, and/or the above first variant thereof, the human Oncoinhibin protein is one whose production has been enhanced by phorbol ester.

By a third variant of this first broad aspect of the present invention, and/or the above variants thereof, the human Oncoinhibin protein is active in a standard bioassay for Oncoinhibin and is substantially-homogeneous to SDS-PAGE analysis.

A second broad aspect of the present invention provides a process for preparing human Oncoinhibin protein having a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity. The process comprises the step of incubating human erythroblastoid cells. The process further comprises the step of inducing the production of Oncoinhibin protein. The process further comprises the step of harvesting

conditioned cell supernatants containing the human Oncoinhibin protein so-produced.

By a first variant of this second broad aspect of the present invention, the process further comprises the step of concentrating the supernatant.

By a second variant of this second broad aspect of the present invention, and/or the above first variant thereof, the process further comprises the step of enhancing the production of the human Oncoinhibin protein with phorbol ester.

By a third variant of this second broad aspect of the present invention, and/or the above variants thereof, the process further comprises the step of selecting the erythroblastoid cells from cell line K562.

By a fourth variant of this second broad aspect of the present invention, and/or the above variants thereof, the process further comprises the step of incubating the cells in RPMP1640 medium.

By a fifth variant of this second broad aspect of the present invention, and/or the above variants thereof, the process further comprises the step of inducing the production of human Oncoinhibin protein in a serum-free medium.

A third broad aspect of the present invention provides human Oncoinhibin protein as described above according to the first broad aspect of the present invention, whenever prepared by the process of the second broad aspect of the present invention.

A fourth broad aspect of the present invention provides a method of purifying human Oncoinhibin protein having a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity. The purification method includes the step of ultrafiltering conditioned cell supernatants containing human Oncoinhibin protein,

thereby to provide ultrafiltered supernatants. The method further comprises the step of dialyzing the ultrafiltered supernatants, thereby to provide dialyzed, ultrafiltered supernatants. The method further comprises the step of performing chromatography on the dialyzed, ultrafiltered supernatants thereby to provide chromatographed dialyzed, ultrafiltered supernatants. The method further comprises the step of performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reverse phase high performance liquid chromatography on the chromatographed dialyzed, ultrafiltered supernatants which have been provided by the immediately previous step.

A fifth broad aspect of the present invention provides a human Oncoinhibin protein as described above according to the first broad aspect of the present invention, whenever prepared by the method of the fourth broad aspect of the present invention.

A sixth broad aspect of the present invention provides a pharmaceutical composition comprising a purified isolated human Oncoinhibin protein as described above according to the first broad aspect of the present invention, and a pharmaceutically-acceptable carrier.

A seventh broad aspect of the present invention provides a use of an effective dose of the composition of the sixth broad aspect of the present invention for inhibiting the growth of a neoplastic cell.

By a first variant of this seventh broad aspect of the present invention, the neoplastic cell is *in vitro*.

By a second variant of this seventh broad aspect of the present invention, and/or the above first variant thereof, the neoplastic cell is selected from the group consisting of lymphomas, breast carcinomas, melanomas, cervical carcinomas, ovarian carcinomas, and hepatomas.

By a third variant of this seventh broad aspect of the present invention, and/or the above variants

thereof, the neoplastic cell occurs in a human, or in non-human animal.

By a fourth variant of this seventh broad aspect of the present invention, and/or the above variants thereof, the use is for preventing recurrence of a neoplastic condition.

By a fifth variant of this seventh broad aspect of the present invention, and/or the above variants thereof, the use is for extending the survival time of a host of the neoplastic cell.

An eighth broad aspect of the present invention provides a use of the purified and isolated human Oncoinhibin protein as described above according to the first broad aspect of the present invention, to treat a disease which is selected from the group consisting of a carcinoma and a lymphoma.

By a variant of this eighth broad aspect of the present invention, the carcinoma is selected from the group consisting of breast carcinomas, melanomas, cervical carcinomas, ovarian carcinomas, and hepatomas.

A ninth broad aspect of the present invention provides an immunomodulator for activating lymphocytes, monocytes and neutrophils to kill tumour cells, which comprises purified and isolated human Oncoinhibin as described above according to the first broad aspect of the present invention, or in the third broad aspect of the present invention or in the fifth broad aspect of the present invention.

A tenth broad aspect of the present invention provides a growth factor for stimulating the growth of normal cells, consisting of purified and isolated human Oncoinhibin as described above according to the first broad aspect of the present invention, or the third broad aspect of the present invention or the fifth broad aspect of the present invention.

By a variant of this tenth broad aspect of the present invention, the normal cell is a fibroblast.

An eleventh broad aspect of the present invention provides a use of a purified and isolated human Oncoinhibin protein as described above according to the first broad aspect of the present invention, in the third broad aspect of the present invention or in the fifth broad aspect of the present invention for preparing a medicament for inhibiting a neoplastic cell.

DESCRIPTION OF THE FIGURES

In the accompanying drawings,

Figure 1 illustrates that K-562 cell conditioned supernatants inhibit the growth of MCF-7 cells.

Figure 2 depicts a standard bioassay for Oncoinhibin.

Figure 3 shows the production of Oncoinhibin by K-562 cells in the presence of serum and in the absence of serum.

Figure 4 depicts the effects of phorbol ester on the induction of Oncoinhibin.

Figure 5 shows the effects of ultrafiltration on Oncoinhibin activity.

Figure 6 shows the characterization of Oncoinhibin by gel permeation chromatography.

Figure 7 shows the elution of Oncoinhibin activity from SDS polyacrylamide gel electrophoresis.

Figure 8 depicts the SDS-PAGE analysis of Oncoinhibin.

Figure 9 shows the binding elution of Oncoinhibin activity from DEAE AFFIGEL™ blue (upper panel) and from a Q-SEPHAROSE™ column (lower panel).

Figure 10 illustrates the dose dependent anti-proliferative effects of Oncoinhibin and TNF.

Figure 11 shows dose dependent proliferative effects of Oncoinhibin on normal human foreskin fibroblasts.

Figure 12 shows the effect of Oncoinhibin on actinomycin D treated murine L-929 cells.

Figure 13 shows a northern blot analysis for TNF and LT of K-562 cells untreated and treated with phorbol esters.

Figure 14 shows the comparison of growth inhibitory effects of Oncoinhibin (panel A) and oncostatin M (panel B) on human melanoma A375 cells.

Figure 15 shows the growth inhibitory effects of oncostatin M and IL-6 on normal fibroblasts.

Figure 16 shows the effect of IL-6 on human breast tumor (MCF-7) cells.

Figure 17 depicts the effects of antibodies against interferon-Ƭ on the Oncoinhibin activity on human breast tumor cells (MCF-7).

Figure 18 shows the inhibitory effects of interferon-Ƭ on TNF (upper panel) but not Oncoinhibin (lower panel) on human foreskin fibroblasts.

Figure 19 shows the growth rate of human breast tumor MCF-7 cells in the absence and presence of Oncoinhibin.

Figure 20 shows the effect of time of exposure of MCF-7 cells to Oncoinhibin.

Figure 1 shows comparison of the effect of Oncoinhibin with TNF on the morphology of human breast tumour MCF-7 cells.

It is to be noted, however, that these drawings illustrate preferred embodiments of the invention and therefore are not to be considered limiting of their scope.

AT LEAST ONE MODE FOR CARRYING OUT THE INVENTION

Various cell lines including mouse connective tissue cell line L929 (CCL 1), K-562 (CCL-243), U-937 (CRL-1543), HL-60 (CCL-240), Raji (CCL-86), Jurkat (CRL-8163), BT 20 (HTB-1 9), MCF-7 (HTB-22), SK-BR-3 (HTB-30), ZR-75-1 (CRL-1500), RPMI 7951 (HTB-66), A375 (CRL-1619), A-431 (CRL-1555), ME-1 80 (HTB 83), OVCAR-3 (HTB-161), He La (CCL-2), Hep-2 (HB-8065), and NIH 3T3 (CRL-1618) were obtained from American Type Culture Collection, (Rockville, MD). TNF-resistant NIH 3T3 cells were isolated as described by K. Totpal, R. LaPushin, H. N. Ananthaswamy and B. B. Aggarwal, Lymphokine and Cytokine Res. 10 (1991) 359-367. Cells were tested for mycoplasma contamination using the DNA-based assay kit purchased from Gen-Probe (San Diego, CA).

All cell cultures were maintained in continuous exponential growth by weekly passage. Some of the cells were subcultivated twice a week. Cells were routinely grown in RPMI 1640 medium supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and fetal bovine serum (10%) in a humidified incubator in 5% CO_2 in air.

The conditioned supernatants of human erythroblastoid cell line K-562 produces an activity which is growth inhibitory to human breast tumor cell line MCF-7. Due to its ability to inhibit the growth of tumor cells and not that of normal cells, this activity is termed "Oncoinhibin".

For the production and induction of Oncoinhibin, human erythroblastoid cell line K-562 was

grown in RPMI 1640 medium containing 10% fetal bovine serum supplemented with glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). Cells were harvested by centrifugation when a density of 0.8×10^6 cells/ml was reached, the cells were washed once with medium without serum and transferred to serum-free conditions in RPMI-1640 medium containing glutamine, penicillin and streptomycin. For production of Oncoinhibin, 1×10^6/ml of these cells were incubated for 48 hours in T175 flask (FALCON™) under stationary culture conditions in RPMI 1640 medium without serum and then treated with phorbol ester (100 ng/ml) for 48 hours at 37° C. Thereafter, the conditioned cell supernatants were harvested by centrifugation, filtered through 0.22 micron filter (FALCON™) and stored at 4°C until further characterization. In order to concentrate the Oncoinhibin conditioned media from K-562 cell lines was ultrafiltered by PM-10 membrane (Amicon Corp.) and then dialyzed with 20 mM Tris, pH 8.0.

With reference to Figure 1, inhibitory activity of Oncoinhibin on tumor cell growth was examined by three separate methods. These methods included (1) counting cells on hemocytometer after trypan blue staining; (2) crystal violet dye-uptake method; and (3) by tritiated thymidine incorporation method. Oncoinhibin clearly inhibits the growth of MCF-7 cells by all three methods in a dose dependent manner. Due to the convenience and sensitivity, MCF-7 cell line was used as a target to develop the bioassay for Oncoinhibin. The inhibition of tritiated thymidine incorporation by Oncoinhibin was found to be a highly sensitive method to detect this cytokine.

The standard bioassay for Oncoinhibin consists of plating 5 x 10^3 cells in 96 well flat bottom well plates in 0.1 ml of RPMI 1640 medium with 10% FCS overnight at 37°C in a CO_2 incubator. Then the media is removed, a two-fold serial dilution of the test sample is added in a total final volume of 0.1 ml and incubation is continued for 24 hours at 37°C. During last 6 hours, tritiated thymidine (0.5 μCl/0.05 mi/ well) is added. At the end of 24 hours incubation period, media is poured-off and cells are detached with 0.1 ml of Trypsin (0.5%) and EDTA (5.3 mM) treatment for 30 minutes at 37° C. Cells are harvested by using PHD Cambridge cell harvester and cell-incorporated radioactivity is determined by beta counter. The data was expressed as % relative viability which is defined as amount of dpm taken up by cells in the presence of Oncoinhibin divided by the dpm incorporated in the presence of the media alone, multiplied by 100. The amount of Oncoinhibin required to inhibit the viability by 50% was defined as one unit of the cytokine. As is seen in Figure 1, Oncoinhibin exerts a dose-dependent inhibition of tumor cell growth as illustrated by all three methods.

With reference to Figure 2, a clear dose-dependent response by MCF-7 to Oncoinhibin could be observed within 24 hours. The reciprocal of the dilution of the sample needed to achieve 50% inhibition in thymidine incorporation was defined as one unit of Oncoinhibin.

With reference to Figure 3, the production of Oncoinhibin under serum-free conditions was examined. Serum-free conditions were used due to the difficulty in purifying the proteins from samples containing serum. These results clearly indicate that the

Oncoinhibin is secreted by K-562 cells even in the absence of serum.

We examined whether different agents can induce production of Oncoinhibin. Calcium ionophore, Concanavalin A, Phytohemagglutinin, and phorbol ester were examined. Figure 4 shows that phorbol ester can increase the production of Oncoinhibin. Thus, phorbol ester can be used to optimize the production of Oncoinhibin from K-562 cells. An approximately four fold increase in the production of Oncoinhibin was observed when cells were exposed to phorbol ester (100 ng/ml). Optimum induction of Oncoinhibin was observed when cells were incubated with phorbol ester for 48 hours (Table I) and at a cell-density of 1 x 10^6 cells per ml of the media (Table II).

TABLE I

Time Course of Induction of Oncoinhibin from Human K-562 Cells by Phorbol Ester

Time (hrs)	Uninduced	Induced
	Relative Cell Viability (%)	
0	94	-
6	100	60
24	84	33
48	67	16
72	41	40

K-562 cells (1x10^6/ml) were cultured in serum-free media (RPMI-1640) either in the presence or absence of the phorbol ester (100 ng/ml) at 37°C in a CO_2 incubator for different times and then the

conditioned media was harvested by centrifugation. Samples were tested on MCF-7 cells at 1:2 fold-serial dilutions as indicated in Materials and Methods.

TABLE II

Optimization of Production of Oncoinhibin in Presence and Absence of Phorbol Ester at Different Cell-Densities*

Cell Number($x10^6$/ml)	Uninduced	Induced
	Relative Cell Viability (%)	
0.01	76	83
0.1	89	56
0.5	65	41
1.0	37	26
1.5	50	28

*K-562 cell were incubated at the indicated cell-density in serum-free media (RPMI-1640) for 72 hrs at 37°C in a CO_2 incubator. The conditioned media was harvested by centrifugation and tested at two-fold serial dilution on MCF-7 cells as described in Materials and Methods.

With reference to Figure 5, to purify and characterize Oncoinhibin, the cell conditioned media as concentrated by ultrafiltration using a 10,000 molecular weight cut-off (PM-10) membrane. The activity in the fraction not retained by the filter (Flow through or FT) was lower whereas that in the retained fraction or concentrate (C) was proportionately higher than that of the standard buffer (starting material, SM). The results indicate that Oncoinhibin activity is retained and concentrated.

These results also indicate a molecular weight higher than 10,000 for Oncoinhibin.

In the gel permeation fast protein liquid chromatographic experiment of Figure 6, panels a and b, a sample of Oncoinhibin was applied onto a SUPEROSE™-6 column (Pharmacia) pre-equilibrated with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.01% sodium azide. The column was run at room temperature with a flow rate of 0.5 ml per minute and the size of each fraction was 0.5 ml. The column was calibrated with molecular weight standards (Schwarz/Mann, Cambridge, MA). The latter included Apoferritin (480 kDa), Alpha Amylase (20 kDa), Gamma-Globulin (160 kDa), Bovine Serum Albumin (67 kDa), Ovalalbumin (45 kDa), Chymotrypsinogen (24 kDa) and Cytochrome C (12.4 kDa).

With reference to Figure 6, it appears that Oncoinhibin has an approximate molecular weight of 25 kDa. The molecular weight of Oncoinhibin was examined by gel permeation Fast Protein Liquid Chromatography on SUPEROSE™-6 column under nondenaturing conditions. The results of gel filtration run in phosphate buffered saline, pH 7.4 show two major peaks of activity, one coinciding with the excluded volume and the second peak corresponding to an approximate molecular weight of around 25 kDa (Figure 6, panel B).

In the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) experiments of Figures 7 and 8, a 15% polyacrylamide gel was run essentially according to U. K. Laemmli (Nature 227 (1970) 680-685) and proteins were visualized by silver staining. For preparative gel electrophoresis and elution of activity, a portion of the gel before fixing and staining was sliced with a

razor blade into over 40 different slices, eluted in a test tube with 50 mM ammonium bicarbonate by diffusion overnight, the fractions were dialyzed against 20 mM Tris, pH 8.0 and then assayed for biological activity.

With reference to Figure 7, to further confirm the molecular weight of Oncoinhibin, SDS-PAGE analysis was performed. After electrophoresis, the gels were sliced, eluted in 50 mM ammonium bicarbonate overnight and assayed for Oncoinhibin activity. Greater than 50% of the applied Oncoinhibin activity was recovered in the molecular weight region of around 30 kDa. Less than 10% activity was also found in an area near the dye front.

With reference to Figure 8, a rerun of the biologically active fraction on SDS-PAGE and silver staining of gels showed a single major band at a molecular weight of around 28 kDa.

With reference to Figure 9, panels a and b, Oncoinhibin binding to and elution from anion exchange resins was examined. One column (1 cm x 5 cm) was packed with an anion exchange resin Q-SEPHAROSE™ and then equilibrated with 20 mM Tris, pH 8.0 (equilibration buffer). A sample of Oncoinhibin dialyzed against the equilibration buffer was loaded onto the column with a flow rate of 0.5 ml/minute. The column was rinsed with the equilibration buffer and then resolved with a step-up gradients of NaCl (0-1 M). Various fractions were analyzed for protein concentration and for bioactivity.

DEAE AFFIGEL™ Blue Chromatography: A second column (1 cm x 5 cm) was packed with DEAE AFFIGEL™ Blue resin and then equilibrated with 20 mM Tris, pH 8.0 (equilibration buffer). A sample of Oncoinhibin pre-equilibrated against the equilibration buffer by

dialysis was loaded onto the column with a flow rate of 0.5 ml/minute. The column was rinsed with the equilibration buffer and then resolved with a step-up gradients of NaCl (0-1M). Various fractions were analyzed for protein concentration and for bioactivity.

Panels a and b of Figure 9 show that Oncoinhibin binds and can be eluted from anion exchange resins. To both the Q-SEPHAROSE™ and DEAE AFFIGEL™ Blue resins, Oncoinhibin activity bound in 20 mM Tris, pH 8.0 buffer. The bound Oncoinhibin could be eluted with 0.2 M NaCl in 20 mM Tris buffer from DEAE AFFIGEL Blue and with 0.5 M NaCl from Q-SEPHAROSE™.

Oncoinhibin inhibits the growth of wide variety of tumor cells. (Table III) The antiproliferative activity of Oncoinhibin was examined by tritiated thymidine incorporation.

TABLE III

Differences in tumor cell specificity between TNF and Oncoinhibin

Cell Lines	Relative Cell Viability (%)	
	TNF	Oncoinhibin
Erythroblastoid cell line (K562)	94	2
Histiocytic Lymphoma (U-937)	54	1
Histiocytic Lymphoma (U-937-CF- 1)	13	0
Promyelocytic Lymphoma (HL-60)	100	1
Burkitt Lymphoma (Raji)	76	0
T cell Lymphoma (Jurket)	74	36
Myelogenous Leukemia (KG-1)	43	8
Myelogenous Leukemia (KG-1a)	60	13
Myelogenous Leukemia (ML-1a)	59	0.1
Myelogenous Leukemia (ML-1b)	35	0.1
Monocytic Leukemia (THP-1)	81	1
Breast Carcinoma (BT20)	24	2
Breast Carcinoma(BT20TNFR)	60	3
Breast Carcinoma(MCF7)	1	0
Breast Carcinoma (SK-BR3)	52	0
Breast Carcinoma (ZR-75-1)	6	1
Melanoma (RPMI 7951)	73	0
Melancma (A375)	76	0
Epidermoid Carcinoma (A-431)	82	0
Cervical Carcinoma (ME-1 80)	20	0
Ovarian Carcinoma (OVCAR-3)	27	1
Cervical Carcinoma (HeLa)	55	14
Hepatoma (HepG-2)	83	20
Retinoblastoma (Weri-Rb-1)	60	33
Retinoblastoma (Y-79)	92	46
Glioblastoma (LG)	84	2.3
Murine Fibroblasts (NIH 3T3)	11	35
Murine Fibroblasts (LTR1 000)	85	27
Murine Fibroblasts (L929)	0	1
Normal Human Foreskin Fibroblasts	311	189

Tumor cells (5000/well) were incubated with TNF (0.2 μg/ml) or Oncoinhibin (induced by phorbol ester and concentrated) for 72 hours at 37°C and then relative cell viability (%) was determined by Thymidine incorporation as described previously.

With reference to Figure 10, panels A-D, the dose response effects of Oncoinhibin on some of the cell lines listed in Table III were examined and compared to TNF. It is clear from the results shown in Table III and Figure 10 that, besides MCF-7, Oncoinhibin can inhibit the growth of several different types of leukemias, melanomas, carcinomas and hepatomas. The growth of murine cells was also inhibited. Thus, it appears that, unlike interferons, Oncoinhibin is not species-specific.

With reference to Figures 11 and 12, for growth assays, cells were plated for overnight in 0.1 ml of the medium (RPMI-1640 with 10% FBS) in 96-well FALCON™ plates. Thereafter, the medium was removed and a serial dilution of human Oncoinhibin was layered in 0.1 ml of the volume. After 72 hours of incubation at 37°C, the medium was removed and viable cells were monitored by crystal violet staining according to the procedure as described by B. B. Aggarwal, Human lymphotoxin, Meth. of Enzymol., 116:441-448 (1985).

A dye uptake method to examine cell viability correlates with cell number determined by detachment with a trypsin solution and microscopic counting with hemocytometer. The percentage of relative cell viability was calculated as optical density in the presence of the test sample divided by optical density in the absence of the test sample (medium) multiplied by 100. For LT and TNF, cytotoxicity assays were carried out similar to growth inhibition assays except that 20 x 10^3 L-929 cells were treated with actinomycin D (1 μg/ml) along with the cytokine for 24 hours.

Cell Growth-Stimulatory Assays. Cell growth-stimulatory assays were carried out essentially

according to the procedure described by Vilcek et al., J. Exp. Med. 163:632-643 (1986).

Briefly, confluent human diploid foreskin fibroblasts at passage level 12-16 (corresponding to approximately population doubling levels 24-32) were used for cell growth stimulatory assays. To determine the effect of human Oncoinhibin, cells (8 x 10^3 /well) were plated in 0.1 ml of the medium (RPMI-1640 + 10% FBS) in 96-well FALCON™ plates. After overnight incubation in a CO_2 incubator at 37°C, the medium was removed and a serial dilution of the cytokine was layered in 0.2 ml of the volume. After 5 days incubation, media was decanted-off and cells were stained with crystal violet. All determinations were made in triplicate. Percent relative cell viability was calculated as indicated for growth-inhibitory assays.

For [^{3}H] TdR incorporation assays, human fibroblasts were cultured and treated with the cytokine for 5 days. During the last 24 hours, tritiated thymidine (6.7 Ci/mmole; New England Nuclear, Boston, MA) was added to each well (0.5 μCi/well). Thereafter, the culture medium was removed, the wells were washed twice with phosphate-buffered saline and the cells were detached by the addition of a solution of trypsin (0.5%) with EDTA (5.3 mM). The cell suspension was then harvested with the aid of PHD cell harvester (Cambridge Technology Inc. Watertown, MA) and lysed by washing with distilled water. Radioactivity bound to the filter was measured in a liquid scintillation counter (Model 1600 TR; Packard Co., Meriden, CT). Thymidine incorporation in human fibroblast determined by this method correlates with cell growth. In tumor cell growth inhibition studies, cells were incubated

with the cytokine for 3 days in a total of 0.1 ml final volume and then monitored for thymidine incorporation.

With reference to Figure 11, Oncoinhibin appears to stimulate the growth of normal human foreskin fibroblasts. While inhibiting the growth of tumor cells, Oncoinhibin enhanced the proliferation of normal human foreskin fibroblasts.

With reference to Figure 12, similar to TNF and LT, Oncoinhibin cytolyses actinomycin D treated L-929 cells. The antitumor activity of Oncoinhibin against L-929 cells cannot be neutralized by either anti-LT or anti-TNF antibodies (Table IV). That is, no significant amounts of TNF or LT were detected by ELISA assay in Oncoinhibin preparations (Table V). Table III shows that several tumor cell types (e.g; SK-BR-3, HeLa, A-431, OVCAR-3, A375, and RPMI-7951) which are resistant to TNF/LT, are exquisitely sensitive to Oncoinhibin. Oncoinhibin can also be distinguished from TNF/LT on the basis of cell lines isolated for resistance to TNF/LT (NIH 3T3-LTR and BT-20 TNFR), were found to be sensitive to Oncoinhibin. (Table III)

TABLE IV

Lack of Neutralization of Oncoinhibin Activity by Monoclonal Antibodies Against Tumor Necrosis Factor and Lymphotoxin*

Sample	Antibody	Relative Cell Viability %	% Neutralization %
Oncoinhibin	None	58	0
	+Anti-TNF	58	0
	+Anti-LT	60	0
	+Anti-TNF+Anti LT	56	4
TNF	None	39	0
	+Anti-TNF	100	100
	+Anti-LT	44	5
LT	None	35	0
	+Anti-LT	90	90
	+Anti-TNF	38	0

*Oncoinhibin, TNF and LT were incubated with either Anti-TNF or Anti-LT antibodies at 37°C for 1 hour and then directly assayed for remaining nonneutralized TNF or LT activity on Actinomycin D treated L-929 cells. Such assay is described in Aggalwal, B.B. Human Lymphatoxin "Method of Enzymology" 116:441, 448, 1985.

TABLE V

Determination of Various Cytokines in a Crude Preparation of Oncoinhibin Derived from K-562 Cells*

Cytokine	Levels (pg/ml)
TNF-α	3±6
TNF-β (LT)	2+0
IL-1β	0
IL-6	155±7
IL-8	>2000

Serum-free K-562 cell-conditioned media was used as a source of Oncoinhibin and the level of various cytokines were determined by standard ELISA assays (R&D System). ND is not determined

TNF and LT are products of monocytes and lymphocytes, respectively, and inhibit the growth of a wide variety of cells. Similar to Oncoinhibin, lymphotoxin and TNF inhibit the growth of MCF-7 cells, but large concentrations of TNF and LT (10,000 units/ml) are needed.

To further confirm that Oncoinhibin is neither TNF nor LT, a Northern blot analysis was performed to look for the gene for either of the cytokines. In the northern blot analysis, phorbol ester treated and untreated K-562 cell cultures seeded at 1 x 10^6 cells/ml in 75-cm flasks were incubated with protein kinase C activator for 24 hours and then harvested by centrifugation. Total RNA was extracted

from cells by the guandinium isothiocyanatephenol-chloroform method shown by Chirgwin, et al., *Biochem.* 18:5294-5299 (1979) and Maniatis, et al., *Molecular Cloning* 188-209 (1982).

Routinely, RNA with 260 nm/280 nm absorbance ratio of greater than 1.9 and yield of approximately 100 μg RNA per 20 X 10^6 cells was obtained.

For electrophoresis, 20 μg of RNA was fractionated on 0.8% agarose gels containing 2.2 M formaldehyde at 75-100 V for approximately 3 hours. Thereafter, gels were exposed to diethyl pyrocarbonate-treated water at 68° C for 1 hour, and then the RNA transferred to HYBOND™ nylon membranes (Amersham Corp., Arlington Heights, IL.). After transfer (3 hours), the filter was rinsed twice with SSC (SSC: 0.15 M sodium chloride, 15 mM sodium acetate, 15 mM sodium citrate, pH 7.0) and placed in a microseal bag.

Prehybridization was carried out at 65° C for 1 hour in a buffer containing 7% SDS, 500 mM sodium phosphate, 1 mM EDTA, pH 7.2 (hybridization buffer). Filters were then hybridized with TNF or LT cDNA probes (specific activity 2 x 10^8 cpm/μg DNA). After hybridization, membranes were washed several times at 65° C in hybridization buffer containing salmon sperm DNA (200 μg/ml). The filters were exposed to KODAK™ XAR-5 film at -70° C for 1-3 days. Procedures for sequential cycles of prehybridization, hybridization, washes and filter stripping were performed. Equal loading of lanes was demonstrated by examination of gels after ethidium bromide staining and also by rehybridization of same filters with cDNAs for either actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Band densitometry was performed by either

scanning the filter for radioactive counts with blot analyzer BETASCOPE™ 603 (Betagen Corp., Waltham, MA.) or **by scanning autoradiogram for optical density by using Scanning Densitometer (Helena Laboratories Inc.** Beaumont, TX.). As shown in Figure 13, no expression of mRNA for either LT or TNF was observed in K-562 cells. Furthermore, gel filtration and SDS-PAGE experiments confirm that the molecular weight of Oncoinhibin is different from that of either TNF or LT.

With reference to Figure 19, human breast tumor cells grow rapidly in culture in the media. When Oncoinhibin was added to the culture, however, no growth of these cells was observed. In order to determine the time of exposure needed to inhibit the growth of these cells, the cells were exposed to Oncoinhibin for different times. With reference to Figure 20, the growth-inhibitory effects of Oncoinhibin are terminated when the cytokine is removed from the media. These results suggest the need for continuous presence of the Oncoinhibin.

With reference to Figure 21, the morphology of the MCF-7 cells was examined after treatment with Oncoinhibin and compared to TNF. The results indicate a difference in the method by which TNF and Oncoinhibin inhibit the growth of breast tumor cells. TNF induces rounding-up of the cells which leads to their detachment from the dish, while Oncoinhibin induces enlargement or swelling of cells. The latter may result from effects of Oncoinhibin on the permeability of the cells. Oncoinhibin also inhibits the colony formation of human breast tumor cells.

Oncoinhibin Stability Studies. Oncoinhibin was treated with organic solvent (acetonitrile or methanol or propanol), acidic solvents (HCl,

trichloroacetic acid or acetic acid), alkaline solvents (NaOH, Ammonium hydroxide) or detergents (SDS, TWEEN™ 10) for two hours at room temperature, then dialyzed against 20 mM Tris-HCl, pH 8.0 overnight in a cold room and assayed for biological activity. (Tables VI and VIII).

For thermostability experiments, Oncoinhibin was exposed to different temperatures for different times and the biological activity was determined directly. The biological activity of Oncoinhibin was found to be stable to 80°C for 60 minutes but approximately 50% loss of activity was observed when exposed to 100°C for 30 minutes (Table VI).

TABLE VI

Thermostability of Oncoinhibin at Different Temperatures

		Activity Remaining	
Temperature(°C)	Time(min)	U/Ml	%
4	60	256	100
80	30	210	82
	60	230	90
90	30	200	78
	60	190	74
100	30	128	50
	60	110	43

In Table VII, Oncoinhibin was treated with pronase E, trypsin, chymotrypsin and V8 Staph protease and then analyzed for its biological activity. The results indicate that Oncoinhibin activity can be

partially sensitive to trypsin treatment and completely resistant to chymotrypsin and V8 protease. Deoxyribonuclease also had no effect on the activity of Oncoinhibin.

TABLE VII

Effect of Proteolytic Enzymes on the Activity of Oncoinhibin

Enzymes	Conc.	Activity Remaining (%)
None	10% (w/w)	100
Pronase E	10% (w/w)	0.2
Trypsin	10% (w/w)	50
V8 Staph. Protease	10% (w/w)	100
Chymotrypsin	10% (w/w)	100
Deoxyribonuclease	10% (w/w)	100

Oncoinhibin was incubated with various enzymes at 37°C for 24 hours in 20 mM Tris buffer at pH 8.0, then reaction was stopped by addition of 10% serum and assayed for Oncoinhibin activity.

Stability of Oncoinhibin to Detergents: A sample of Oncoinhibin was treated with different concentrations of either SDS, a negatively charged detergent or TWEEN™-20, a neutral detergent for 2 hours, dialyzed and then assayed for biological activity. Bovine serum albumin treated with the same detergent was used as a control. The results of these experiments is shown in Table V. No loss of biological activity was observed on treatment of the protein either with SDS or TWEEN™-20. In case of SDS, it appears that there is a significant increase in the biological activity of Oncoinhibin in a dose-dependent

manner The increase was not significant with TWEEN™-20

TABLE VIII

Effect of Detergent, pH and Organic Solvent Treatment on the Stability of Oncoinhibin

Agent		Conc.	Activity Remaining U/ml	%
Detergent Stability:				
Sodium dodecyl sulphate		None	19	100
		0.01%	23	124
		0.05%	45	243
		0.10%	61	330
		0.50%	59	319
TWEEN™-20		0.01%	24	130
		0.05%	24	130
		0.10%	16	88
pH Stability:				
Glycine	pH2.0	0.1M	240	300
Acetic acid	pH2.4	1.0M	256	320
Sodium acetate	pH4.0	0.1M	83	104
Sodium acetate	pH6.0	0.1M	76	95
Tris-HCL	pH8.0	0.02M	80	100
NaHCO3	pH10.0	0.1M	92	115
NH4OH	pH11.4	1.0M	185	231
Organic Solvent Stability:			S/P	
None		-	42	100
Methanol		50%	81/2	198
Propanol		50%	56/3	140
Acetone		70%	23/26	117
Ethanol		70%	56/4	143
Acetonitrile		50%	153/4	374

Oncoinhibin samples were treated at room temperature with the various agents for 1 hour in 20 mM Tris buffer pH 8.0, dialyzed overnight and then assayed for biological activity remaining. S and P represents supernatant and pellet fractions.

Stability of Oncoinhibin to reducing agents: Oncoinhibin was treated with different concentrations of dithiothreitol for 2 hours, then dialyzed and assayed for biological activity. The results of these experiments are shown in Table IX. It is clear that the activity of Oncoinhibin is unstable to the treatment of DTT. A 50% loss in activity with 1 mM DTT and 67% loss with 100 mM DTT was observed.

TABLE IX

Stability of Oncoinhibin to Various Treatments

Agent	Conc.	Activity	Percent
Reducing Agents (DTT)	-	256	100
	1mM	128	50
	10mM	96	38
	100mM	84	33
Trichloroacetic acid	5% Sup.	11	4
	Ppt.	256	96
Amm. Sulphate	70% Sup.	11	2
	Ppt.	538	98

Oncoinhibin samples were treated with various agents, dialyzed and then assayed for biological activity.

Oncoinhibin can be concentrated by trichloroacetic acid and ammonium sulphate. Oncoinhibin was treated with different concentrations of either TCA or NH_4SO_4, centrifuged, resuspended, dialyzed and then assayed for biological activity. The results shown in Table IX indicate that all the activity of Oncoinhibin can be precipitated by either 5% TCA or by 70% (SAS)

ammonium sulphate. Thus, these results also suggest the proteinaceous nature of Oncoinhibin.

Amphiregulin is glycoprotein that was isolated from phorbol ester-treated MCF-7 cells and inhibits the growth of A431 cells. Amphiregulin has an apparent molecular weight of 22.5 kDa. Both Oncoinhibin and amphiregulin inhibit the growth of tumor cells. Oncoinhibin, however, is not produced by phorbol-ester treated MCF-7 cells. Secondly, even though both Oncoinhibin and amphiregulin exhibit antiproliferative activity against A431 cells (Table III), amphiregulin, in contrast to Oncoinhibin, is inactive against human melanoma (A375), human adenocarcinoma of the breast (ZR-75-1 or MCF-7), human adenocarcinoma of the lung (A-549), human carcinoma of colon (H3347) human lymphoblastoid T cells (CEM), human EBV transformed B cells, human epidermal carcinoma of larynx (Hep 2), bovine fetal heart endothelial cells (CRL-1395), murine BALB/3T3, and mink lung (CCL-64) cells. Similar to Oncoinhibin, amphiregulin stimulates the proliferation of human fibroblasts. Besides human fibroblasts, amphiregulin also stimulates the growth of certain tumor cells including human pituitary tumor cells (CRL 7386), human ovarian carcinoma cells (HTB 77), African green monkey kidney cells (BSC-1) and rat kidney cells (NRK). The molecular weight of Oncoinhibin, however, is also significantly different from that of amphiregulin and the mature form of the latter is an 84 amino acid long protein.

Oncoinhibin is stable to both acidic and alkaline conditions at a pH range of 2.0-10.0 (Table VIII). Several cytokines have been reported which are stable to pH 2, including IFN-α, IFN-β, IL-2, IL-4, IL-8, CSF-1, GM-CSF, TGF-β, Oncostatin M and amphiregulin.

Oncostatin M, amphiregulin, CSF-1 and IL-4 were also found stable to alkaline conditions (pH 12). Recently, lipolysis promoting factor (LPF), a protein with an approximate molecular weight of 6 kDa, has been isolated from A375 melanoma cell line which is stable to heat 96°C for 10 minutes), protease K (10 μg/ml), trypsin, pronase, RNase, DNase and periodate oxidation. LPF can be precipitated by trichloroacetic acid (10%) without any loss of biological activity. Oncoinhibin was found to be stable to heat up to 80°C for 30 minutes and only partial activity was lost at 100°C (Table VI). A trypsin 10% (w/w) treatment of Oncoinhibin for 24 hours at 37°C lead to a partial loss of biological activity. This is similar to that observed with CSF-1, GM-CSF and LT. The biological activity of Oncoinhibin was also found stable to 0.5% SDS.

Oncoinhibin is distinct from Tumor Killing Factor (TKF), a cytokine identified from human macrophage-monocyte hybridoma in that TKF is a basic protein (pI 8-9.0), has an apparent molecular weight of 56 kDa by gel filtration and can be eluted from Conconavaline A-SEPHAROSE™ with 0.4 M α-methyl mannoside.

Oncostatin M is a cytokine isolated from U-937 cells treated with phorbol ester and inhibits the growth of human melanoma cell line A375 in a thymidine incorporation assay. Unstimulated U-937 cells do not express the gene or secrete this activity. Phytohemagglutinin-activated human peripheral blood T lymphocytes also express the gene and secrete this cytokine. Oncostatin M is a glycoprotein with a molecular weight of 28 kDa by SDS-PAGE and 18 kDa by gel filtration. It synergizes with TGF-β but not with

interferons. Oncostatin M has been shown to inhibit the proliferation of HTB 10 neuroblastoma cells, A-549 lung carcinoma cells, as well as A375 and SKMEL-28 melanoma cells; it does not, however, inhibit the proliferation of L-929 cells. In contrast, Oncoinhibin is produced by K-562 cells (not by U-937 cells) both in the presence as well as in the absence of phorbol ester and affects L-929 cells. Moreover, in contrast to Oncoinhibin, oncostatin M is a relatively weak inhibitor of A375 cells (Figure 14).

Transforming growth factor-ß (TGF-β) is cytokine which is a homodimer with a molecular weight of 25 kDa on SDS-PAGE and inhibits the growth of several cell types of epithelial and mesenchymal origin including human vascular endothelial cells, T and B lymphocytes. A-549, MCF-7 cells are also inhibited by TGF-β in an autocrine manner. However unlike Oncoinhibin, TGF-β is produced by a wide variety of cells including platelets, bone tissue and lymphocytes, and requires acid-activation before its activity can be examined. In addition, TGF-β has a molecular weight of 12.5 kDa on SDS-PAGE under reduced conditions.

Cytokine ELISA assays: A commercially available (R&D Systems) quantitative "sandwich" enzyme immunoassay technique was used to examine the presence of known cytokines (TNF, LT, IL-1, IL-6 and IL-8) in the Oncoinhibin preparation. The standard protocol provided by the supplier was used. Briefly, a monoclonal antibody specific for different cytokines was coated onto the microtiter plates and allowed to set up overnight to immobilize the antibodies. Then the samples were pipetted into the wells and the cytokine if any is captured by the immobilized antibody. After washing away any unbound sample

proteins, an enzyme-linked polyclonal antibody specific for a given cytokine was added to the wells and allowed to bind the cytokine which was bound during the first incubation. Following a wash to remove any unbound polyclonal antibody-enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of cytokine bound in the initial step. Along with the samples tested, a series of wells was prepared using known concentrations of the cytokine standards. A curve plotting the optical density versus the concentration of cytokine in these standard wells was prepared by comparing the optical density of the samples to this standard curve. The concentration of the cytokine in the unknown samples was then calculated (Table V).

Interleukin-1 is a cytokine produced by activated monocytes and fibroblasts, has a molecular weight of 17 kDa and inhibits the growth of tumor cell lines including ovarian carcinoma, A375 melanoma, K-562 and certain breast tumor cell lines. Oncoinhibin, however, differs from Interleukin-1 with respect to its source, molecular weight and tumor specificity. Crude preparations of Oncoinhibin were examined for the presence of IL-I by ELISA. The results shown in Table V demonstrate a lack of presence of the IL-I protein in our Oncoinhibin preparation.

Interleukin-6 is cytokine produced by a wide variety of different cell types in response to highly diverse stimuli and has a molecular weight of 26 kDa on SDS-PAGE. Specifically, IL-6 is also produced by normal human fibroblasts, U-937, human melanoma cell lines A375, RPMI-7951 etc. IL-6 inhibits the growth of myeloid leukemia and breast carcinoma cell lines. Based on the source, method of induction and tumor cell

target specificity, IL-6 appears to be a different cytokine from that of Oncoinhibin. Moreover, MCF-7 cells routinely used as a target for Oncoinhibin are insensitive to the effects of Interleukin-6 (Figure 16). Also in contrast to Oncoinhibin, IL-6 was found to inhibit the growth of normal human fibroblasts (Figure 15).

Interferon-T a cytokine with a molecular weight of 20-25 kDa on SDS-PAGE, is produced by T-lymphocytes when activated with various mitogens and inhibits the growth of certain tumor cell lines. This cytokine is highly sensitive to acidic pH conditions. Oncoinhibin differs from interferon-T with respect to its source, method of induction and pH stability. Interferon-T also differs from Oncoinhibin with respect to its effect on normal human fibroblasts. Oncoinhibin stimulates the proliferation whereas interferon-T inhibits the TNF-induced fibroblast proliferation but not produced by Oncoinhibin (Figure 18). Moreover, antibodies to interferon-T do not reduce the activity of Oncoinhibin but enhance it (Figure 17).

Thus, the present invention provides a novel cytokine exhibiting diverse antineoplastic activity. The cytokine, Oncoinhibin, is secreted by human erythroblastoid cells and has a molecular weight of approximately 28 kDa on SDS-PAGE. Production of Oncoinhibin appears to be enhanced in the presence of phorbol ester. Oncoinhibin appears to be stable to a wide range of substances and stable in a wide pH range and to a high temperature.

Due to its diverse neoplastic activity, it is contemplated that Oncoinhibin will be of therapeutic use in the treatment of a wide variety of neoplastic diseases, including carcinomas and lymphomas.

Oncoinhibin may be supplied to humans or other animals as part of a pharmaceutical composition that would contain a pharmaceutically acceptable carrier. Due to its diverse antineoplastic activity, Oncoinhibin will be useful in preventing recurrence of neoplastic diseases. In addition, administration of Oncoinhibin to hosts having a neoplastic cells will likely extend the survival time of the host. Alternatively, neoplastic cells could be treated with Oncoinhibin *in vitro*, e.g., the treatment and purging of bone marrow containing neoplastic cells. These methods of treating neoplastic cells as described herein are well known in the art of cancer chemotherapy and consequently a person having ordinary skill in this art could, without undue experimentation, determine the appropriate dosages and routes of administration of Oncoinhibin.

Oncoinhibin may also be useful as a novel immunomodulator. Oncoinhibin activates lymphocytes, monocytes and neutrophils to kill tumor cells. In addition, Oncoinhibin may be therapeutically useful as a growth factor. Oncoinhibin stimulates the growth of normal cells.

CLAIMS

1. A purified and isolated human Oncoinhibin protein which is secreted by human erythroblastoid cells, said human Oncoinhibin protein having a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity.

2. The human Oncoinhibin protein as claimed in claim 1, wherein the production of said human Oncoinhibin protein is enhanced by phorbol ester.

3. The human Oncoinhibin protein as claimed in claim 1 wherein said human Oncoinhibin protein is active in a standard bioassay for Oncoinhibin activity, and is substantially homogeneous to SDS-PAGE analysis, wherein said bioassay measures a reduction in tumor cell growth and is selected from the group consisting of counting cells on a hemocytometer after trypan blue staining, crystal violet dye uptake and tritiated thymidine incorporation.

4. A process for preparing human Oncoinhibin protein, which is secreted by human erythroblastoid cells, said human Oncoinhibin protein having a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity comprising the steps of:

incubating human erythroblastoid cells;

inducing the production of said human Oncoinhibin protein; and

harvesting conditioned cell supernatants containing said human Oncoinhibin protein.

5. The process as claimed in claim 4, further comprising the step of concentrating said supernatant.

6. The process as claimed in claim 4 or claim 5, further comprising the step of enhancing the production of said human Oncoinhibin protein with phorbol ester.

7. The process as claimed in claim 4, claim 5 or claim 6, wherein said erythroblastoid cells are from cell line K562.

8. The process as claimed in any one of claims 4 to 7, wherein said step of incubating said cells is in RPMI1640 medium.

9. The process as claimed in any one of claims 4 to 8, wherein said step of inducing the production of said human Oncoinhibin protein is in a serum-free medium.

10. Human Oncoinhibin protein as claimed in any one of claims 1 to 3, whenever prepared by the process of any one of claims 5 to 9.

11. A method of purifying human Oncoinhibin protein having a molecular weight of about 28,000 kDa, is stable in a pH range of from about 2 to about 8 and at a temperature range of from about 4°C to about 100°C, and exhibits anti-neoplastic activity, comprising the steps of:

ultrafiltering conditioned cell supernatants containing human Oncoinhibin protein, thereby to provide ultrafiltered supernatants;

dialyzing said ultrafiltered supernatants, thereby to provide dialyzed, ultrafiltered supernatants;

performing chromatography on said dialyzed, ultrafiltered supernatants, thereby to provide chromatographed dialyzed, ultrafiltered supernatants; and

performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reverse phase high performance liquid chromatography on said chromatographed dialyzed, ultrafiltered supernatants.

12. Purified human Oncoinhibin protein as claimed in any one of claims 1 to 3, whenever prepared by the method of claim 11.

13. A pharmaceutical composition comprising a purified isolated human Oncoinhibin protein as claimed in any one of claims 1 to 3, claim 10 or claim 12, and a pharmaceutically-acceptable carrier.

14. Use of an effective dose of the pharmaceutical composition of claim 13 for inhibiting the growth of a neoplastic cell.

15. The use as claimed in claim 14, wherein said neoplastic cell is *in vitro*.

16. The use as claimed in claim 14,wherein said neoplastic cell occurs in a human or in a non-human animal.

17. The use as claimed in claim 14, claim 15 or claim 16, wherein said neoplastic cell is selected from the group consisting of lymphomas, breast carcinomas, melanomas, cervical carcinomas, ovarian carcinomas, and hepatomas.

18. Use of an effective dose of the pharmaceutical composition of claim 13, for preventing recurrence of a neoplastic condition.

19. Use of an effective dose of the pharmaceutical composition of claim 13, for extending the survival time of a host with a neoplastic cell.

20. Use of the purified and isolated Oncoinhibin protein of any one of claims 1 to 3, claim 10 or claim 12, to treat a disease which is selected from the group consisting of a carcinoma and a lymphoma.

21. The use as claimed in claim 20, wherein said carcinoma is selected from the group consisting of breast carcinomas, melanomas, cervical carcinomas, ovarian carcinomas, and hepatomas.

22. An immunomodulator for activating lymphocytes, monocytes and neutrophils to kill tumour cells, which consists of purified and isolated human Oncoinhibin as claimed in any one of claims 1 to 3, claim 10 or claim 12.

23. A growth factor for stimulating the growth of normal cells, consisting of purified and isolated human Oncoinhibin as claimed in any one of claims 1 to 3, claim 10 or claim 12.

24. The growth factor as claimed in claim 23, wherein said normal cell is a fibroblast.

25. Use of a purified and isolated human Oncoinhibin protein as claimed in any one of claims 1 to 3, claim 10 or claim 12, for preparing a medicament for inhibiting the growth of a neoplastic cell.

FIG. 1A

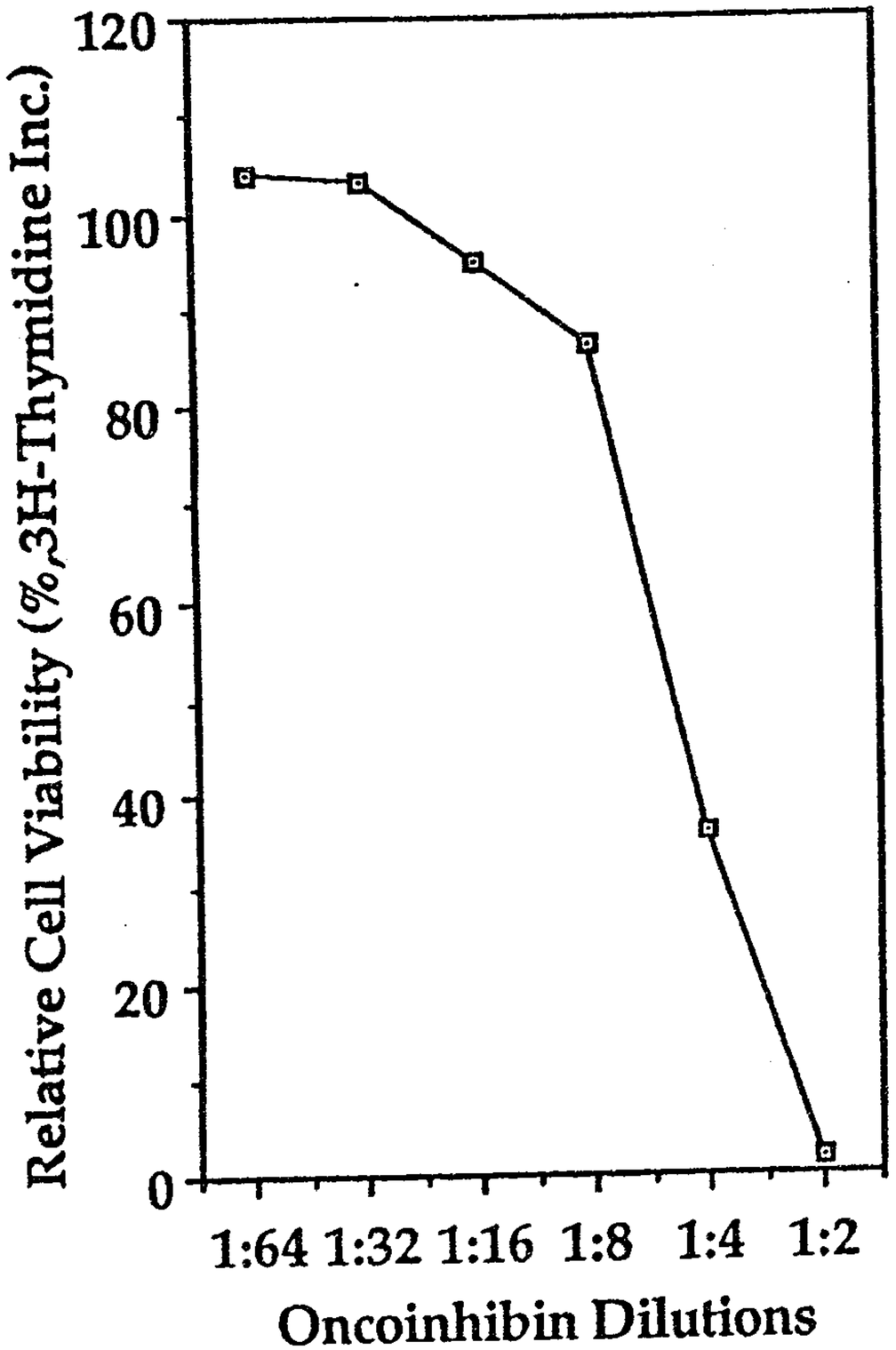


FIG. 1B

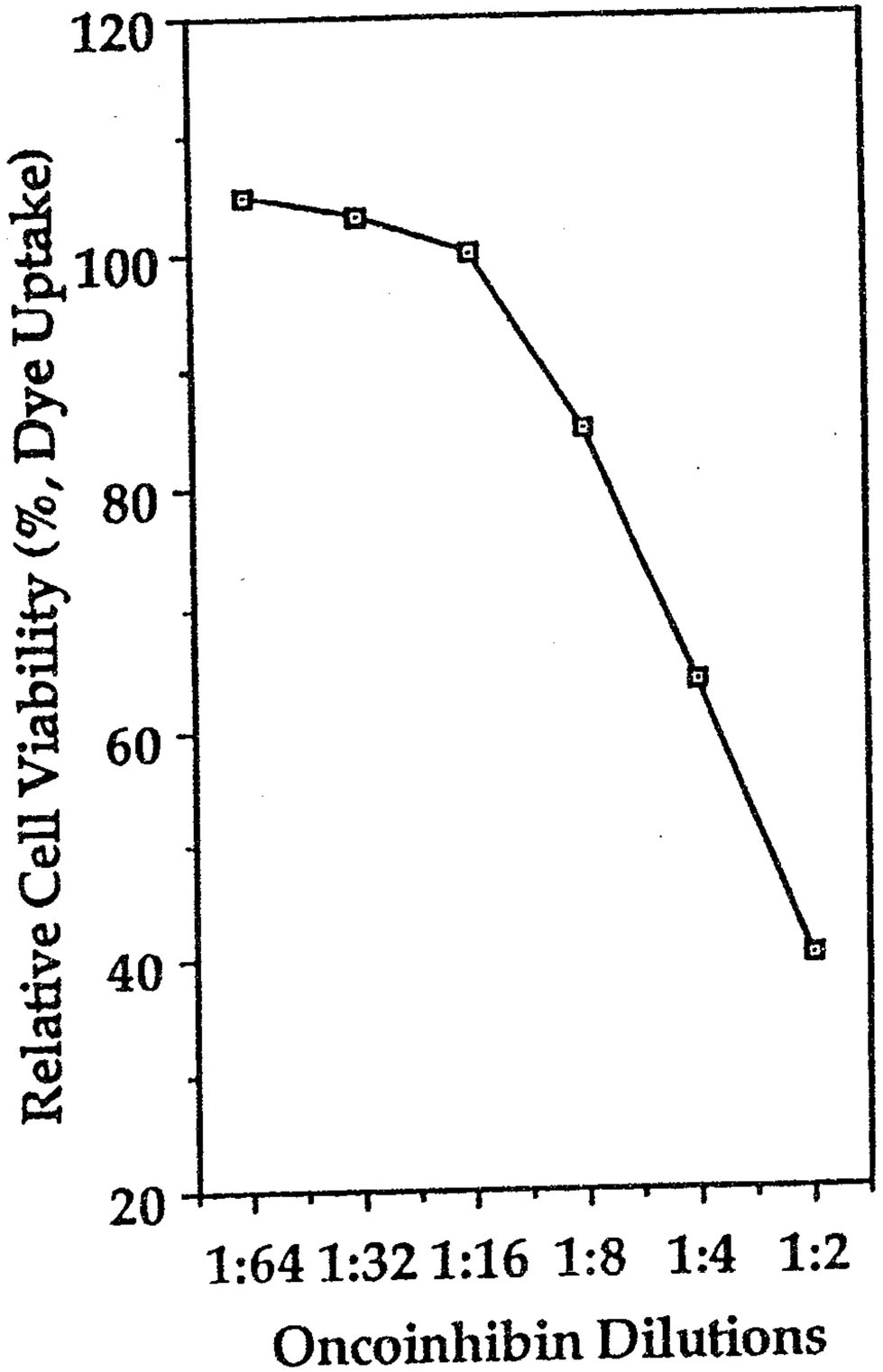
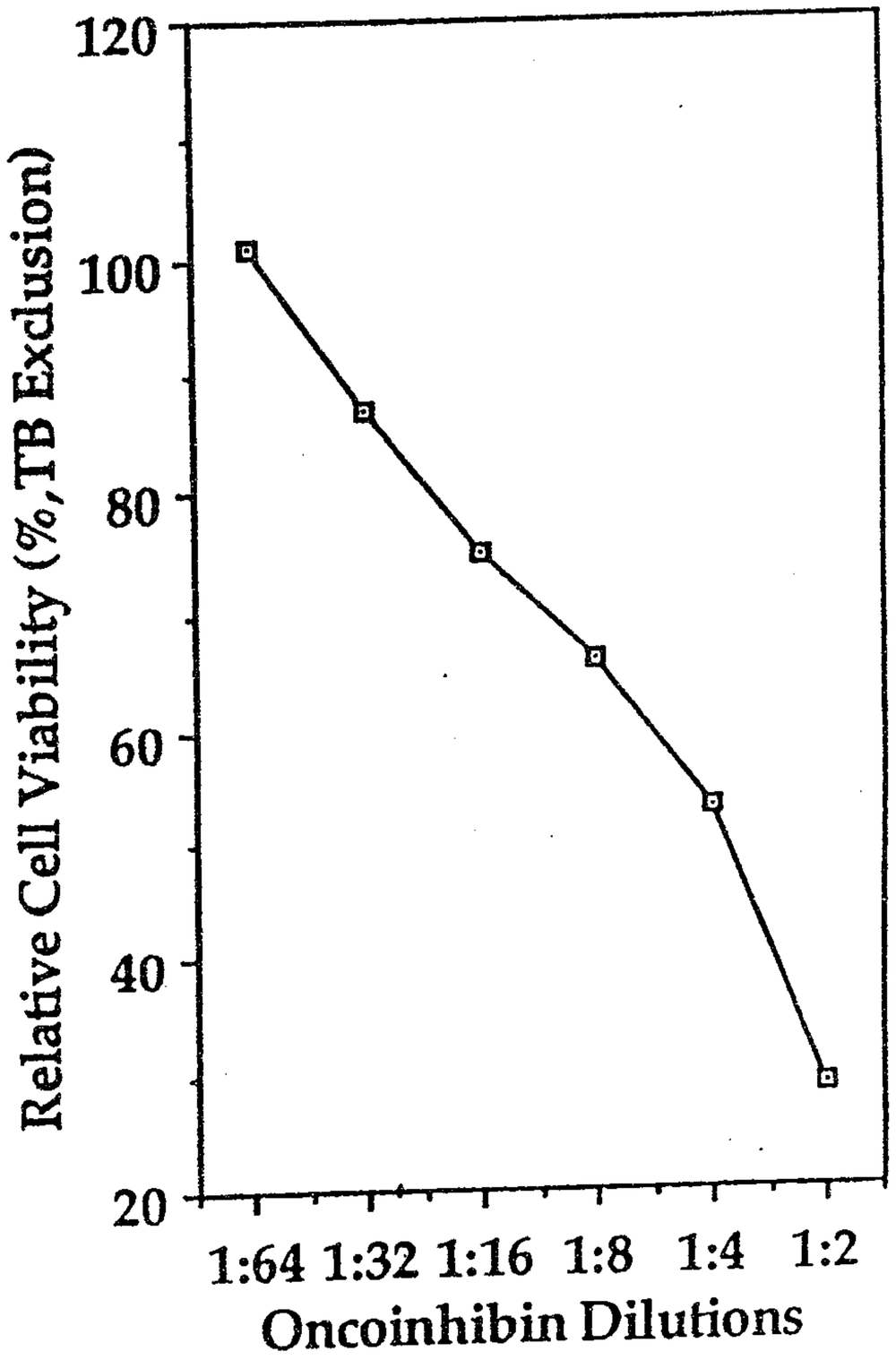


FIG. 1C



2118408

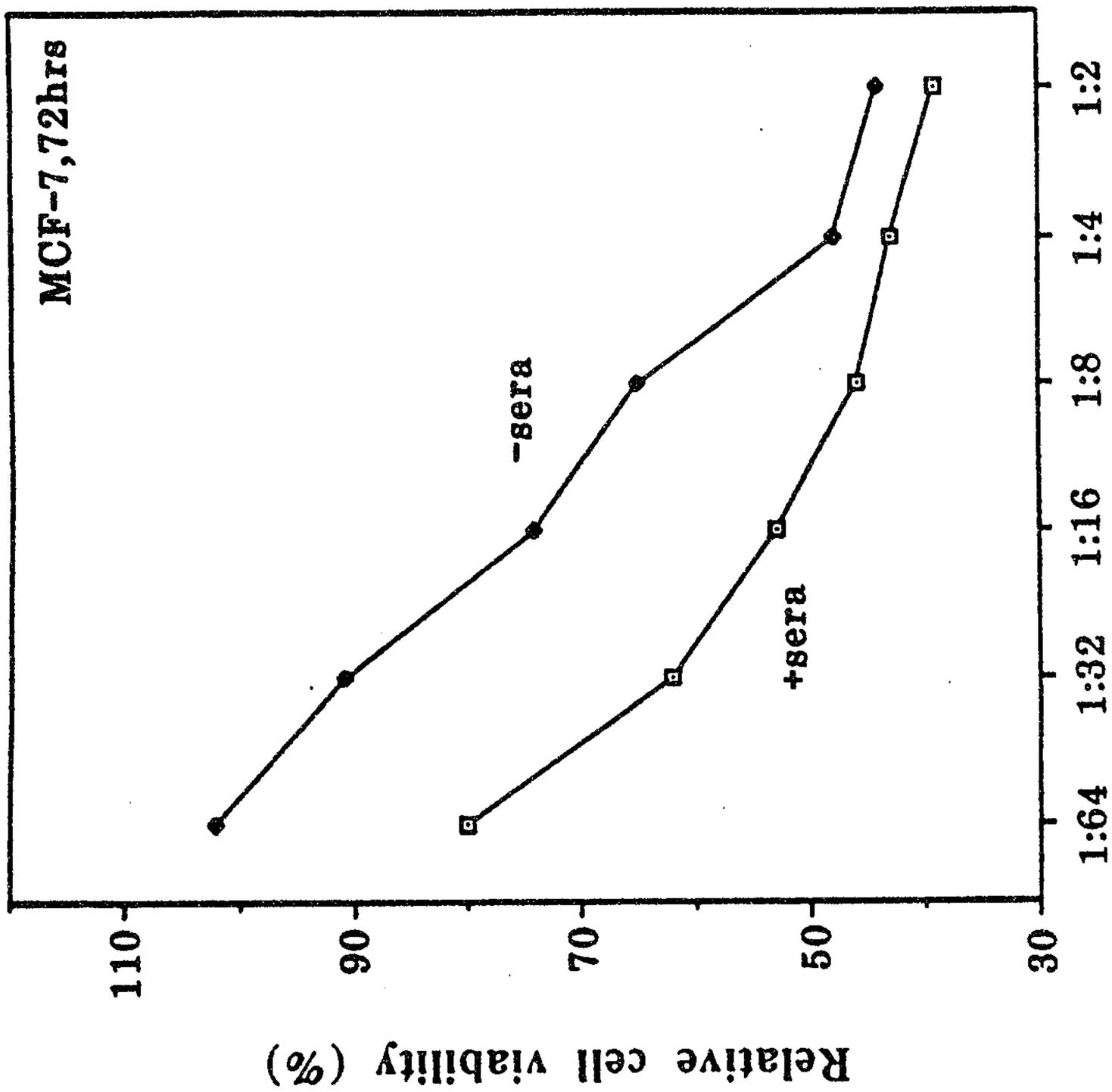

MCF-7,72hrs
-sera
+sera
Relative cell viability (%)
110
90
70
50
30
1:64
1:32
1:16
1:8
1:4
1:2
Oncoinhibin Dilutions

FIG. 3

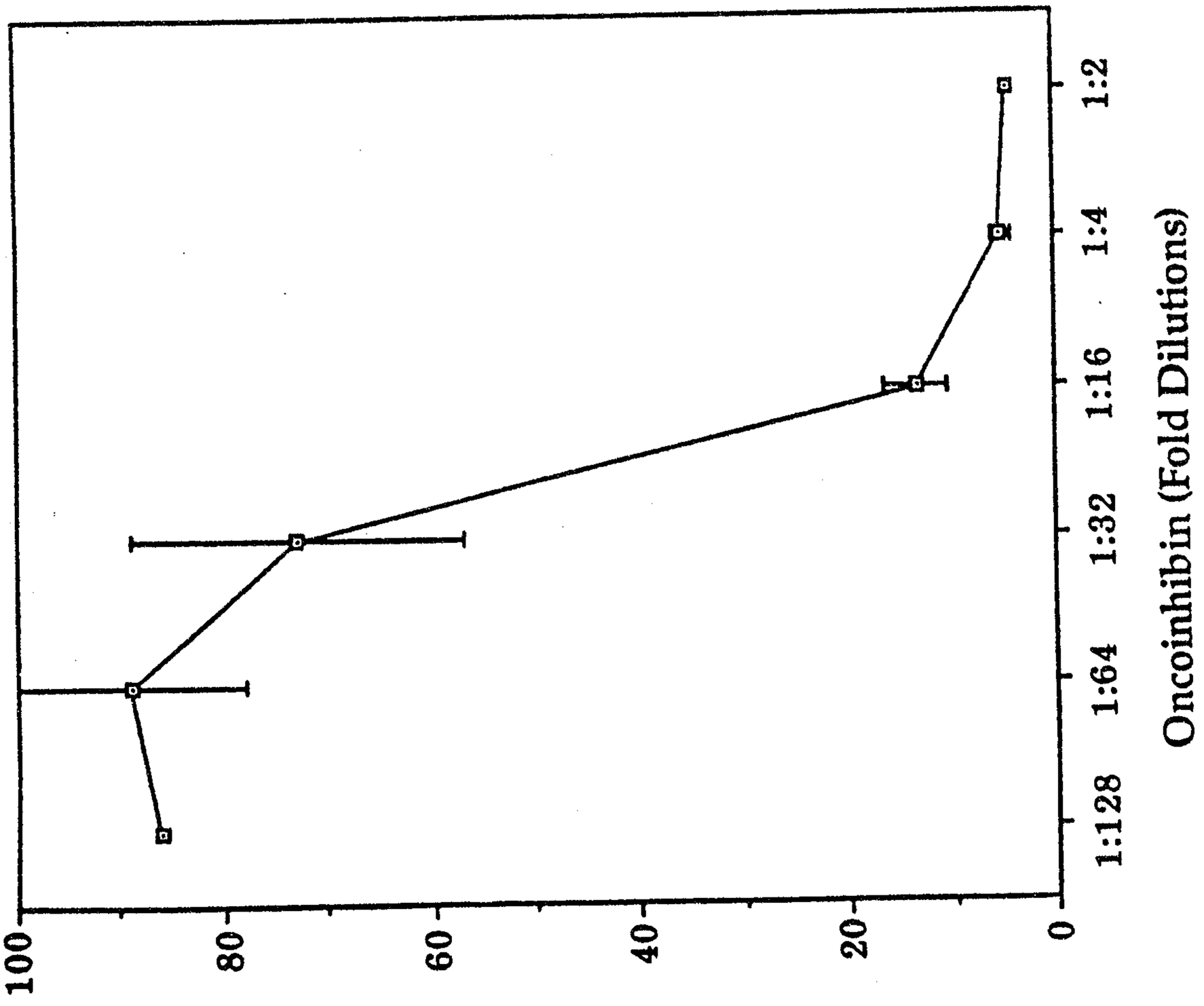

Relative Cell Viability (%)
100
80
60
40
20
0
1:128
1:64
1:32
1:16
1:4
1:2
Oncoinhibin (Fold Dilutions)

FIG. 2

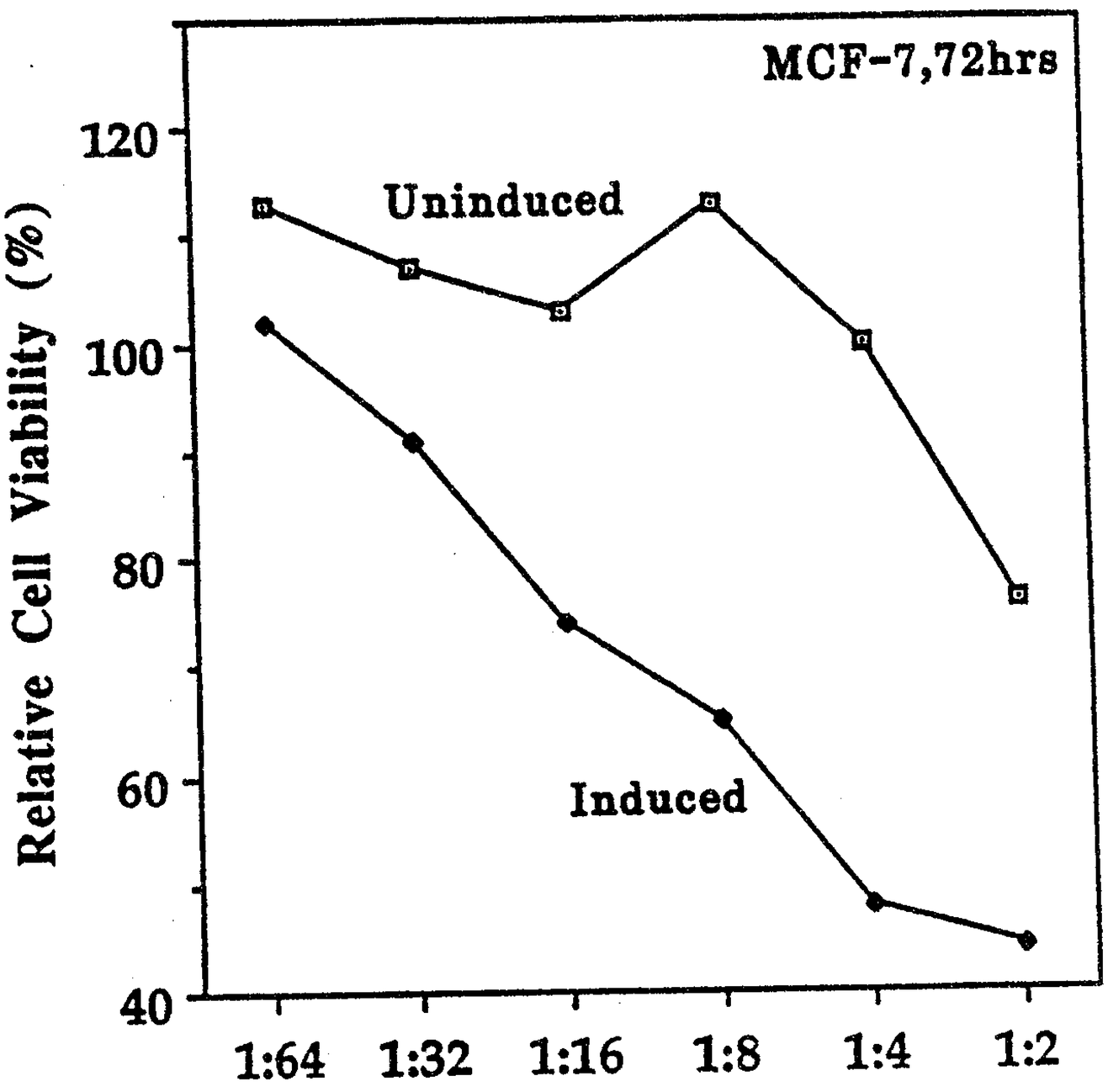

MCF-7,72hrs
Uninduced
Induced
120
100
80
60
40
Relative Cell Viability (%)
1:64
1:32
1:16
1:8
1:4
1:2
Oncoinhibin Dilution

FIG. 4A

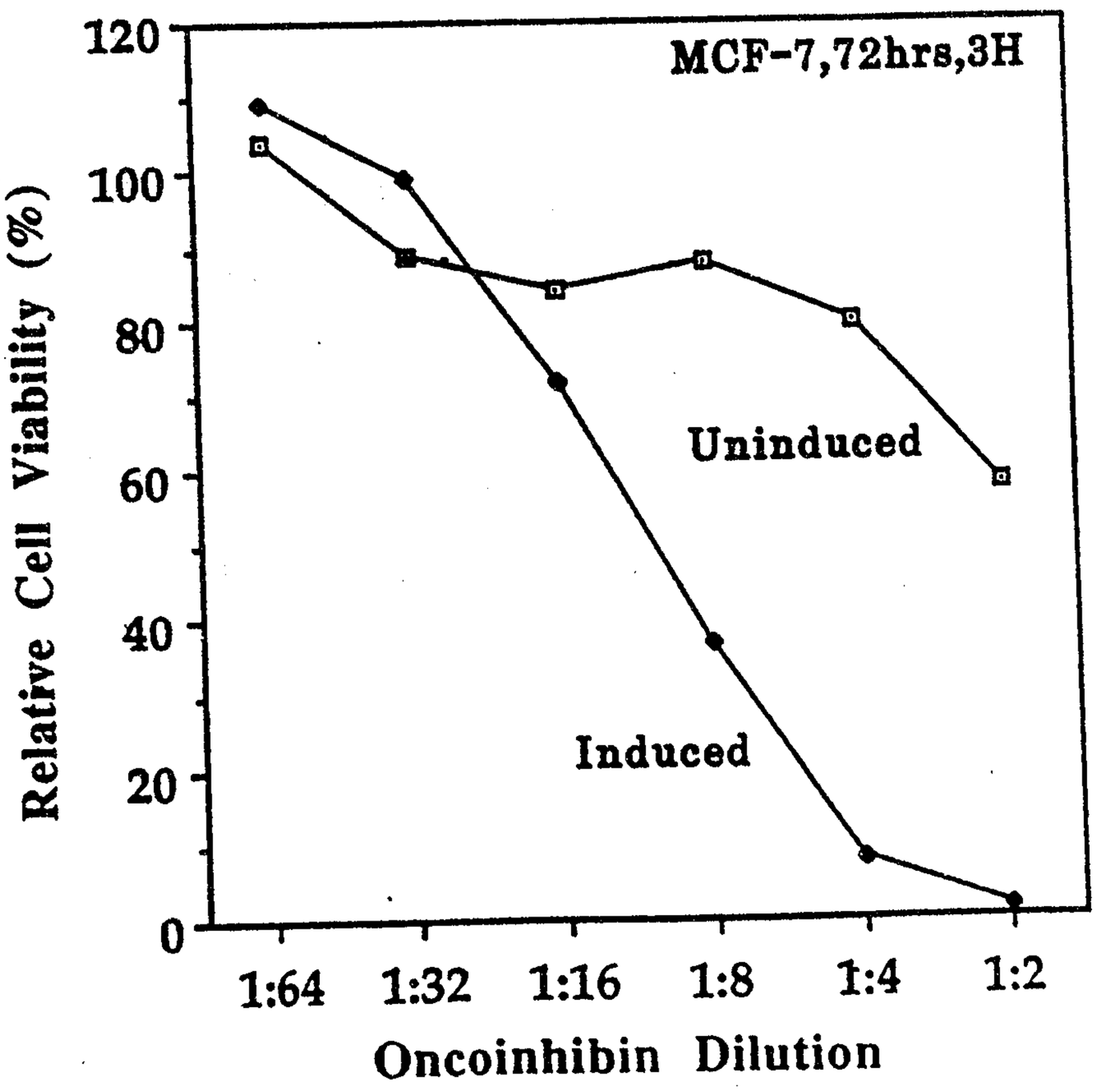

MCF-7,72hrs,3H
Uninduced
Induced
120
100
80
60
40
20
0
Relative Cell Viability (%)
1:64
1:32
1:16
1:8
1:4
1:2
Oncoinhibin Dilution

FIG. 4B

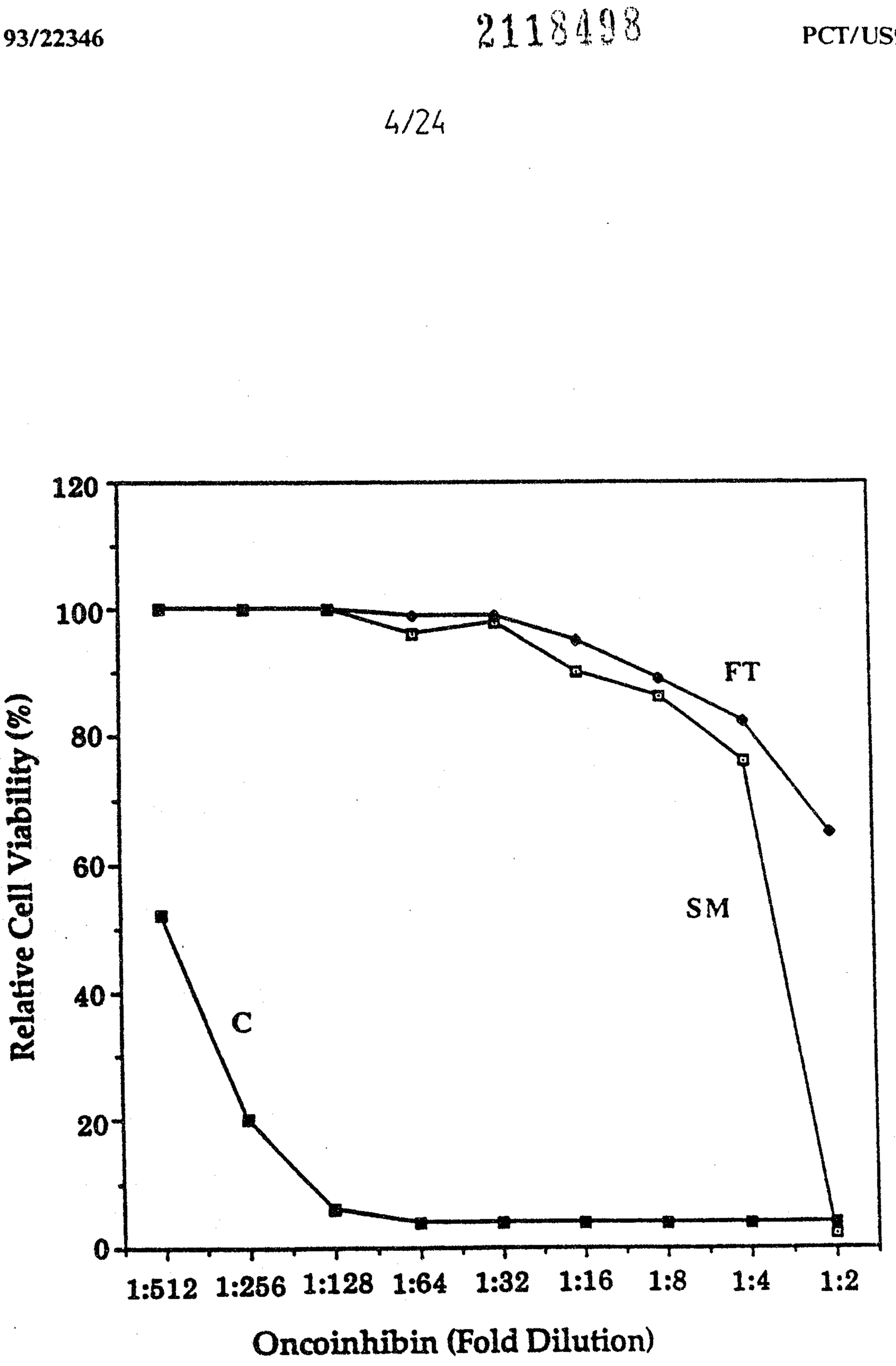

120
100
80
60
40
20
0
Relative Cell Viability (%)
FT
SM
C
1:512
1:256
1:128
1:64
1:32
1:16
1:8
1:4
1:2
Oncoinhibin (Fold Dilution)

FIG. 5

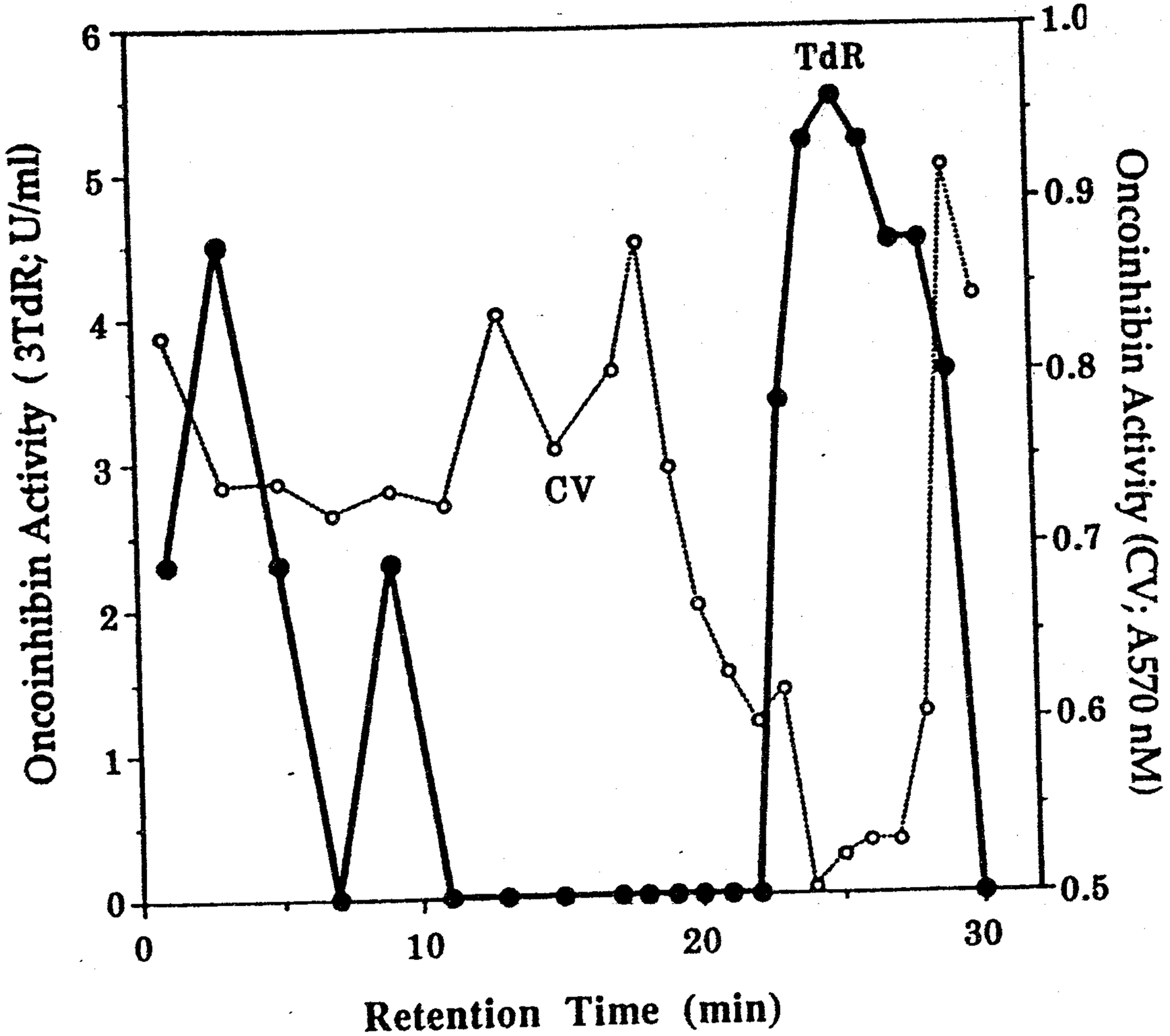

Oncoinhibin Activity (3TdR; U/ml)
Oncoinhibin Activity (CV; A570 nM)
TdR
CV
0
1
2
3
4
5
6
0.5
0.6
0.7
0.8
0.9
1.0
0
10
20
30
Retention Time (min)

FIG. 6A

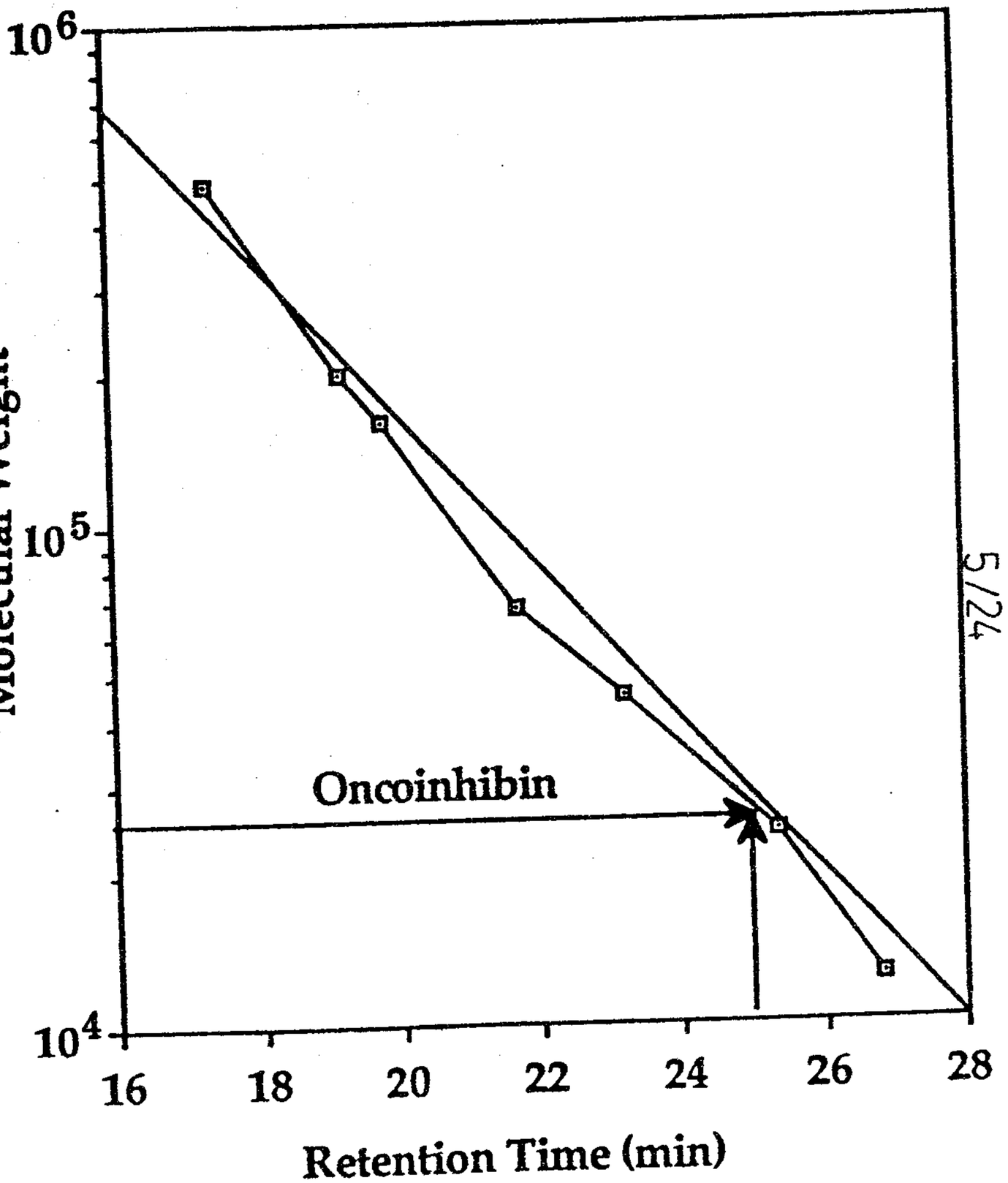

Molecular Weight
10^6
10^5
10^4
Oncoinhibin
16
18
20
22
24
26
28
Retention Time (min)

FIG. 6B

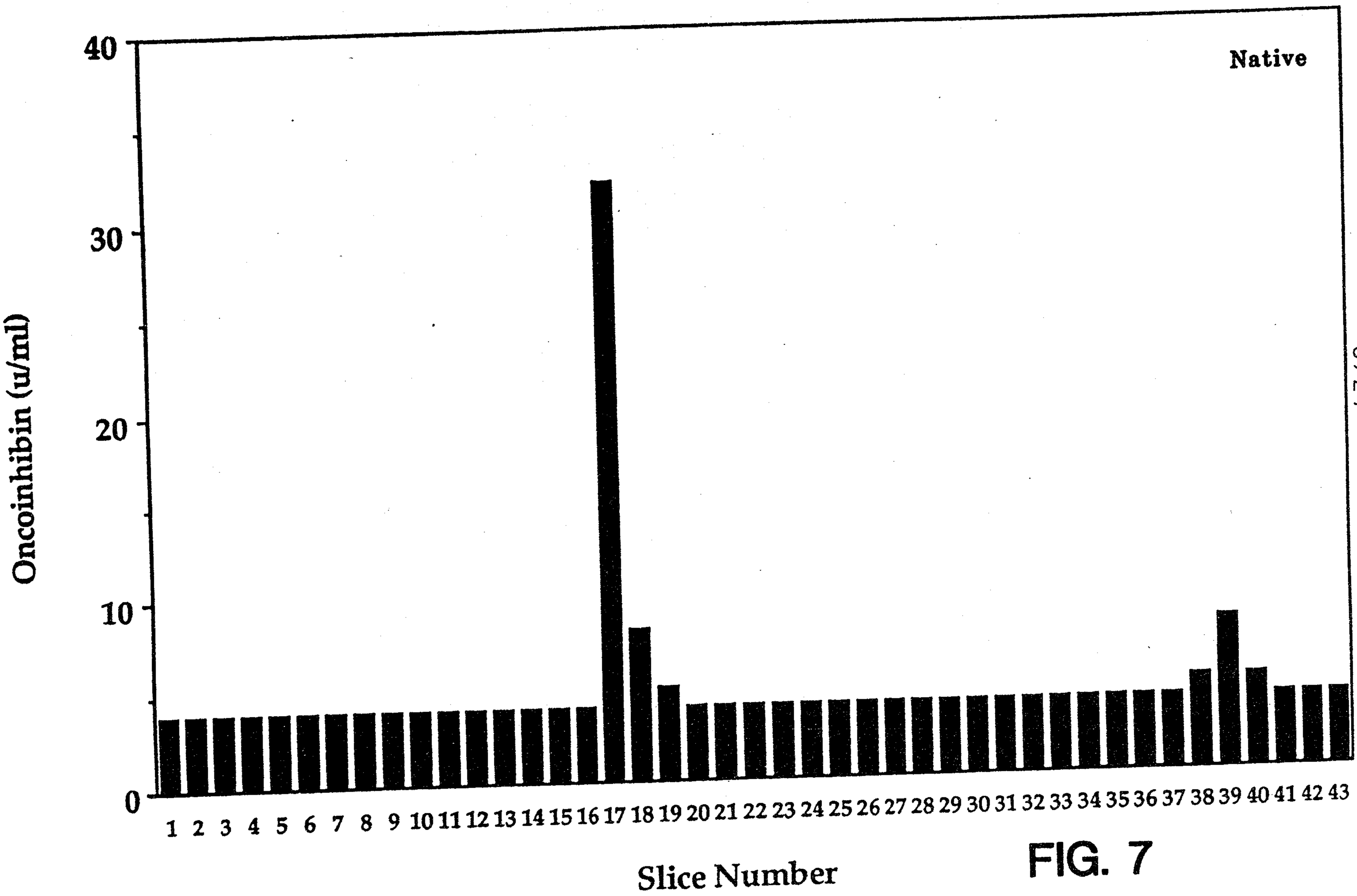
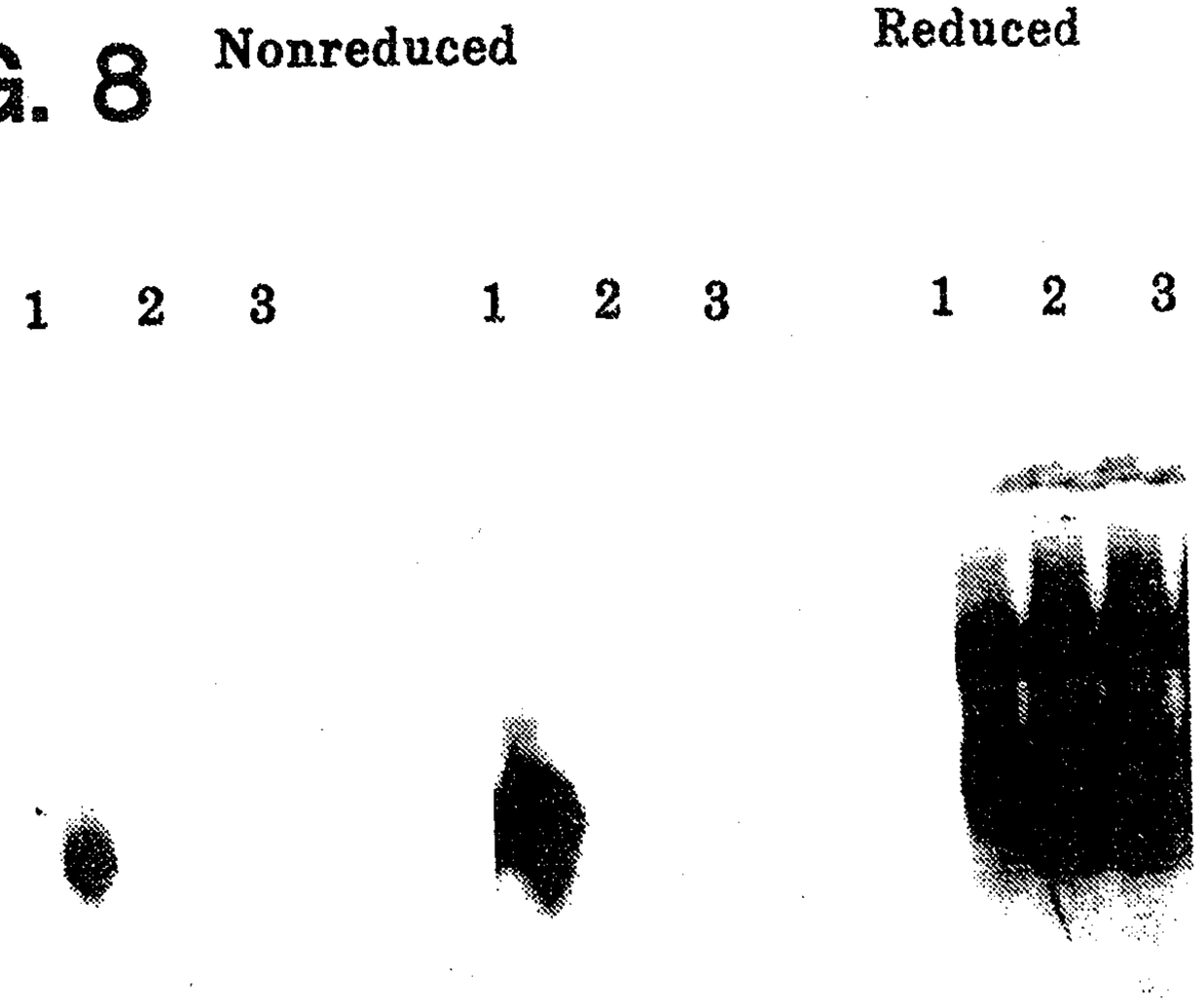

Native
Oncoinhibin (u/ml)
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43
Slice Number

FIG. 7


MW SM 1 2 3 3 2 1 SM
97.4K
66.2K
45.0K
31.0K
21.5K
14.4K

FIG. 8


Nonreduced
Reduced
1 2 3 1 2 3 1 2 3

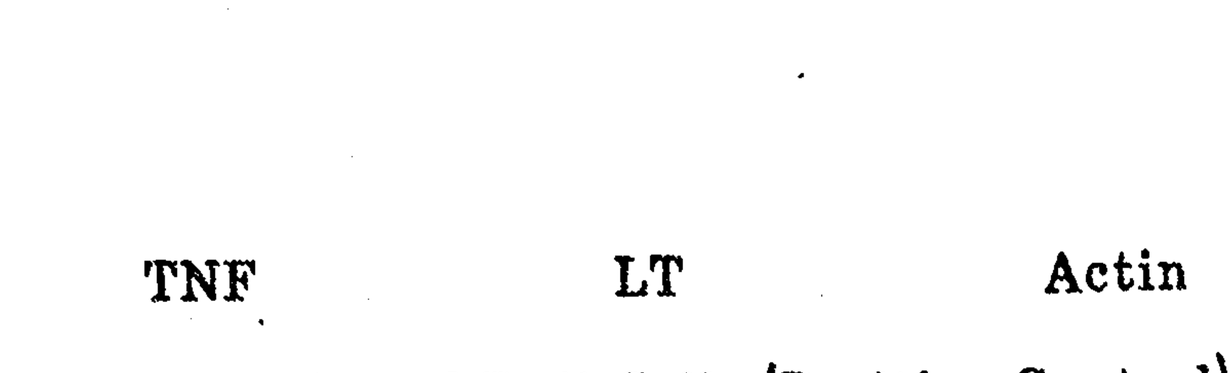

TNF
LT
Actin
1. Tat transfected Raji Cells (Positive Control)
2. PMA induced (24h) K-562 cells
3. Untreated K-562 cells

FIG. 13

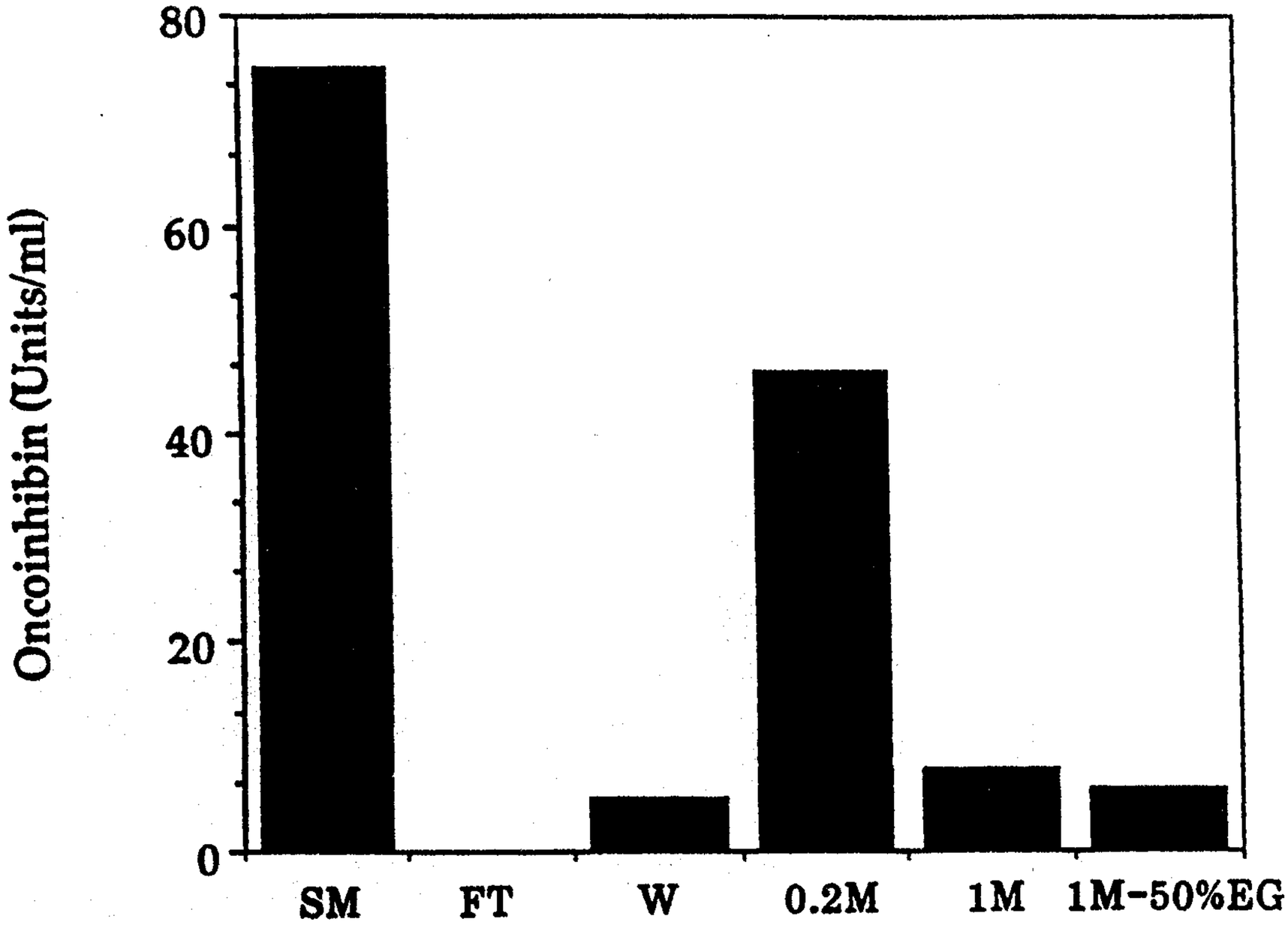

Oncoinhibin (Units/ml)
80
60
40
20
0
SM
FT
W
0.2M
1M
1M-50%EG
ELUTIONS
FRACTIONS

FIG. 9A

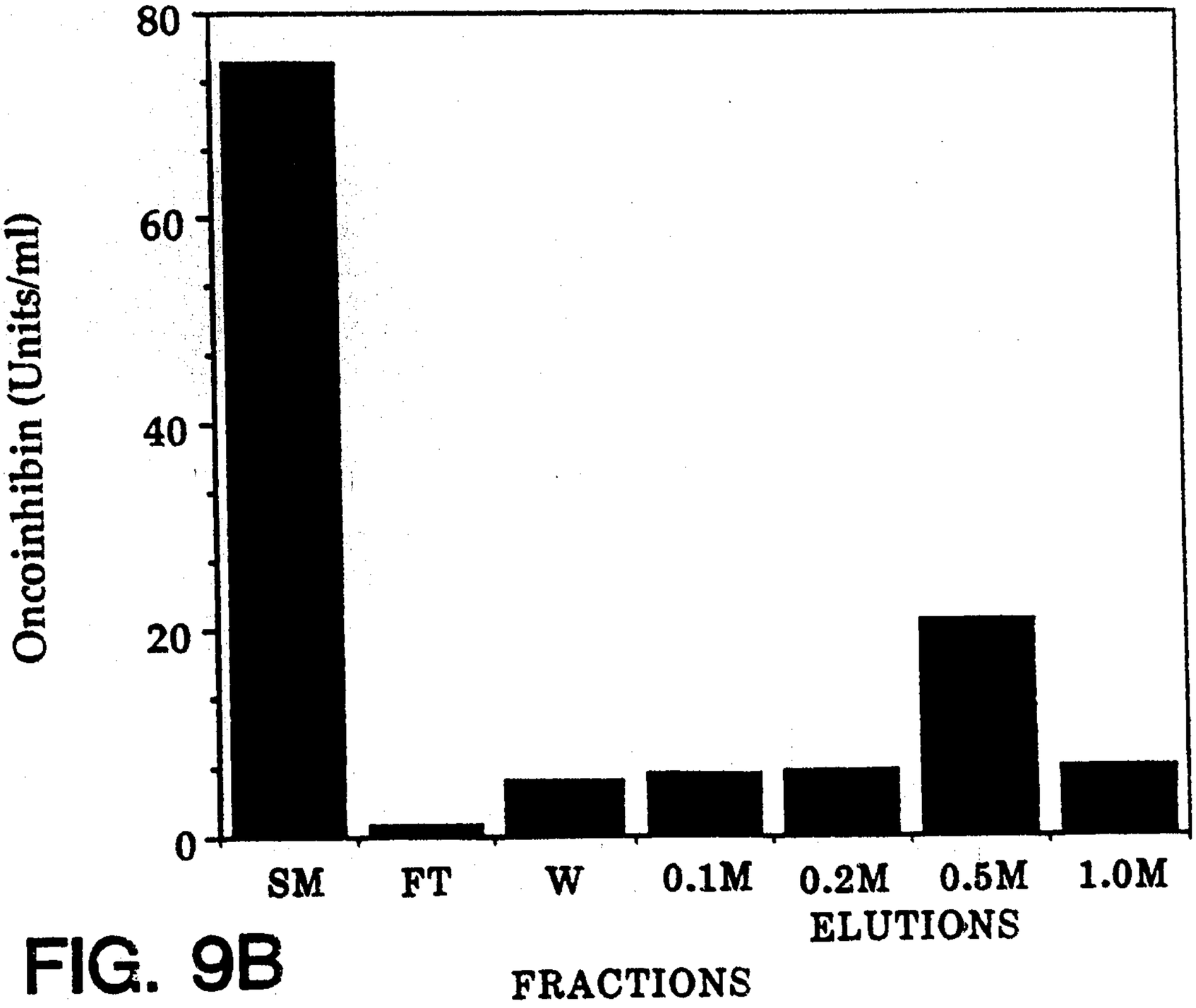

Oncoinhibin (Units/ml)
80
60
40
20
0
SM
FT
W
0.1M
0.2M
0.5M
1.0M
ELUTIONS
FRACTIONS

FIG. 9B

FIG. 10A

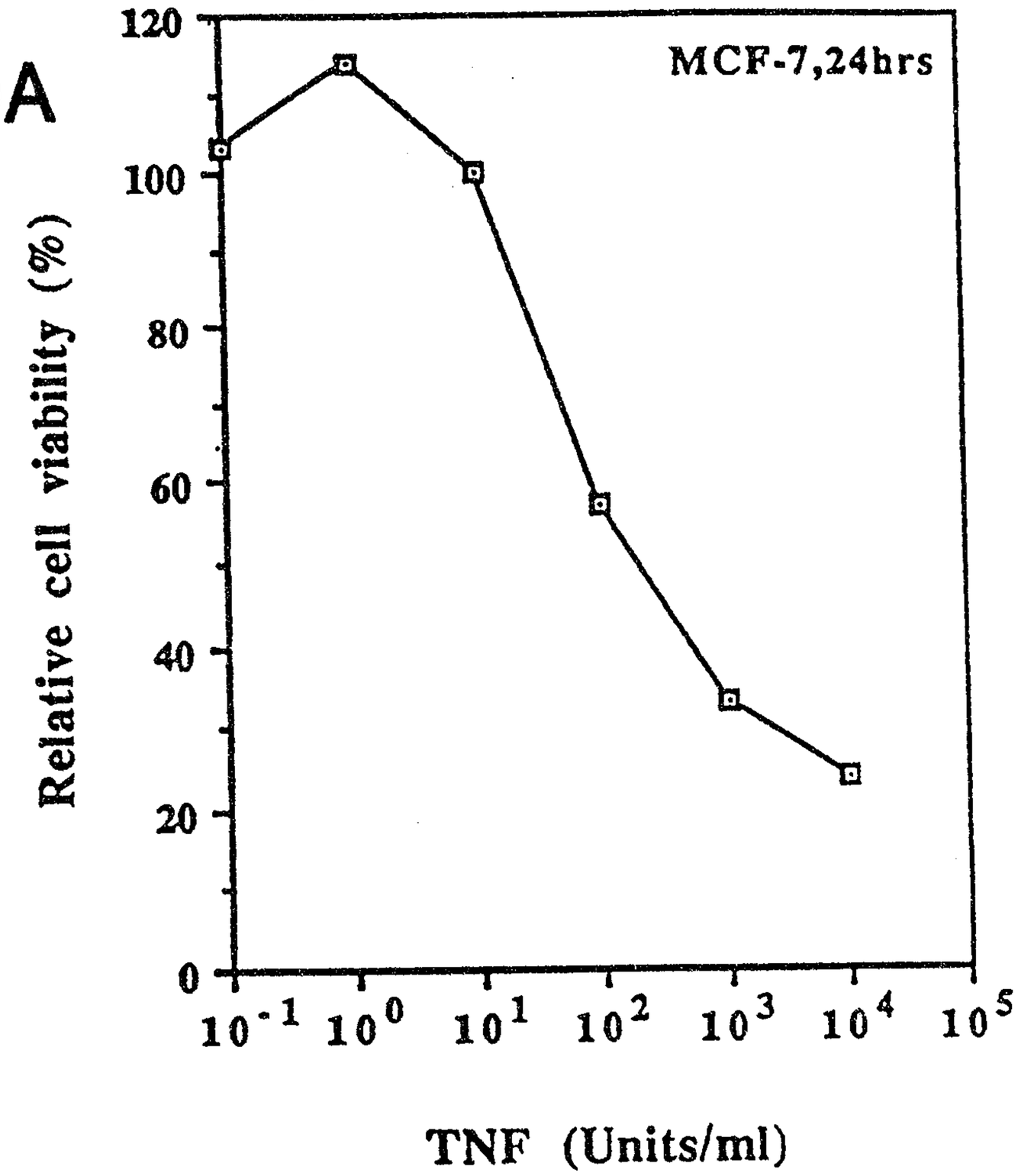


FIG. 10B

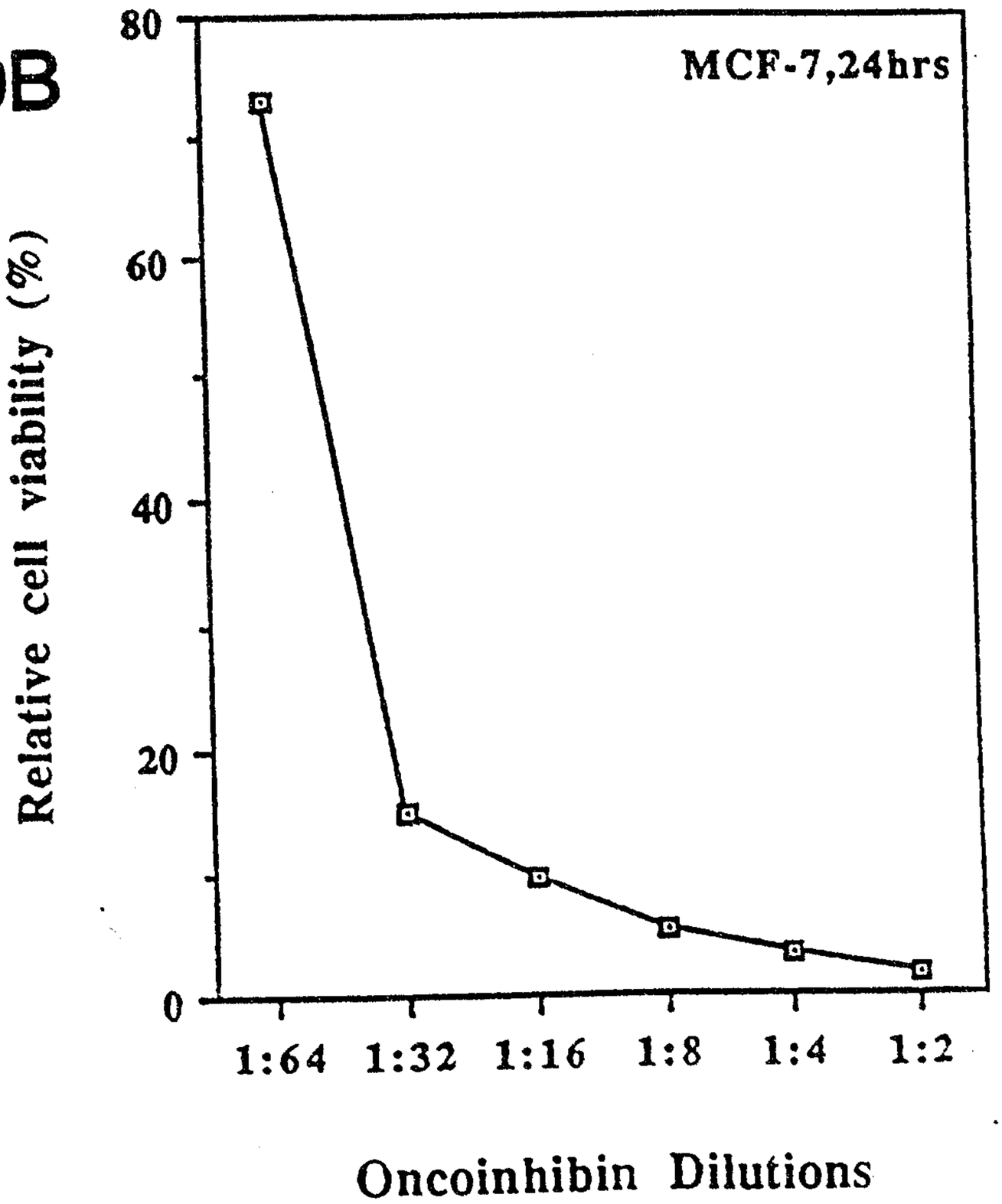


FIG. 10C

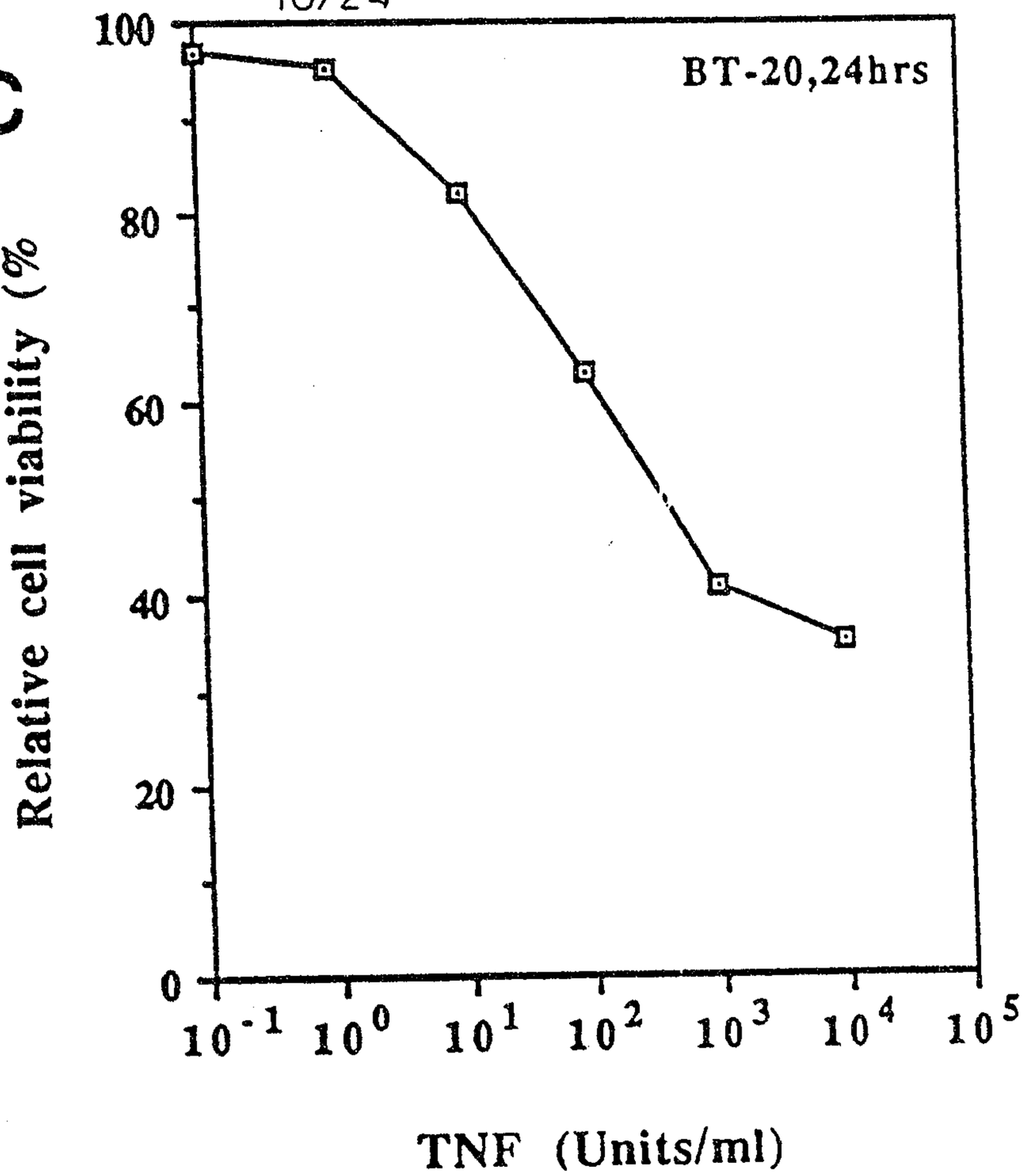


FIG. 10D

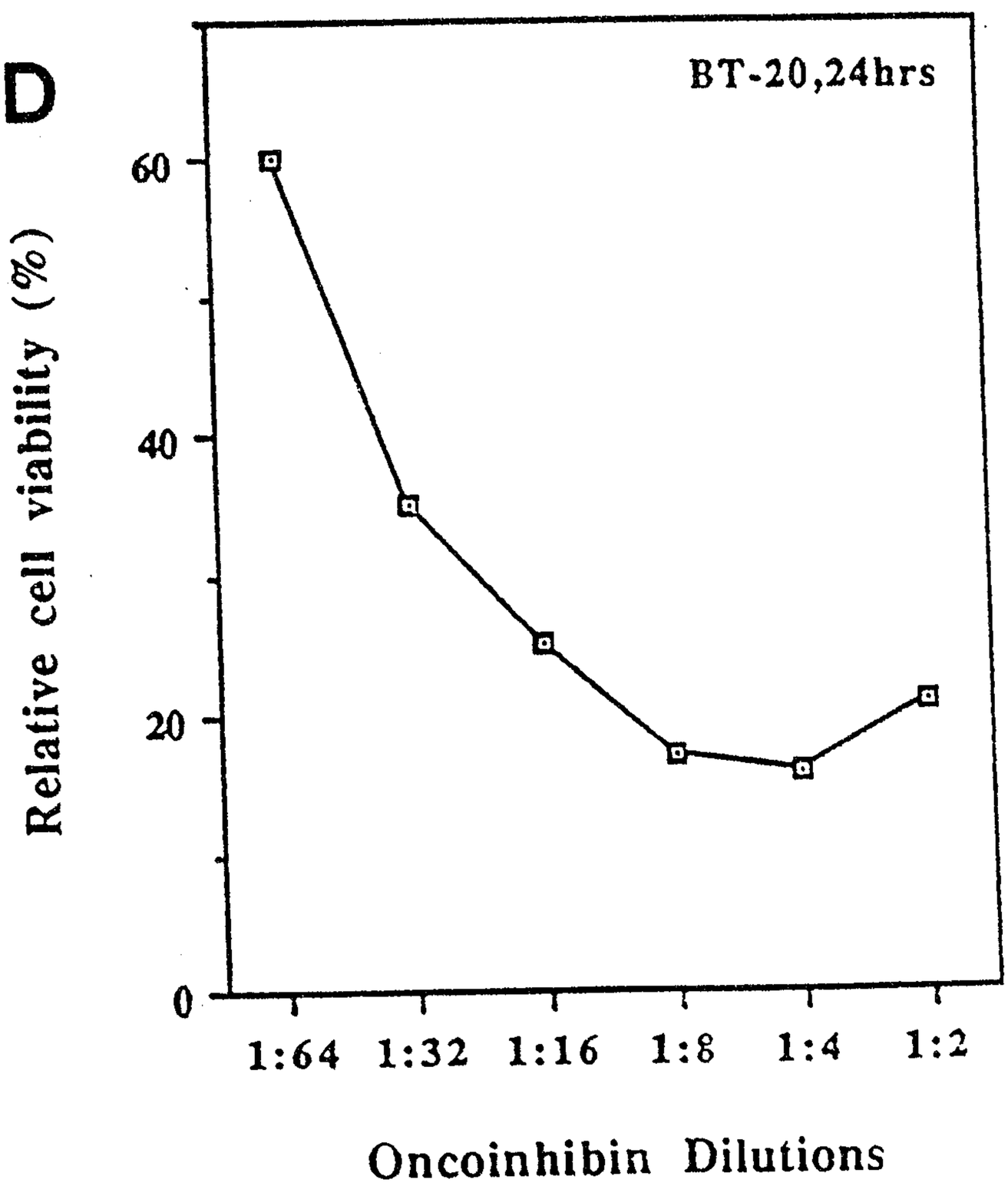


FIG. 10E

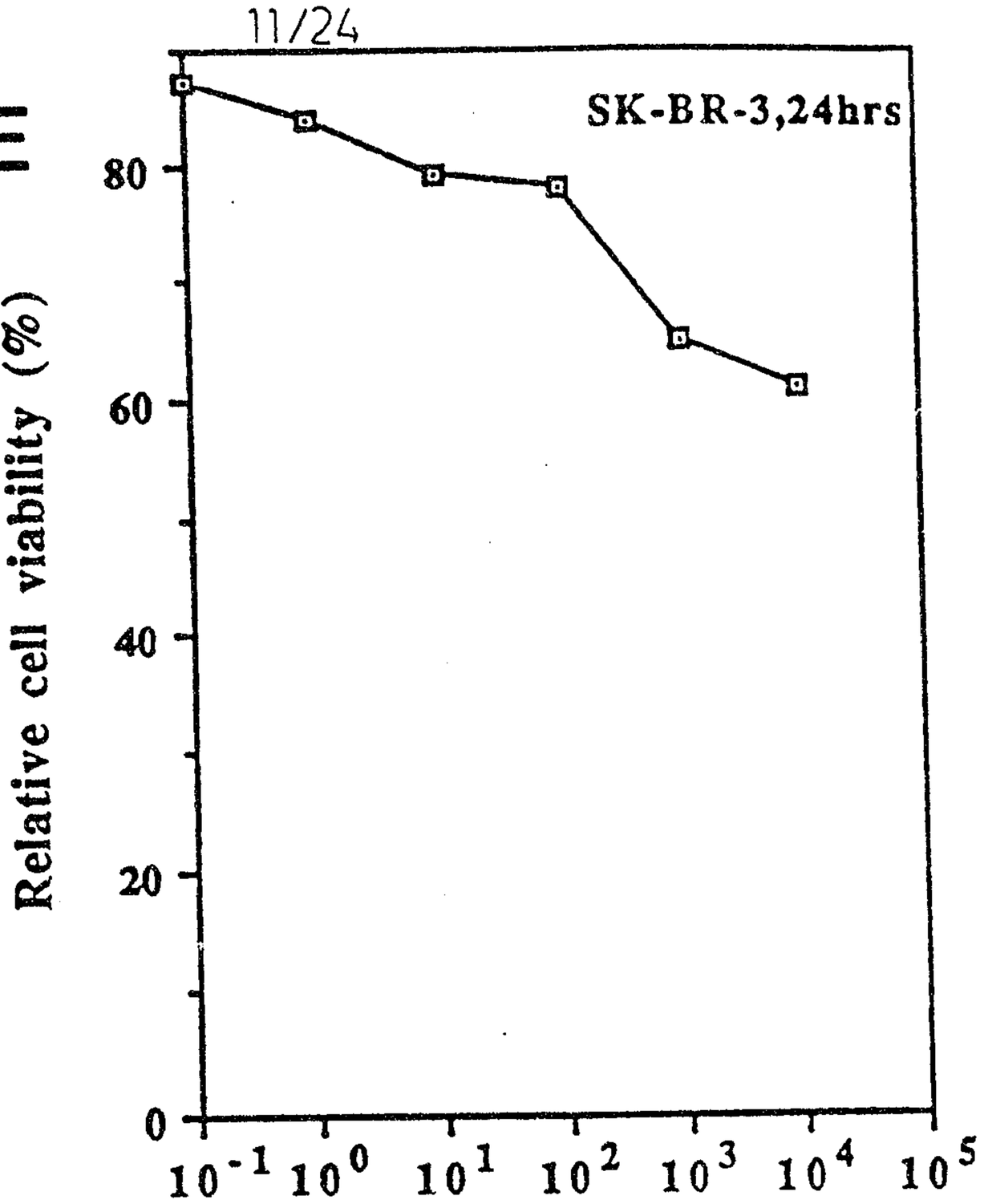


FIG. 10F

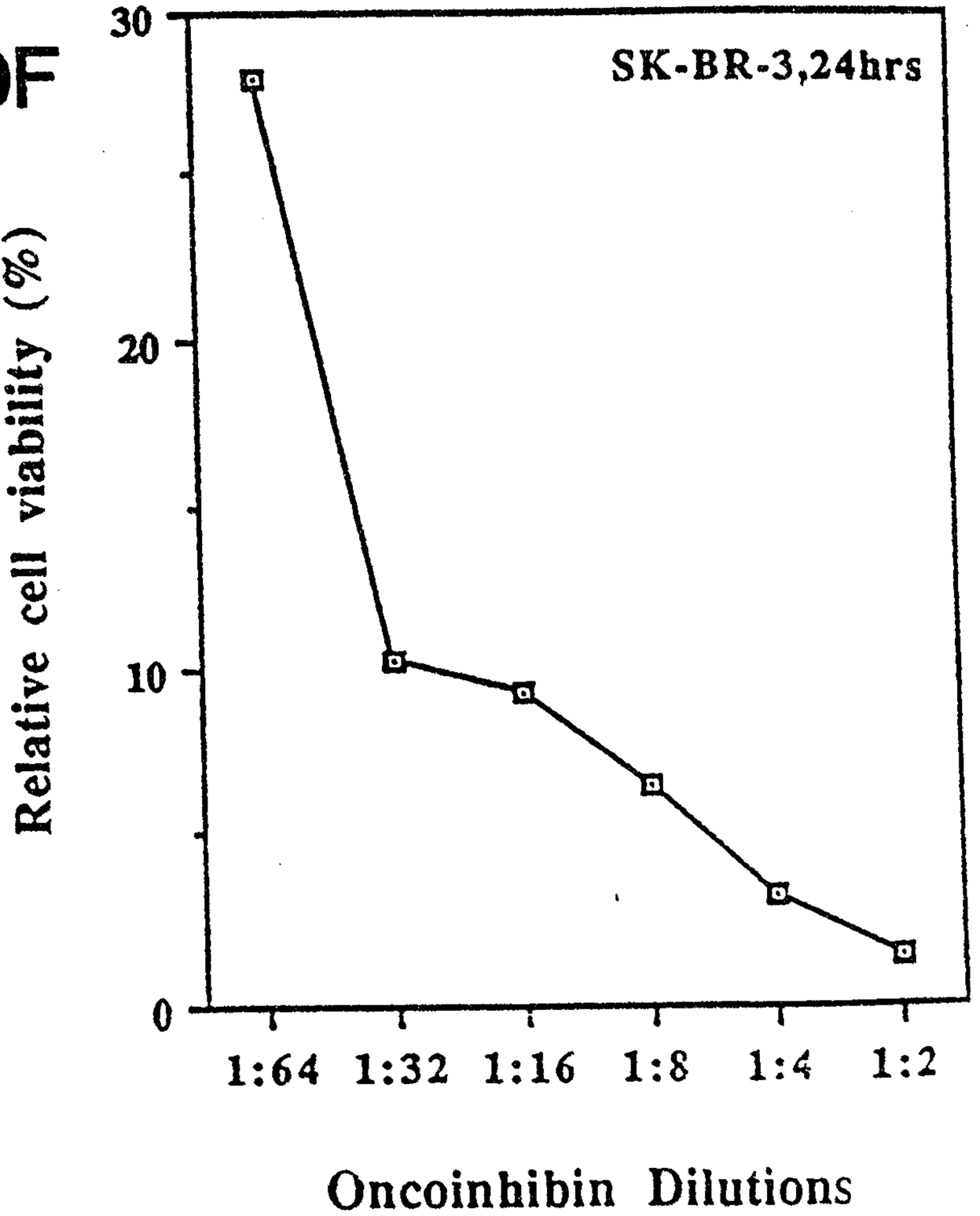


FIG. 10G

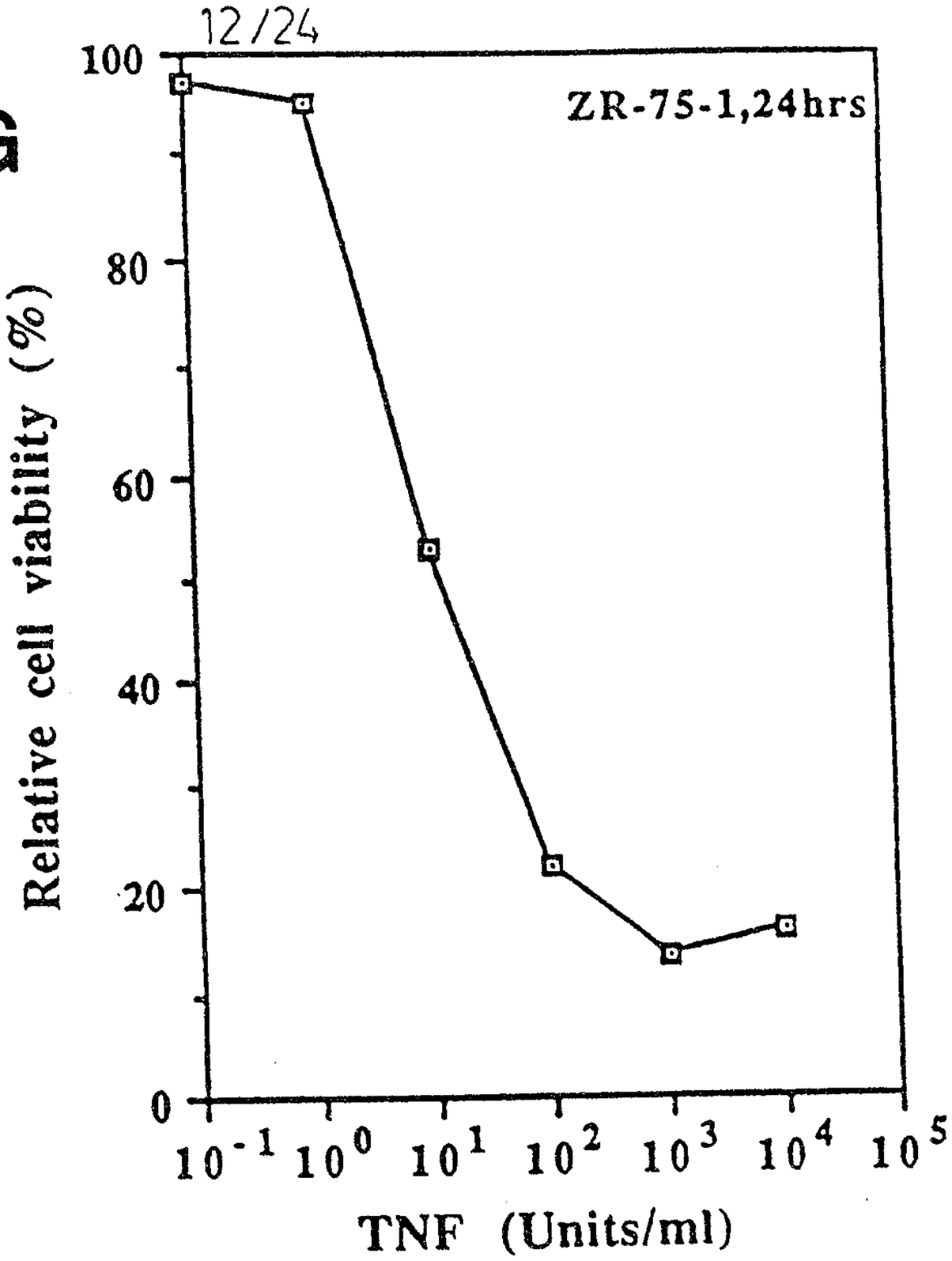


FIG. 10H

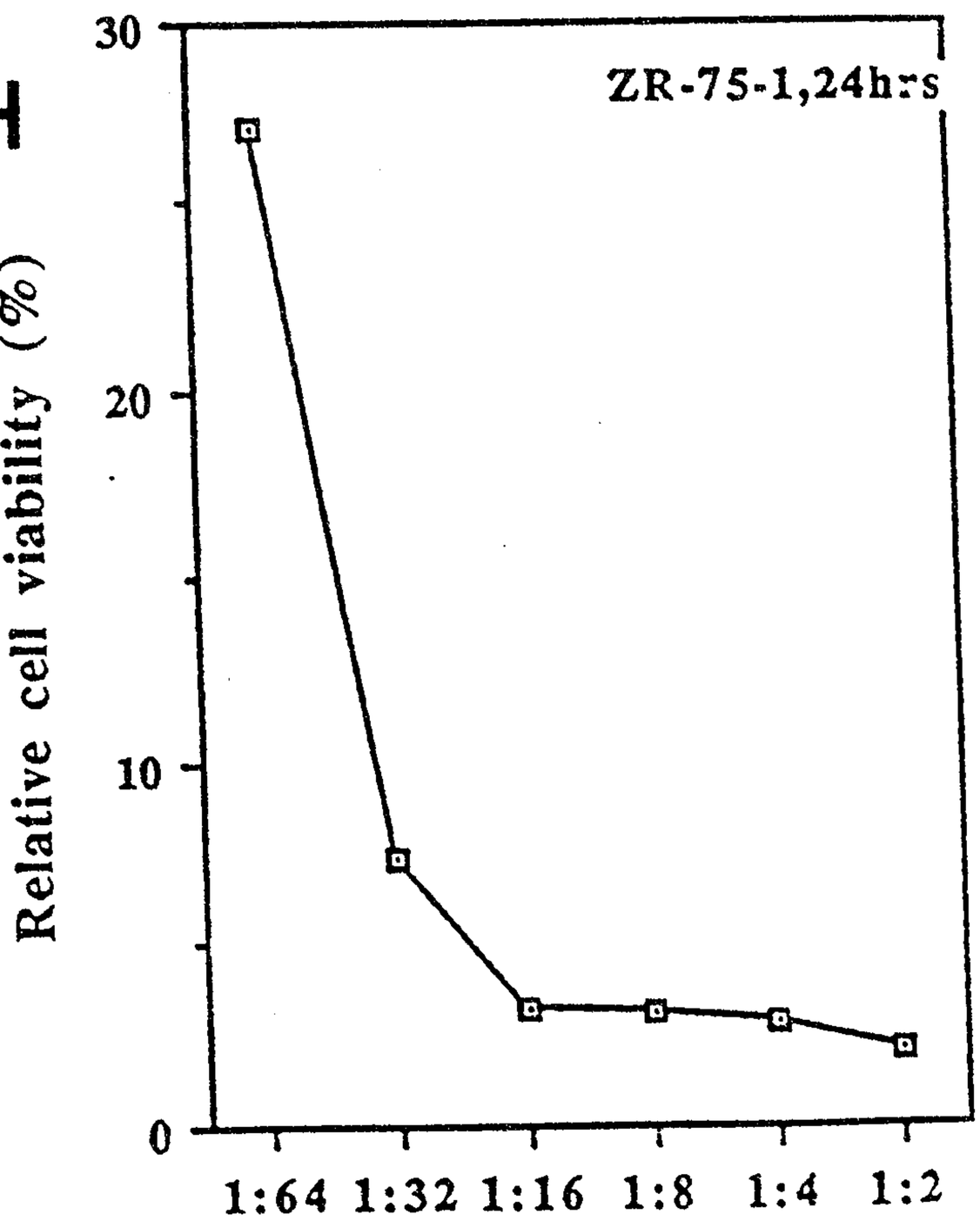


FIG. 10I

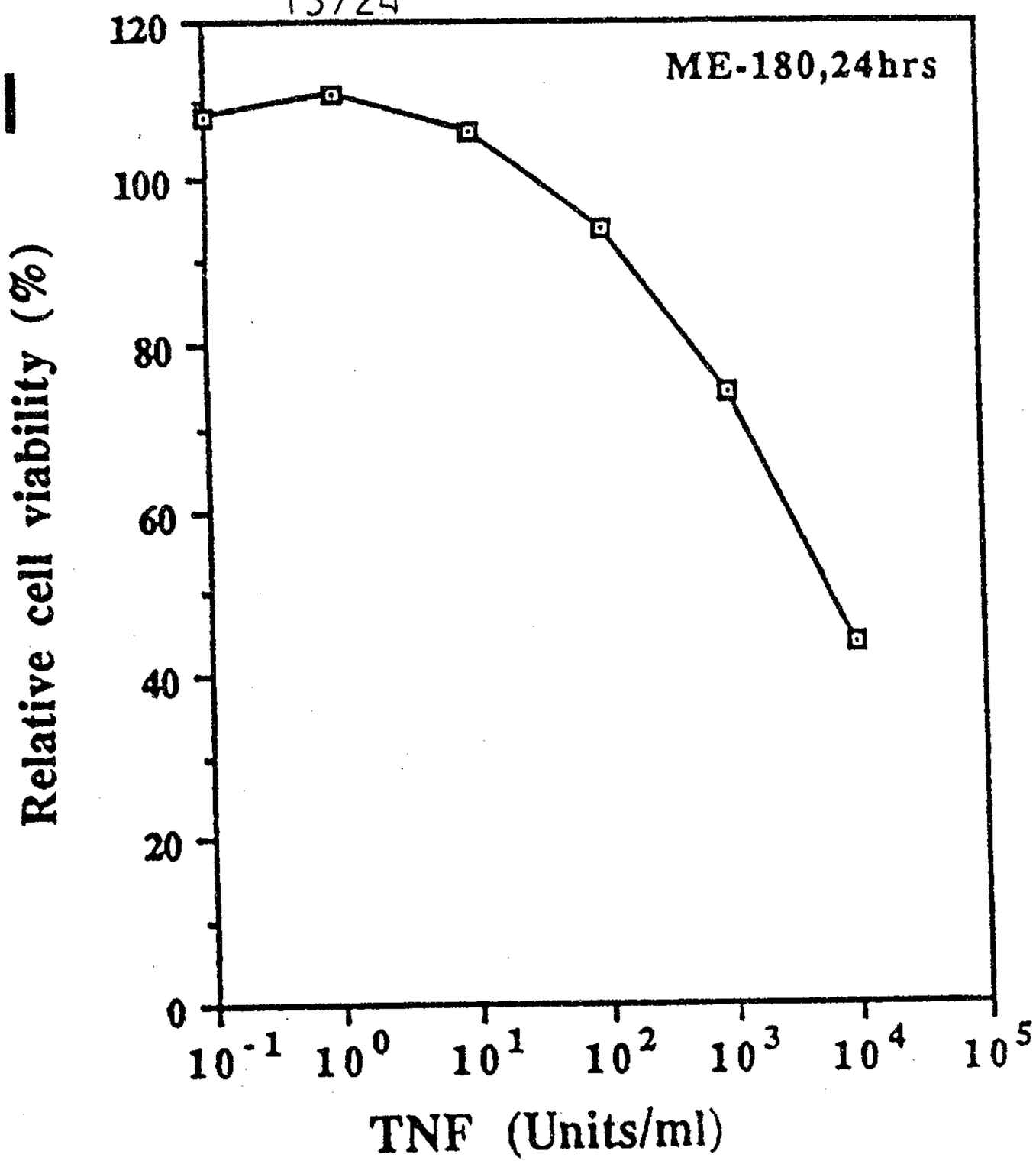


FIG. 10J

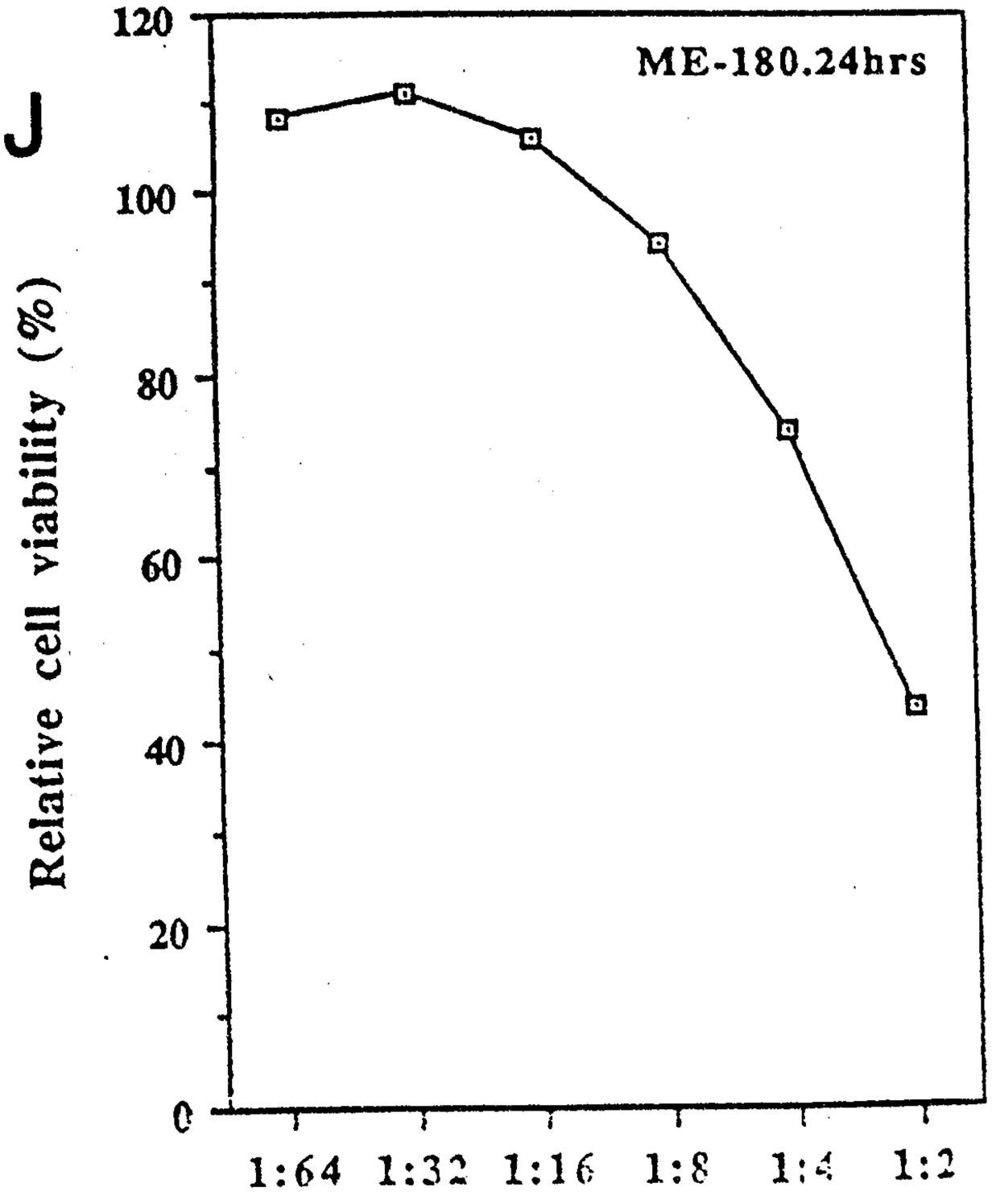


FIG. 10K

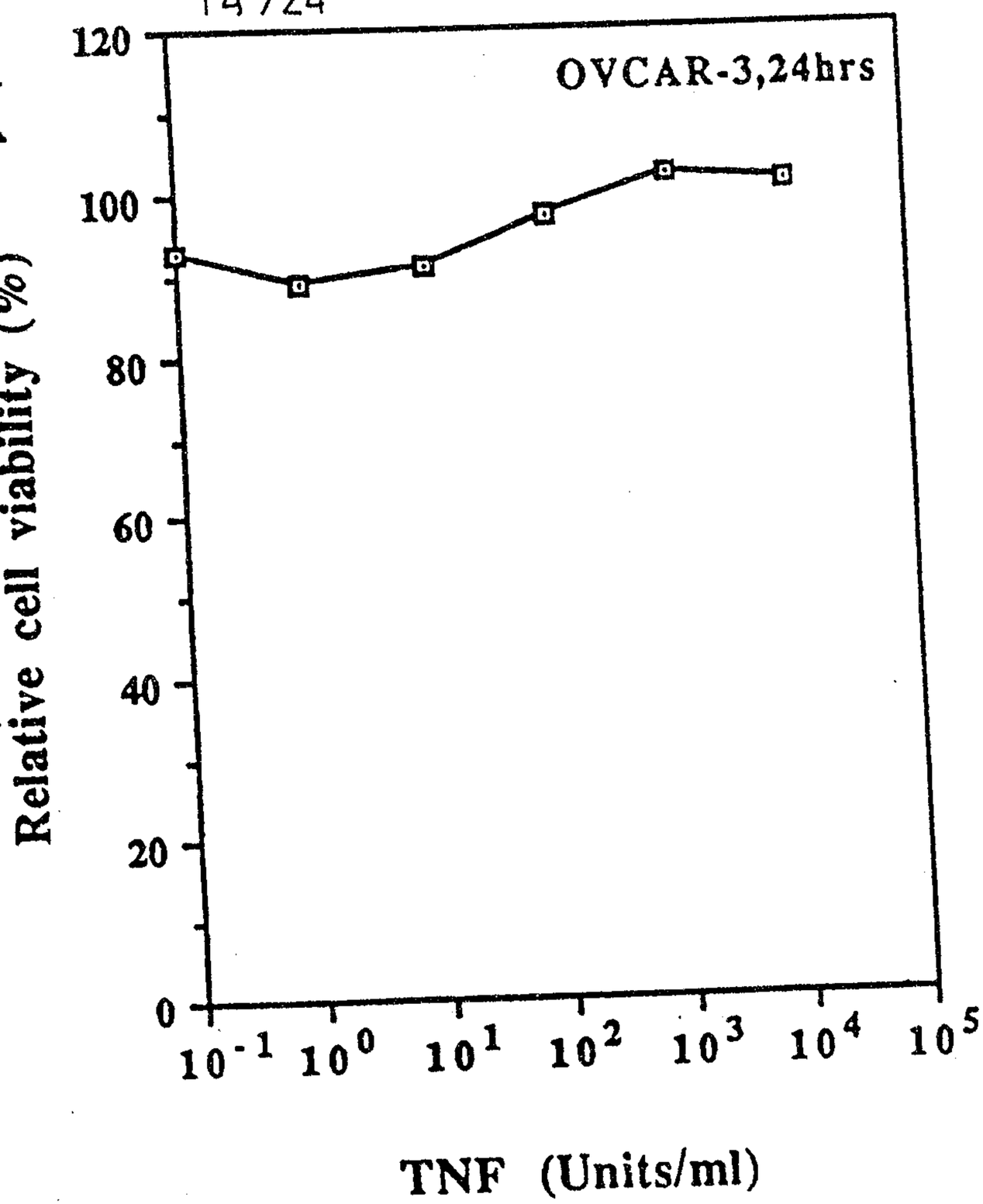


FIG. 10L

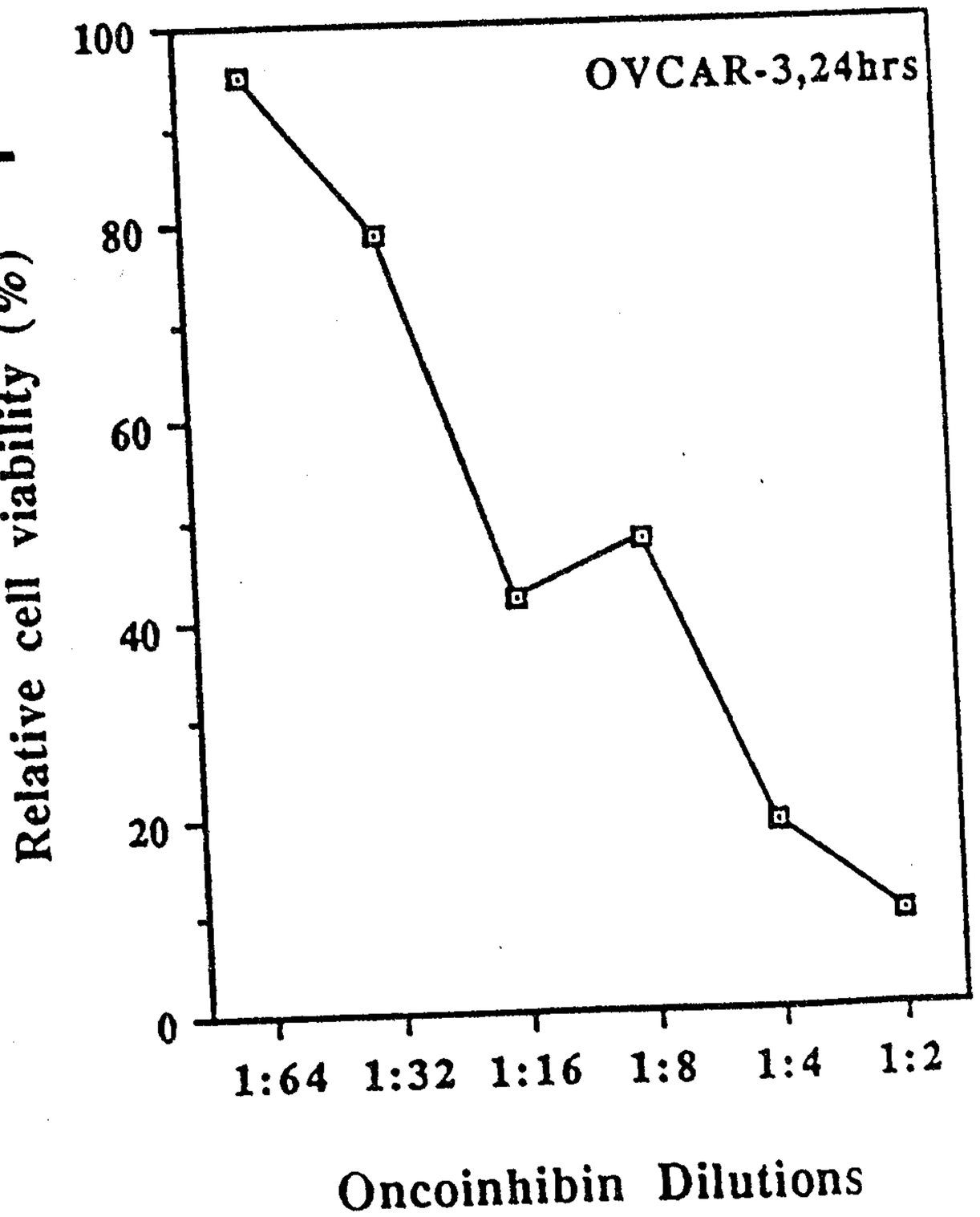


FIG. 10M

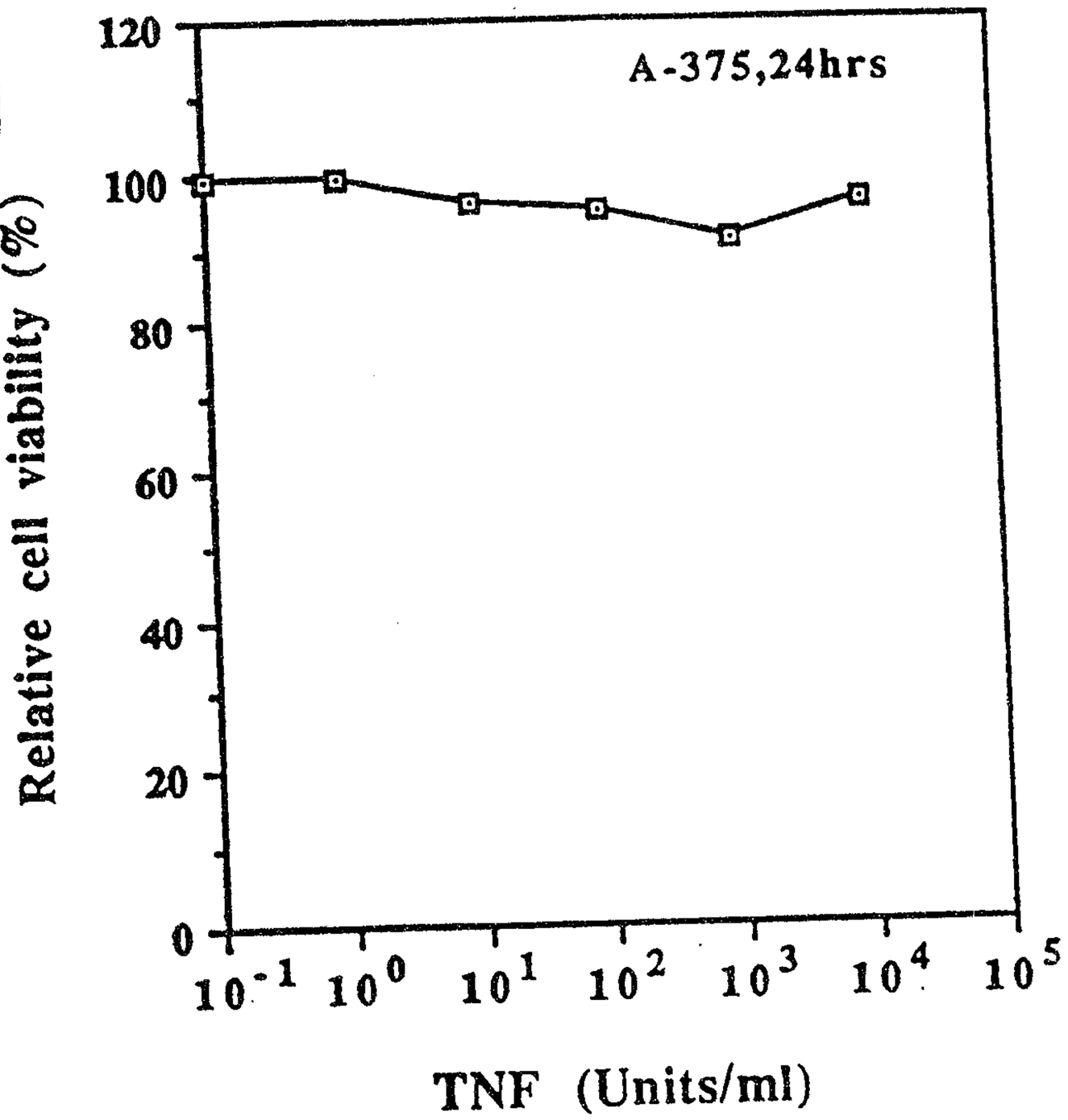


FIG. 10N

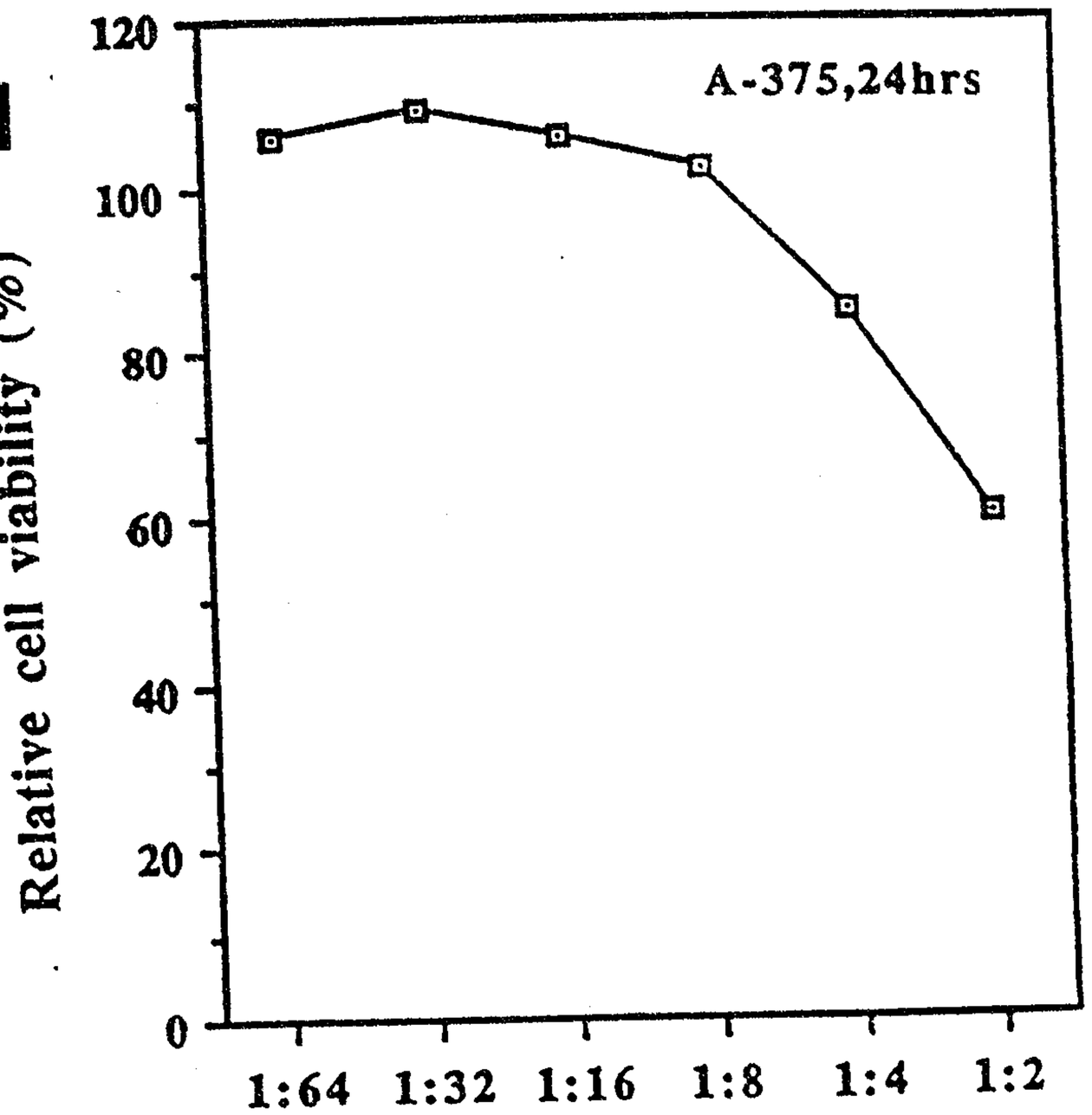


FIG. 10O

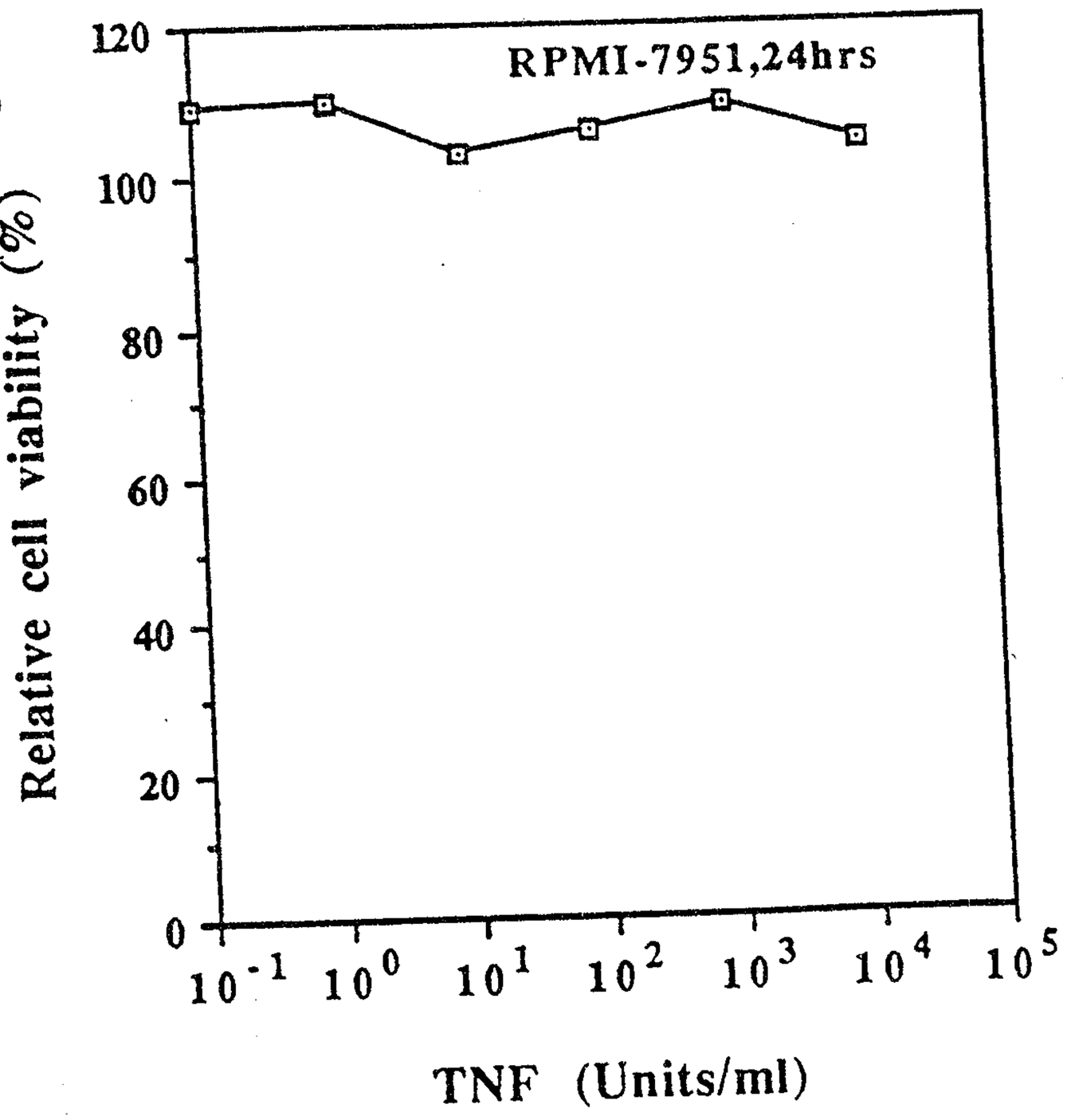
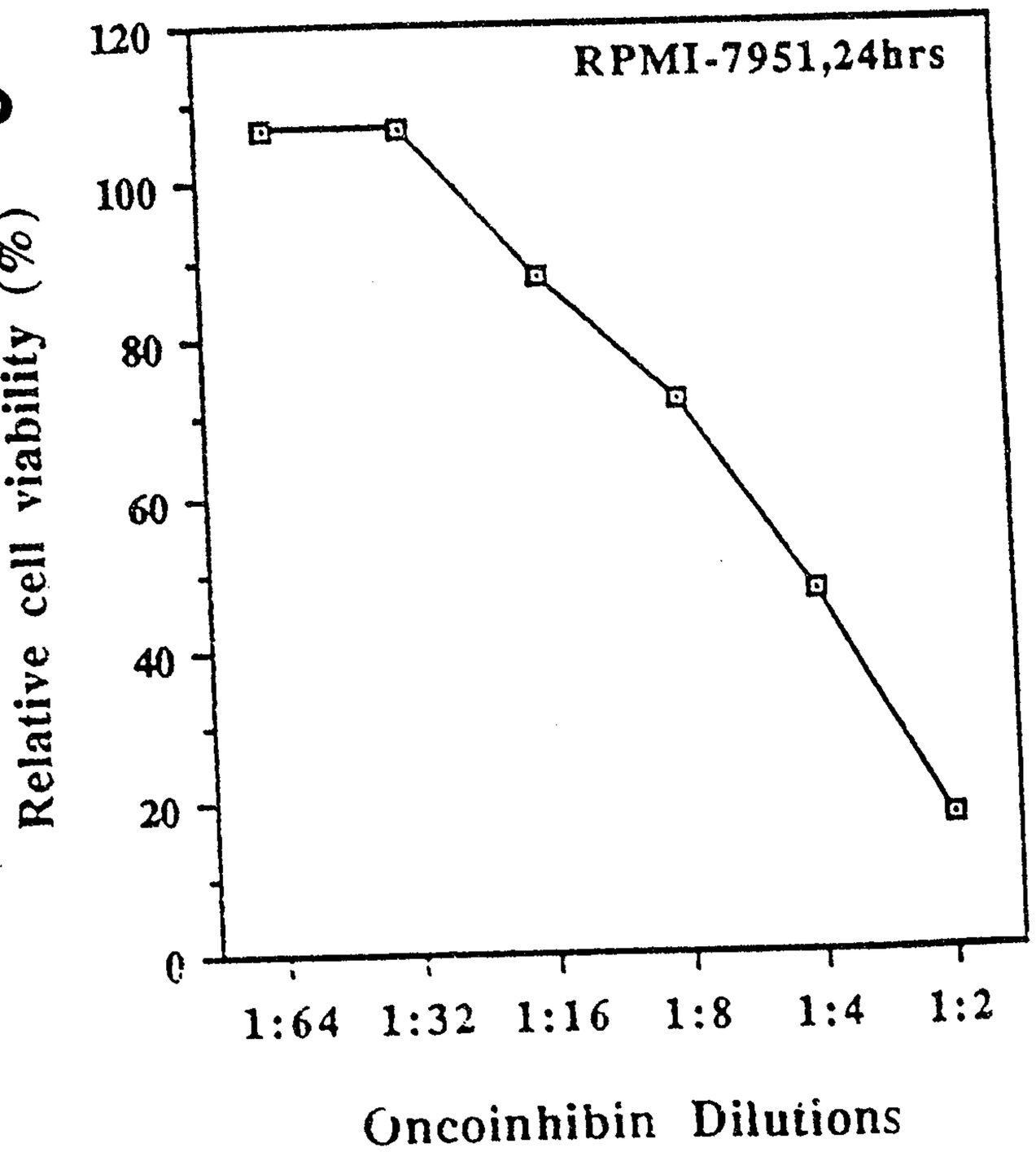


FIG. 10P



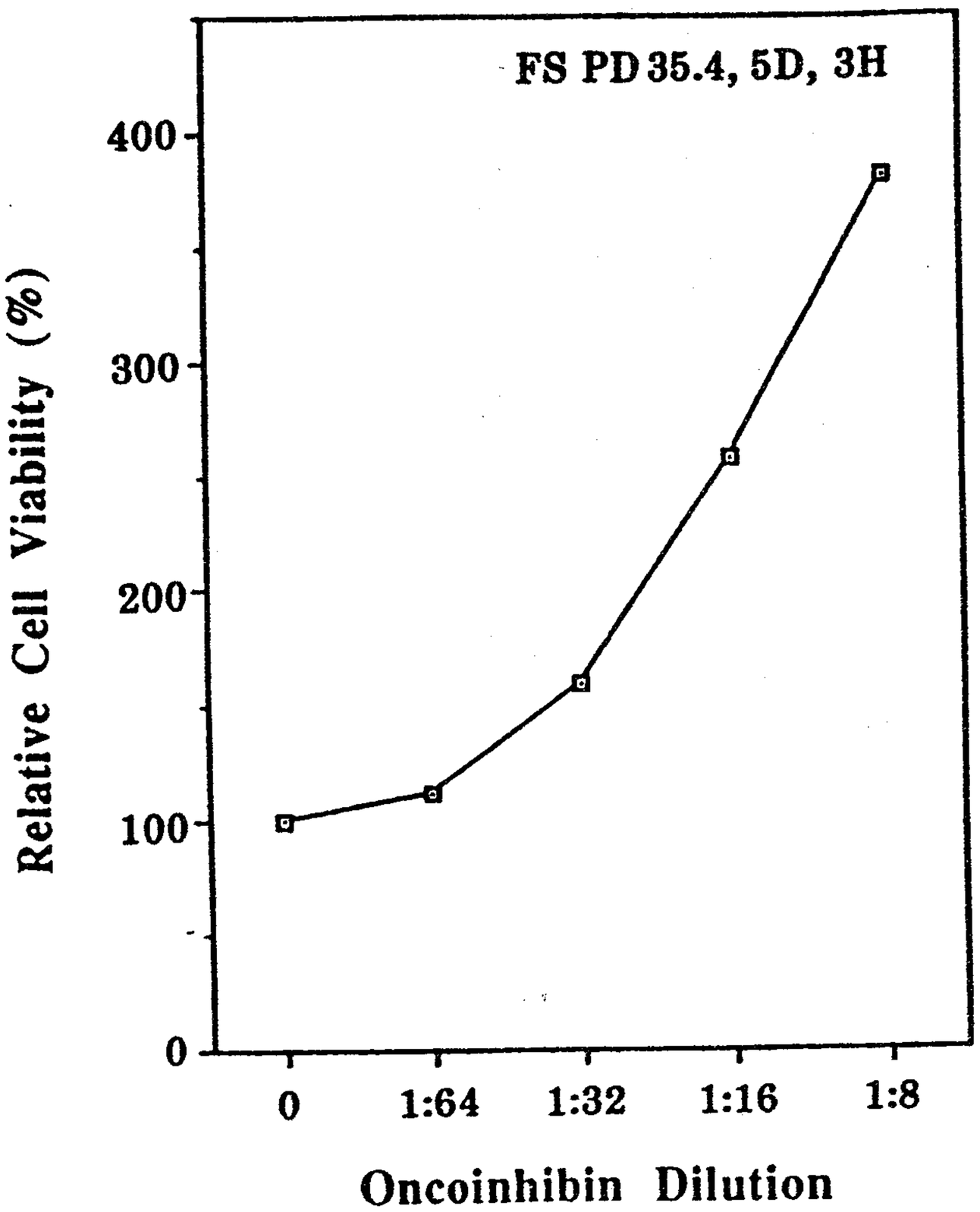

FS PD 35.4, 5D, 3H
Relative Cell Viability (%)
400
300
200
100
0
0
1:64
1:32
1:16
1:8
Oncoinhibin Dilution

FIG. 11

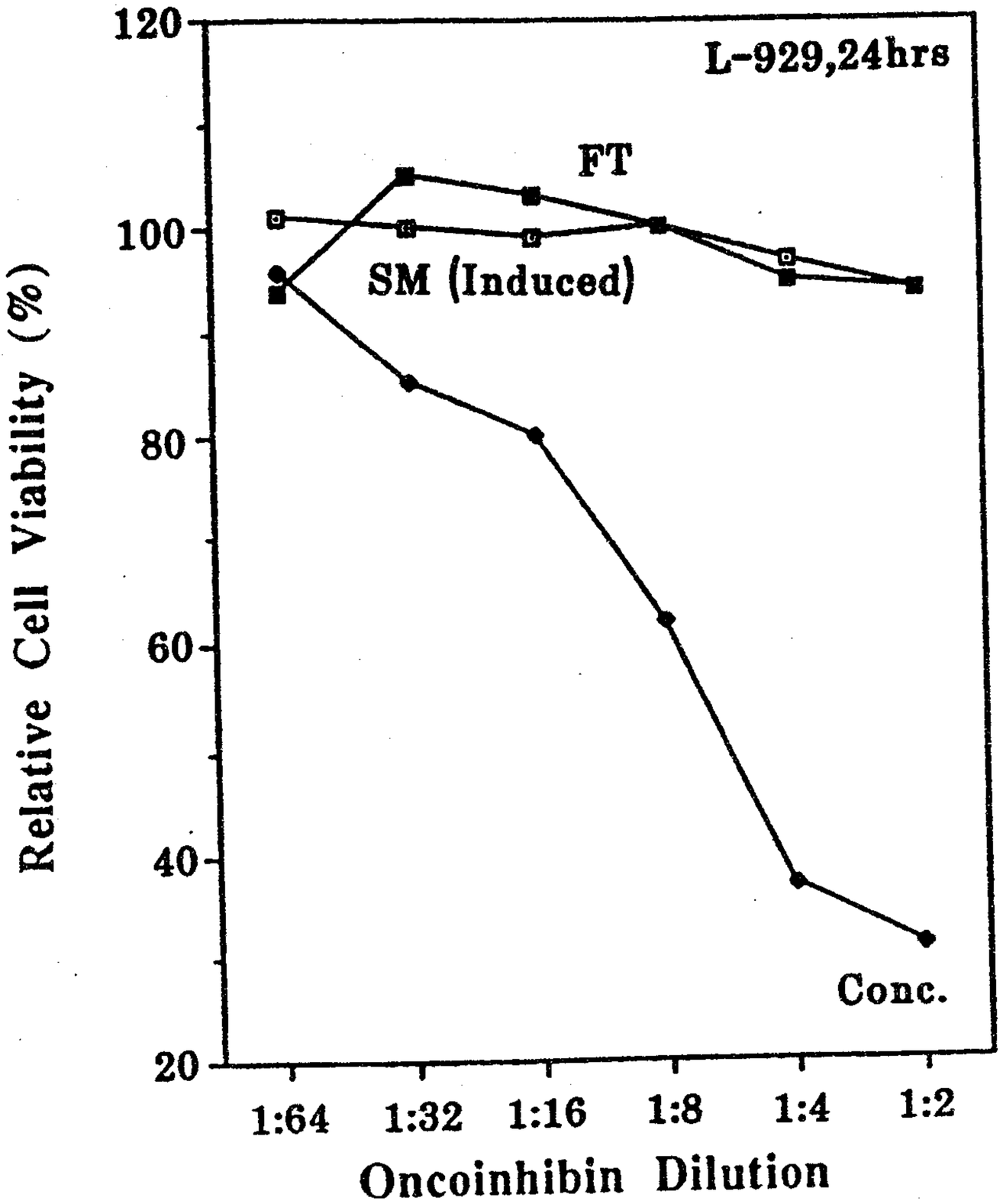

L-929,24hrs
FT
SM (Induced)
Relative Cell Viability (%)
120
100
80
60
40
20
Conc.
1:64
1:32
1:16
1:8
1:4
1:2
Oncoinhibin Dilution

FIG. 12

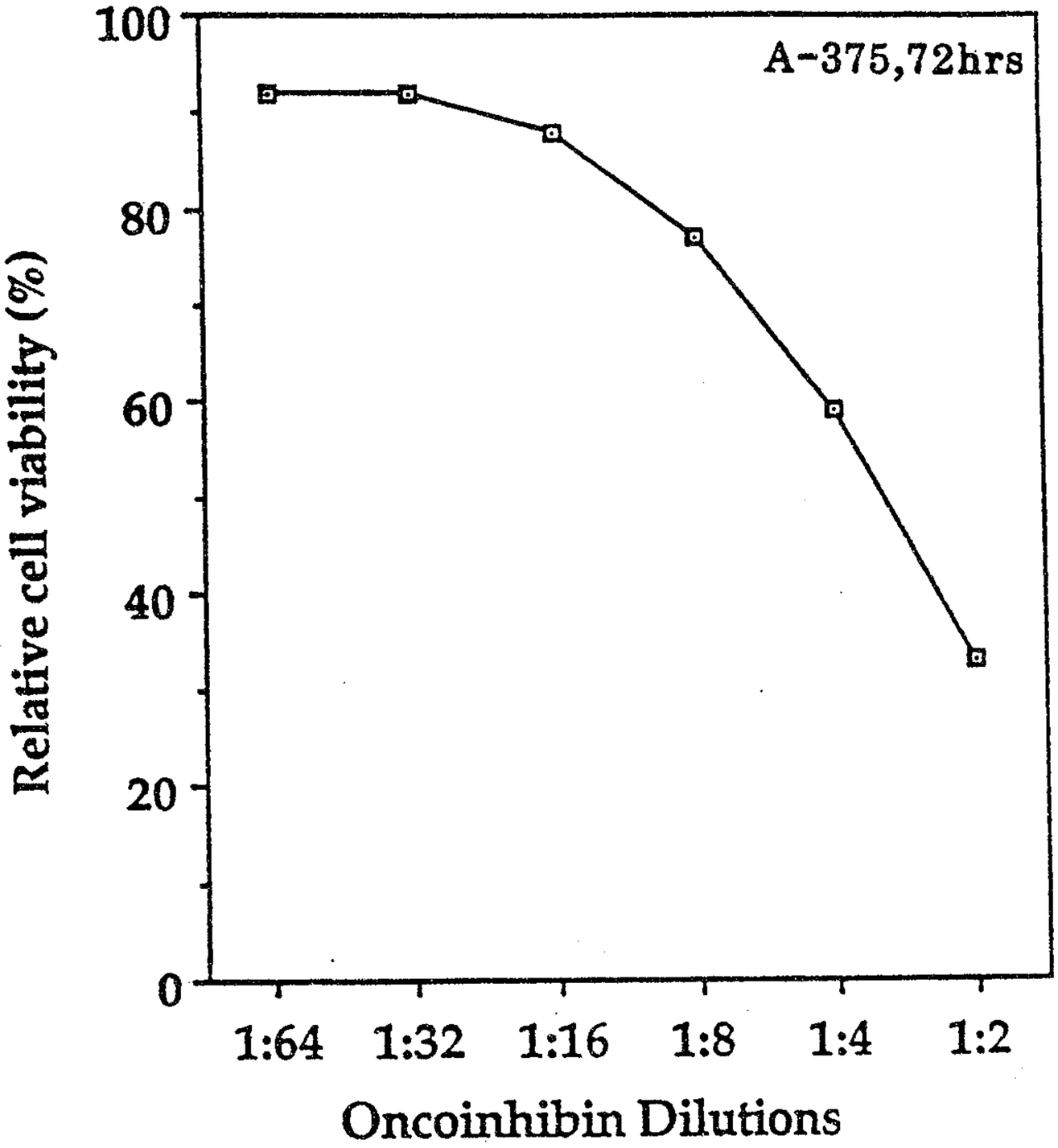


FIG. 14A

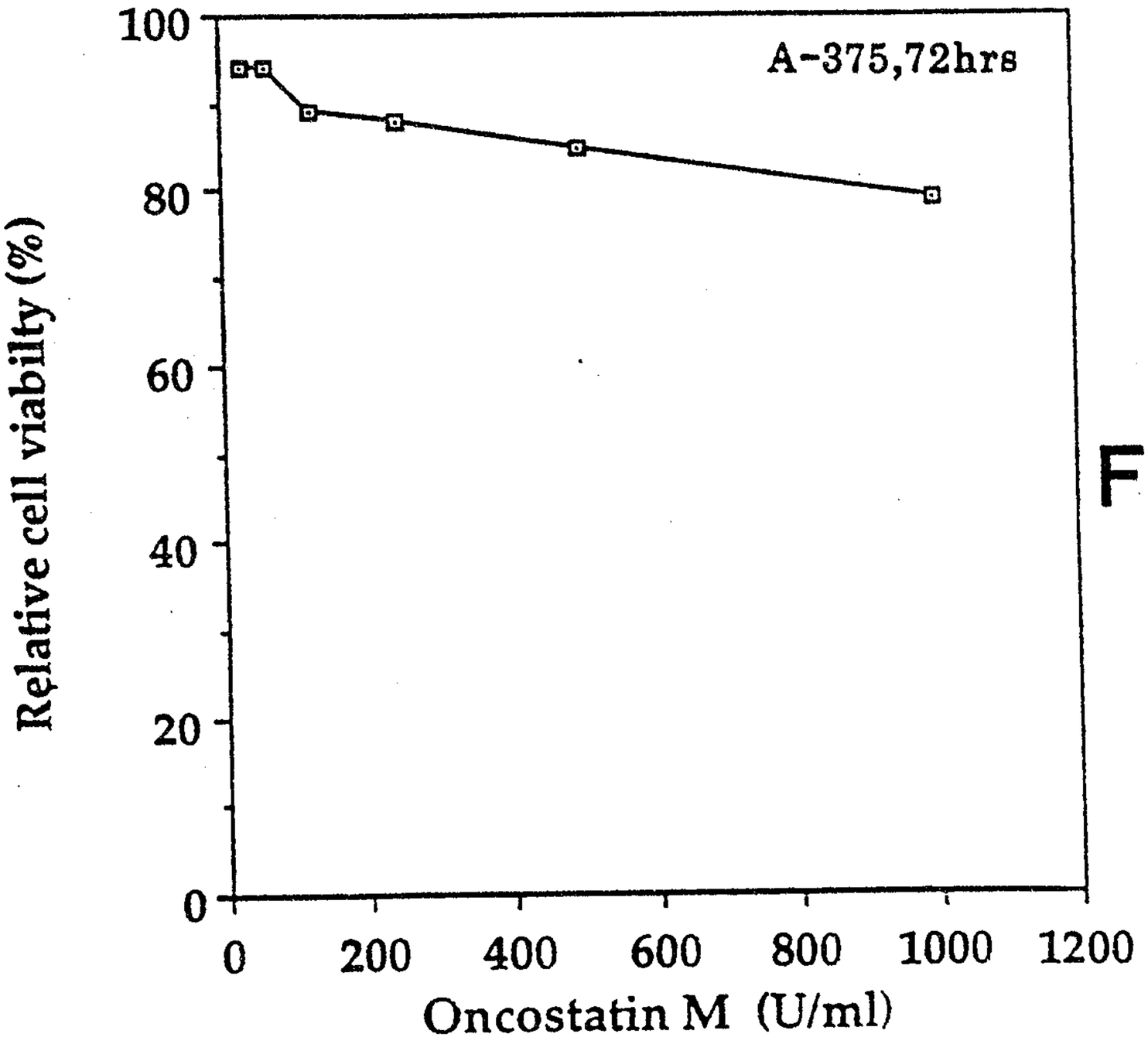


FIG. 14B

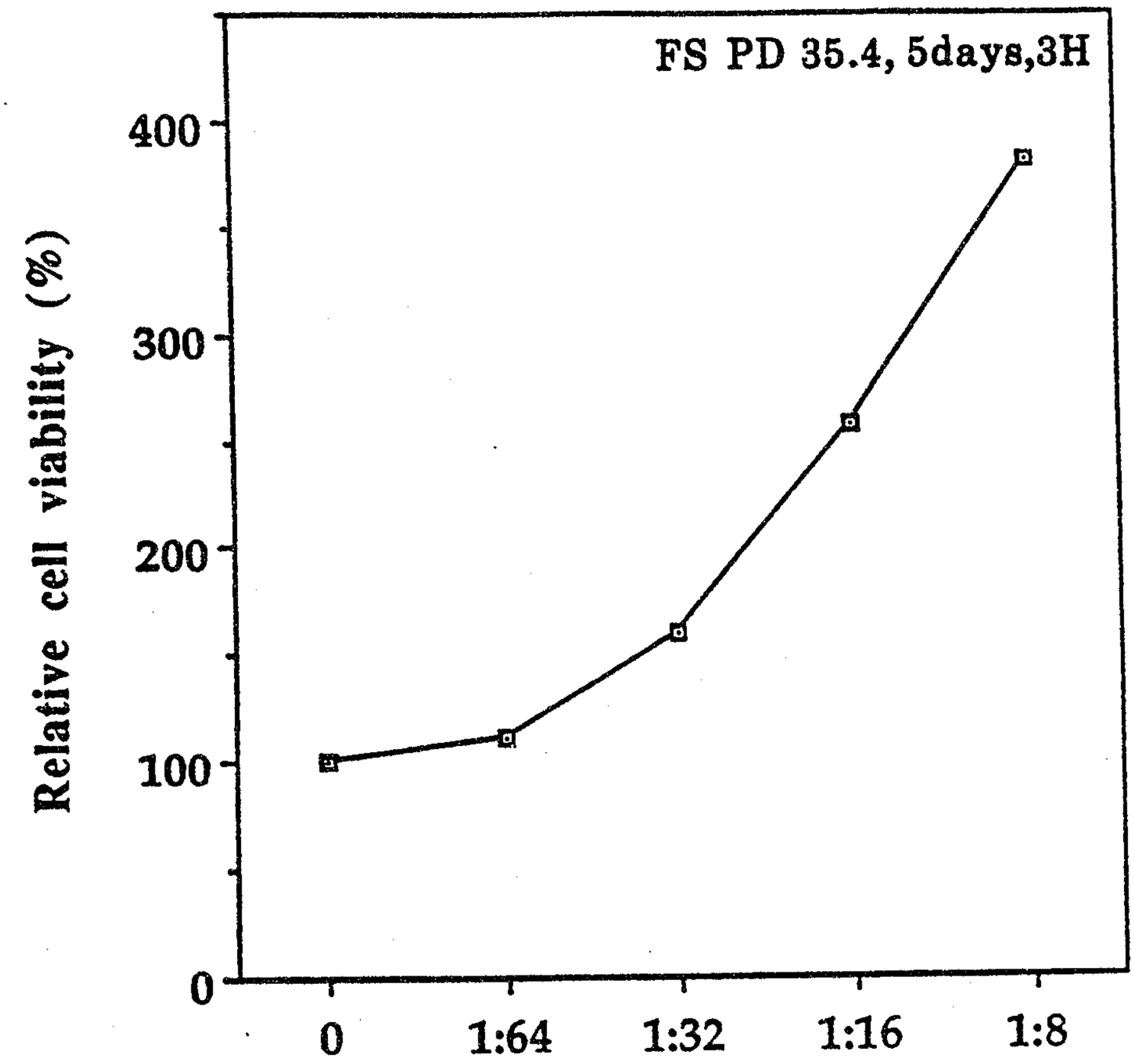


FIG. 15A

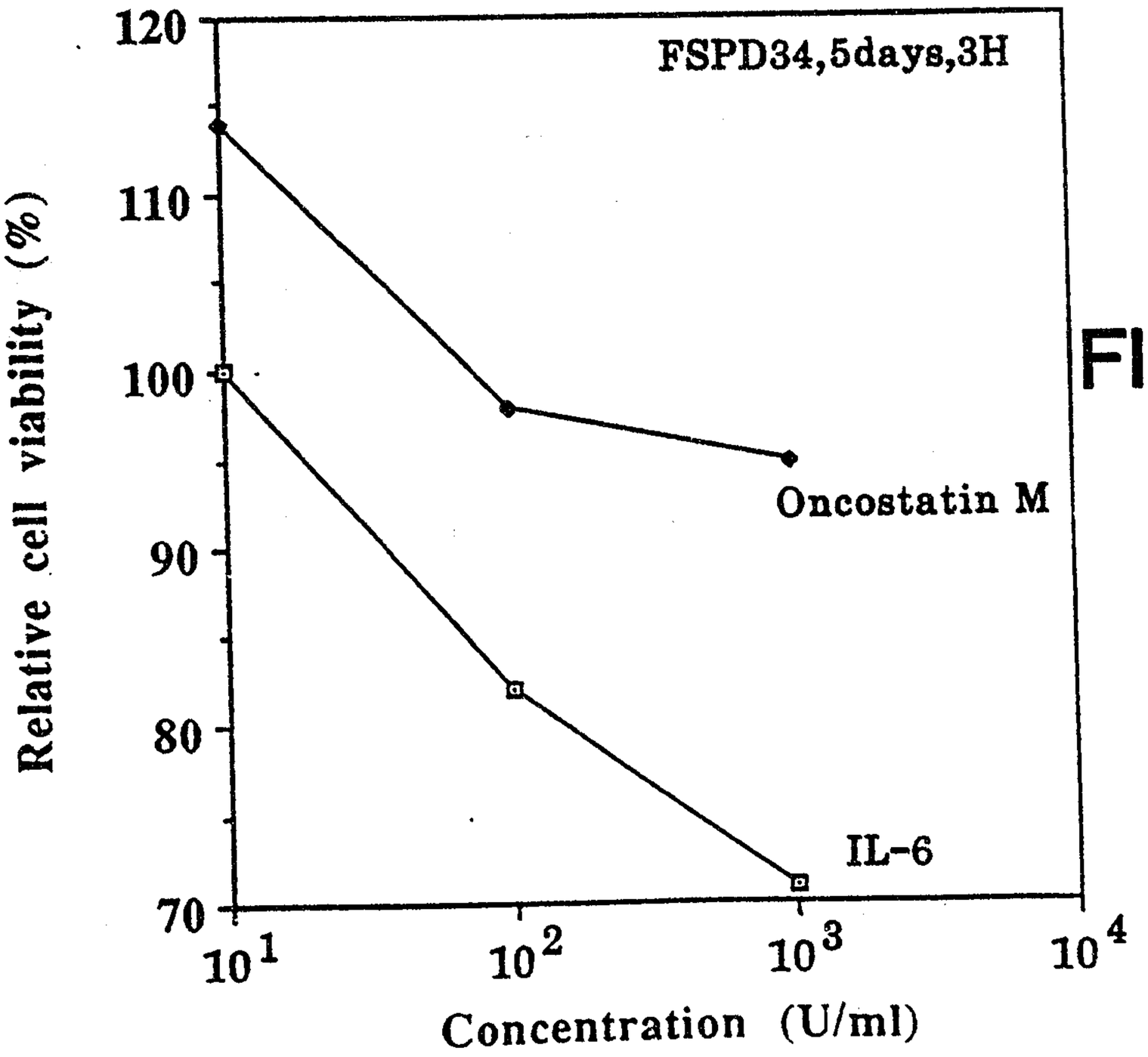


FIG. 15b

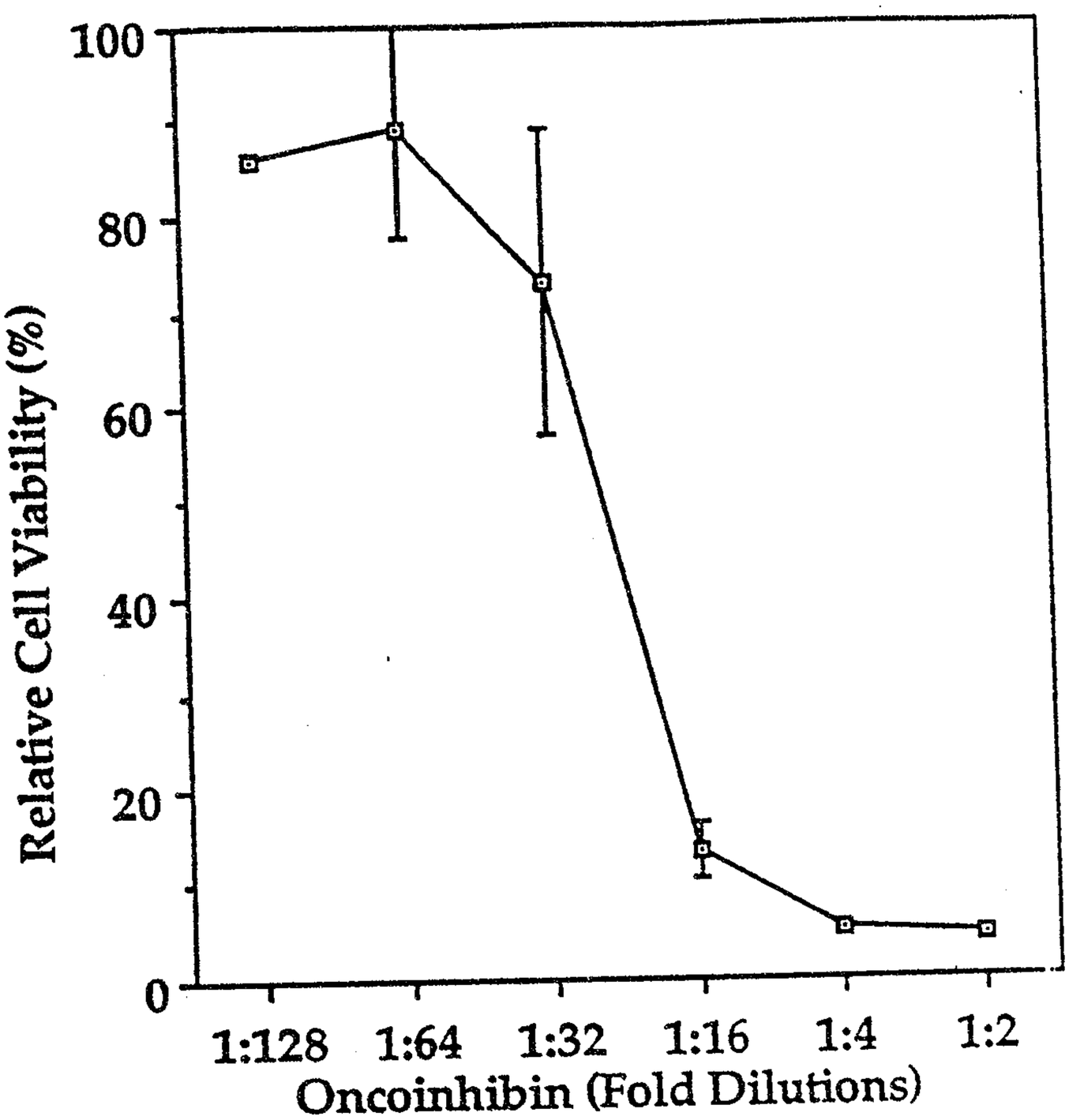


FIG. 16A

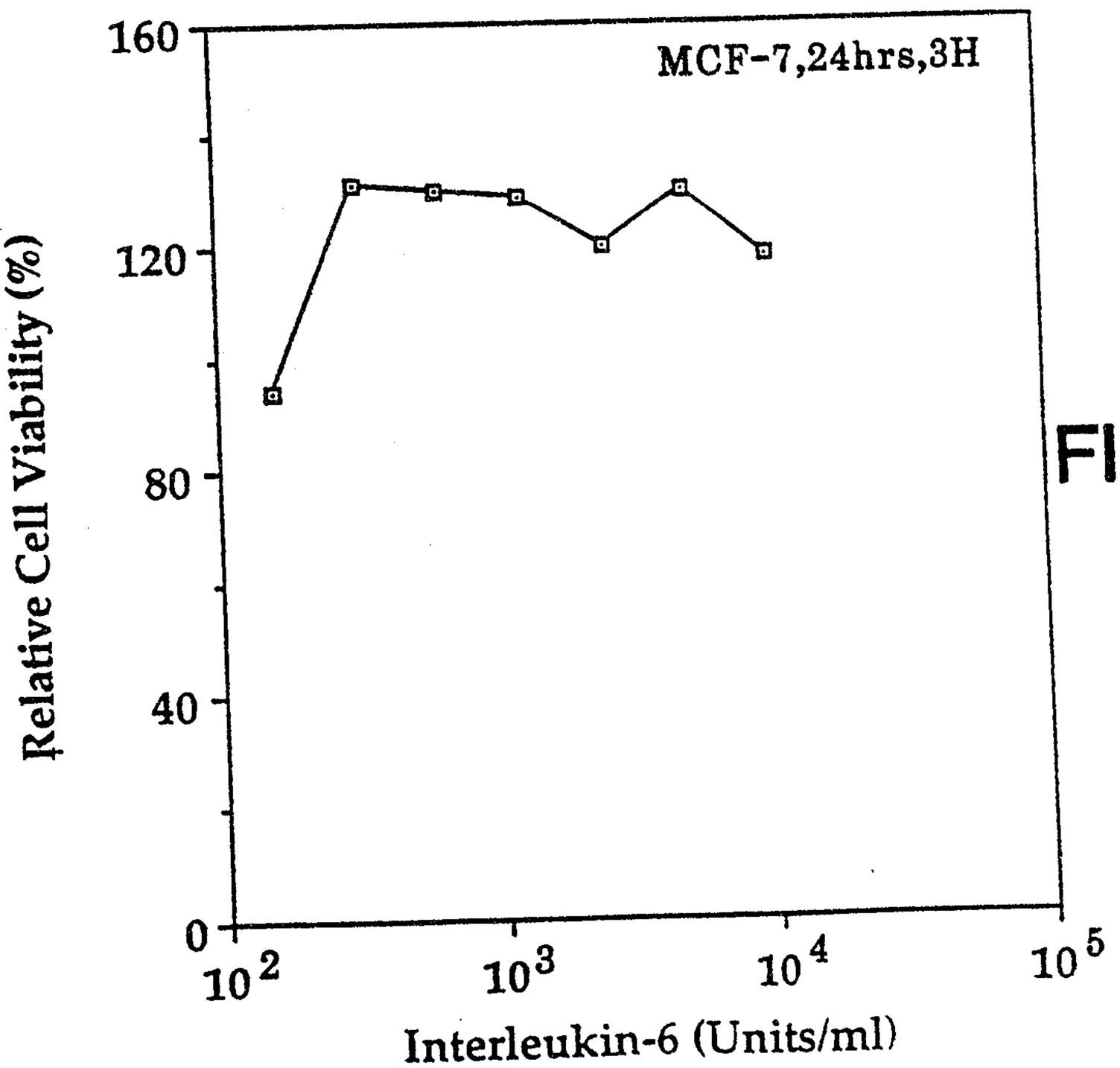


FIG. 16B

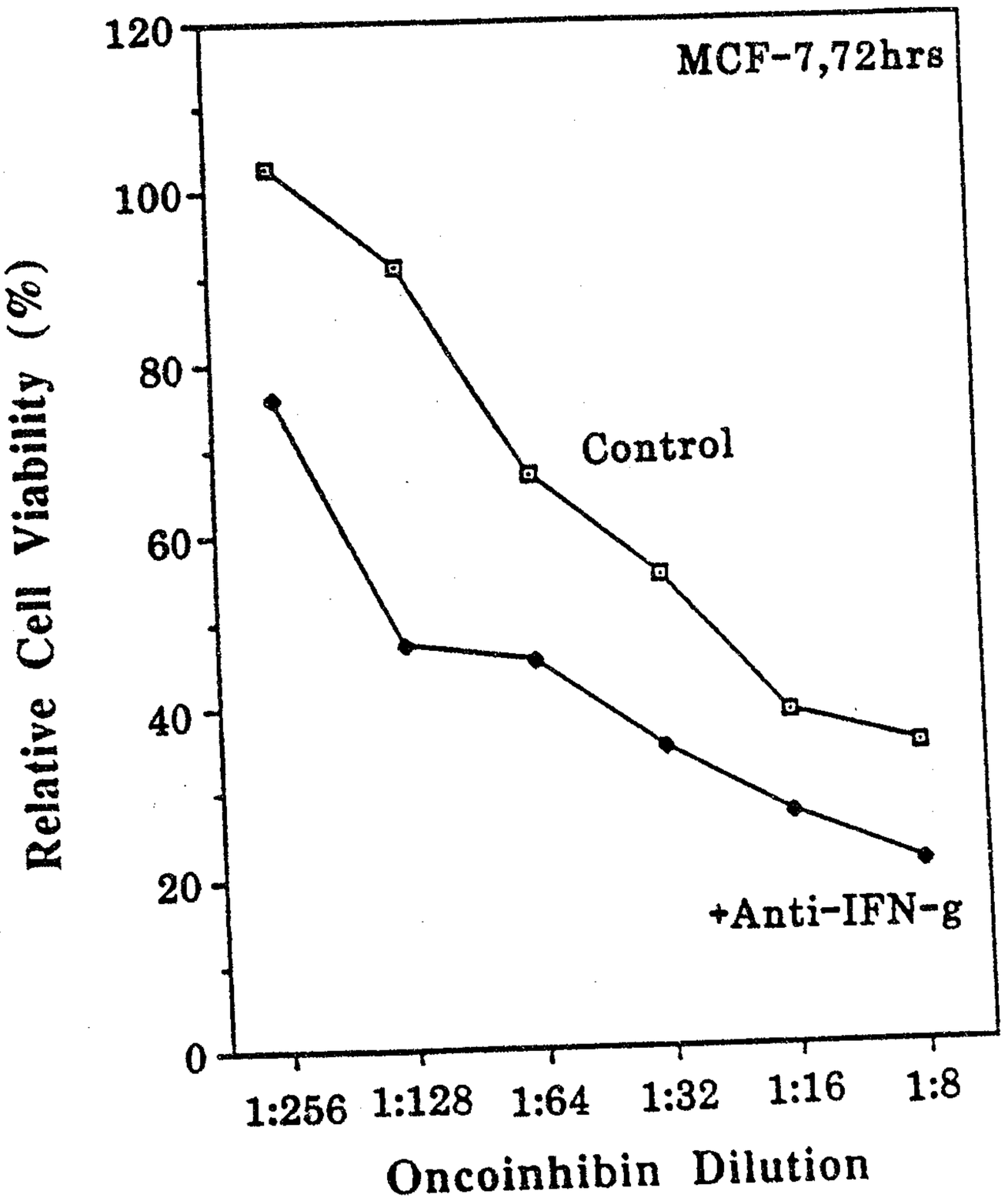

MCF-7,72hrs
Viability (%)
Relative Cell
120
100
80
60
40
20
0
Control
+Anti-IFN-g
1:256
1:128
1:64
1:32
1:16
1:8
Oncoinhibin Dilution

FIG. 17

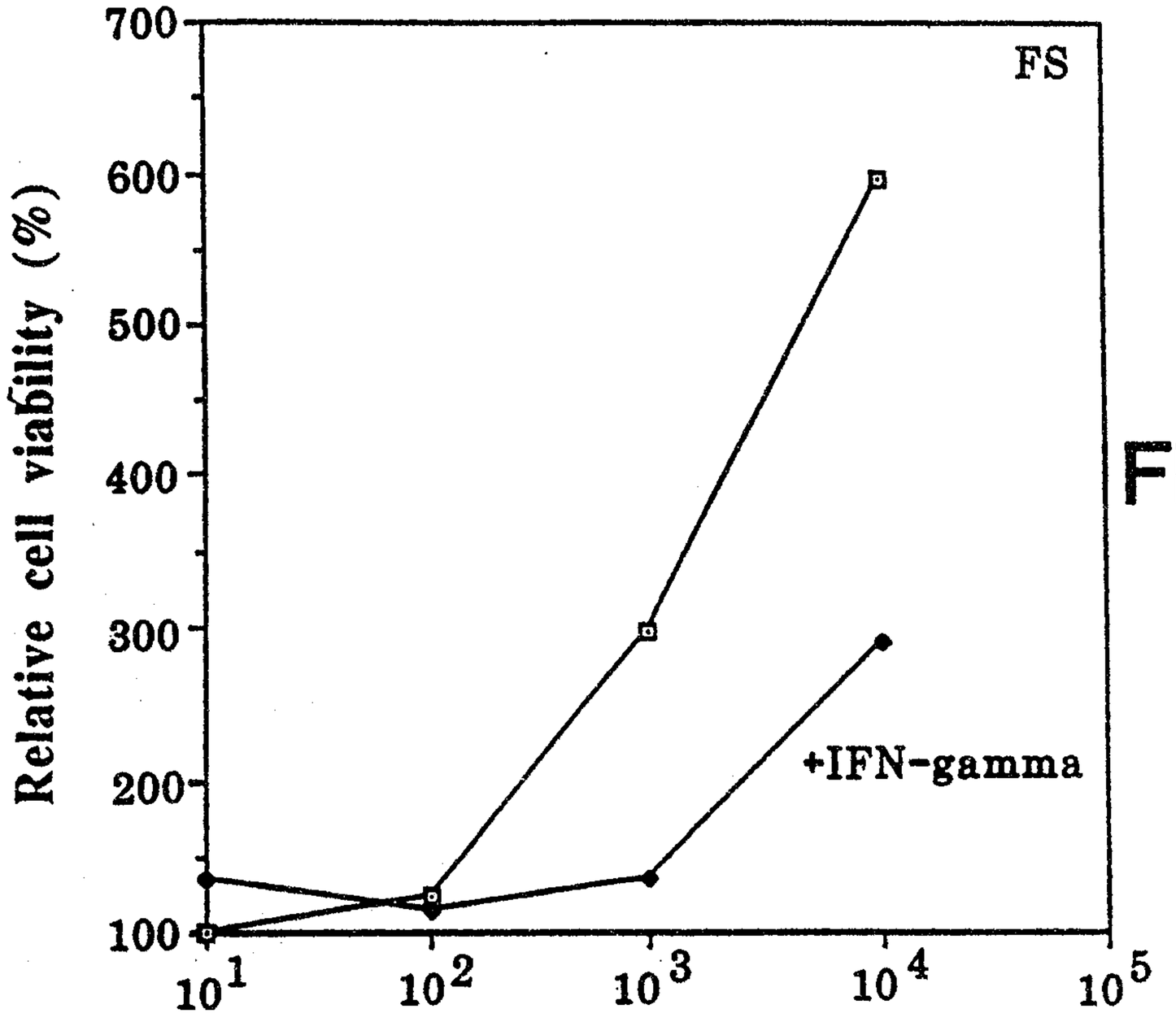


FIG. 18A

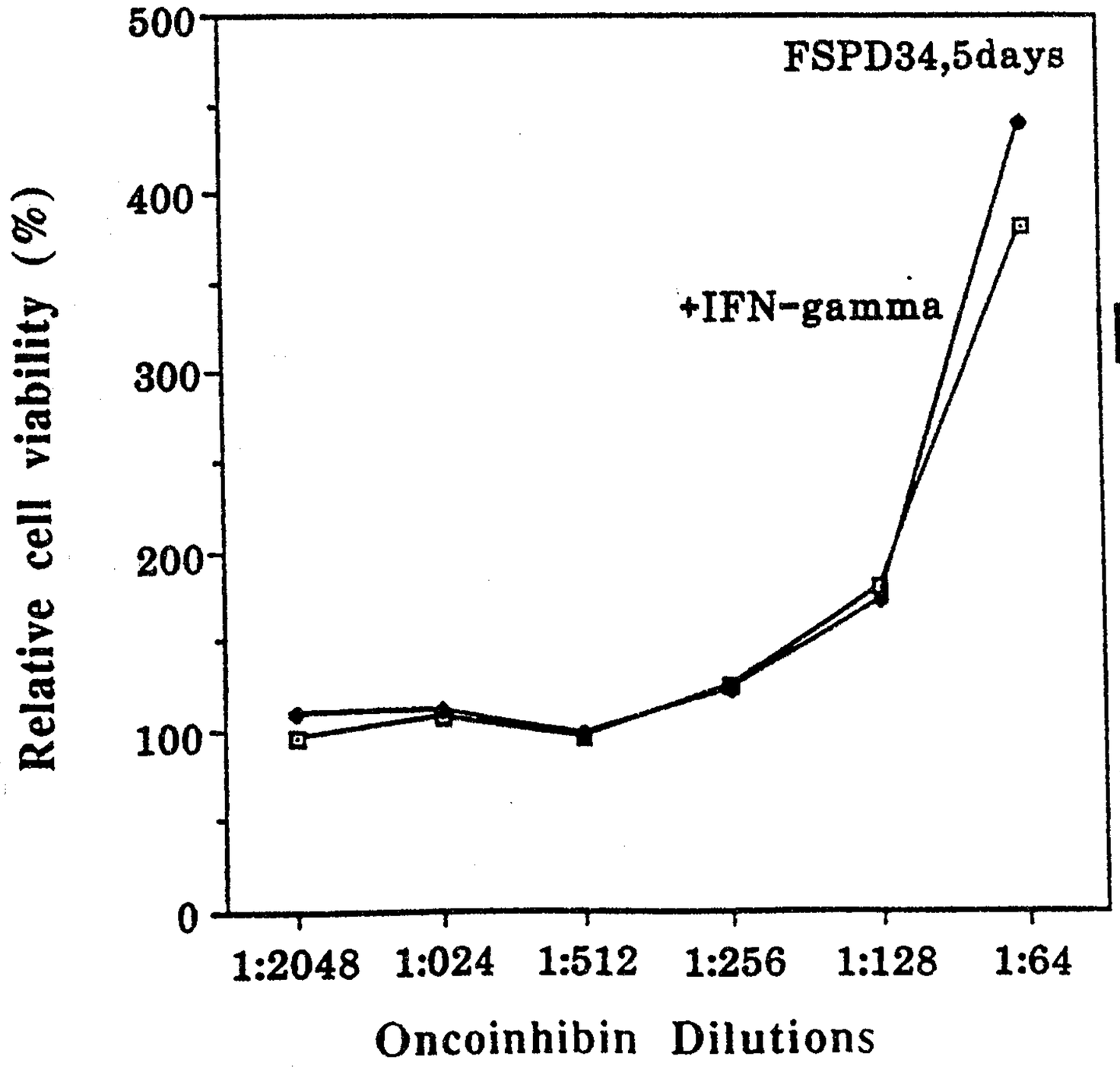


FIG. 18B

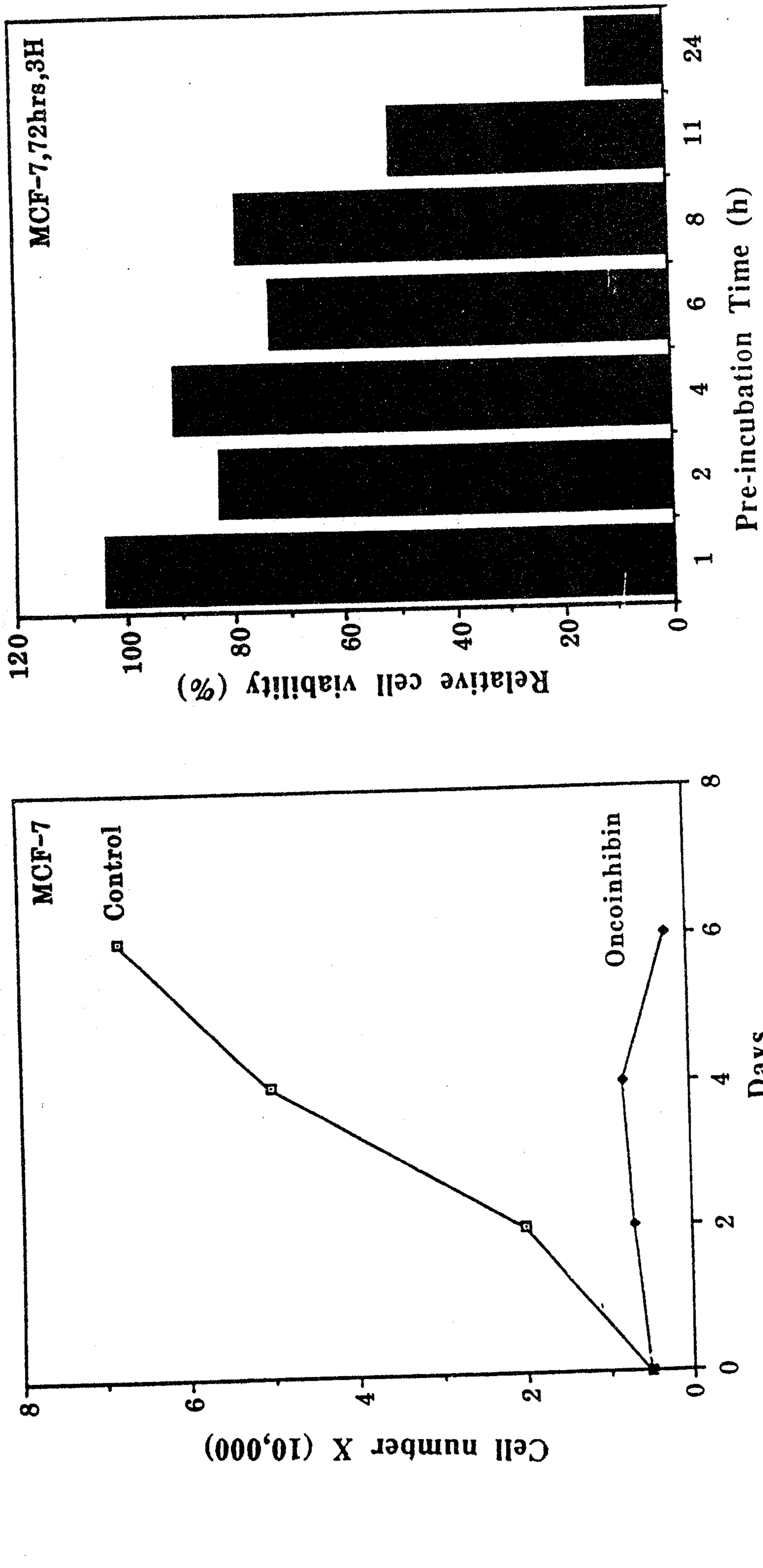

MCF-7,72hrs,3H
Relative cell viability (%)
120
100
80
60
40
20
0
1
2
4
6
8
11
24
Pre-incubation Time (h)

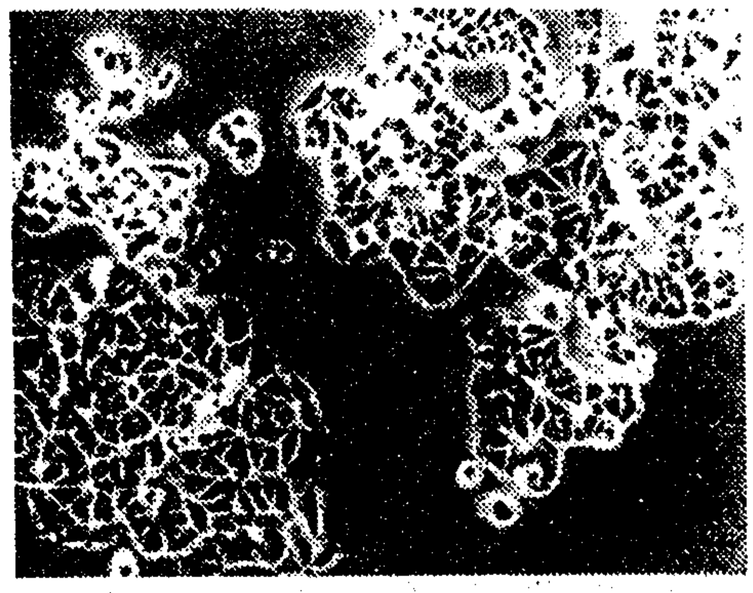
FIG. 20

MCF-7
Control
Oncoinhibin
Cell number X (10,000)
8
6
4
2
0
0
2
4
6
8
Days

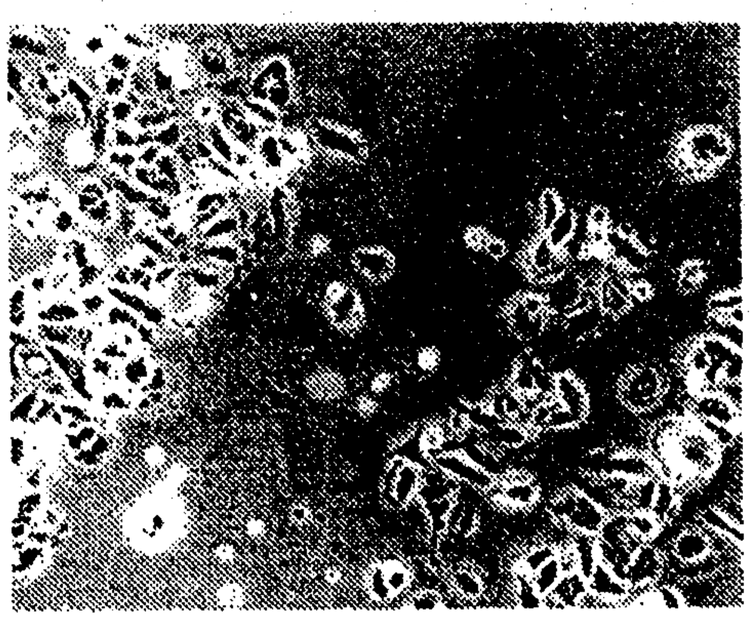
FIG. 19

MCF-7

Control



+TNF



+Oncoinhibin

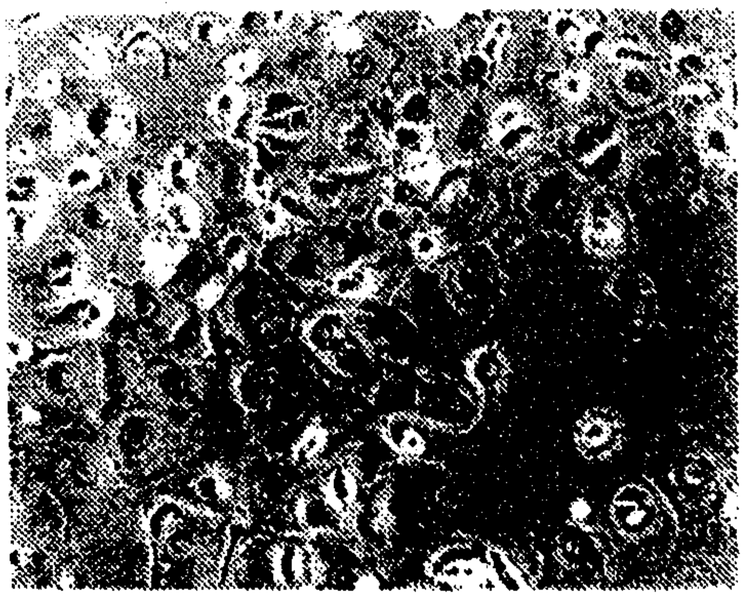


FIG. 21