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Response of NADH oxidation and growth in K-562 cells to the antitumor sulfonylurea, N-(4-methylphenylsulfonyl)-N′-(4-chlorophenyl)urea (LY181984)

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Summary. Growth of K-562 cells in culture is inhibited by the antitumor sulfonylureaLY181984 (N-(4-methylphenylsulfonyl)-N′-(4-chlorophenyl)urea) with an ED_{50} of about 30 µM. LY181984 was shown previously to inhibit NADH oxidation by plasma membranes from HeLa cells and other sources and to influence mitochondrial oxidative phosphorylation. With K-562 cells, NADH oxidation by plasma membranes was transiently stimulated and then inhibited by LY181984. NADH oxidation by whole cells was transiently stimulated and then inhibited by 0.1 to 100 µM LY181984 as well. Both the stimulations and inhibitions of activity were time-dependent. NADH oxidation by lower phase membranes depleted of plasma membranes by aqueous two-phase partition also was inhibited by micromolar and submicromolar concentrations of LY181984. Inhibition did not correlate with mitochondrial presence but rather with membranes that appeared to be fragments of the Golgi apparatus. The oxidation of NADH by whole cells and of plasma membranes that was inhibited by LY181984 was distinguished from mitochondrial NADH oxidation by resistance to inhibition by cyanide and by proceeding under oxygen-depleted conditions or an argon atmosphere. In contrast to the active antitumor agent LY181984, the inactive but chemically-related analog, LY181985 (N-(4-methylphenylsulfonyl)-N′-(4-phenylurea), inhibited neither growth nor NADH oxidation with K-562 cells or cell fractions.

Keywords: Antitumor sulfonylurea; NADH oxidase; Plasma membranes; Growth; Golgi apparatus; Mitochondria; HeLa cells.

Introduction

The antitumor sulfonylureas represent a novel class of antitumor agents with a high degree of efficacy against solid human tumors propagated in immunosuppressed mice (Grindey et al. 1987, Grindey 1988). Their mode of actions differs from that of other common antitumor agents and has been expected to be unique.

Three possible sites of action of the antitumor sulfonylureas have been mentioned in the literature. One site of action involves inhibition of mitochondrial oxidative phosphorylation (Houghton et al. 1990 b). A second site suggested is the inhibition of a growth-related NADH oxidase activity of the plasma membrane of HeLa cells (Morré et al. 1995). Most recently, Golgi apparatus of antitumor sulfonylurea-responsive cell lines have been shown to respond by decreased acidification of trans cisternae and a reduced response to subsequent additions of the monovalent ionophore, monensin (Morré et al. 1994 b).

In this paper, we examine the response of K-562 cells to the antitumor sulfonylurea LY181984 (N-(4-methylphenylsulfonyl)-N′-(4-chlorophenyl)urea). Growth is inhibited in K-562 cells by LY181984 with an ED_{50} of about 30 µM. Therefore, a study was undertaken to investigate the basis for the sensitivity of K-562 cells to LY181984.

Materials and methods

LY181984 and LY181985 were provided by Dr. Warren MacKellar of Lilly Research Laboratories, Indianapolis, IN. The sulfonylureas were dissolved in DMSO. Monensin (Sigma) was dissolved in ethanol.

Growth of cells

K-562 cells (American Type Culture Collection) were grown as a suspension culture in 150 cm^3 flasks in RPMI-1640 (Gibco BRL) in

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a humidified atmosphere of 5% CO_2 in air, pH 7.4, at 37 °C with 10% bovine calf serum (heat-inactivated) plus 50 mg/l gentamycin sulfate (Sigma).

Cells were treated in 3 cm plastic dishes in 2.5 ml culture medium. Sulfonylureas were dissolved in DMSO and added in 2.5 µl of DMSO to yield a final DMSO concentration of 0.1%. Controls received 2.5 µl of DMSO. Growth was determined from cell number estimated after 24, 48, 72, and 96 h of treatment.

Purification of plasma membranes

Cells were collected by centrifugation for 6 to 15 min at 150 to 1,000 **g**. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10 to 30 min to swell the cells. Homogenization was with a Polytron homogenizer for 30 to 40 s at 10,500 rpm using a PT-PA 3012/23 or ST-probe in 7 to 8 ml aliquots. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely.

The homogenates were centrifuged for 10 min at 1,000 rpm (175 **g**) to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 **g** min (e.g., 1 h at 23,500 **g**) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of approximately 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis (Navas et al. 1989, Morré et al. 1994 a). The upper phase, enriched in plasma membranes, was diluted 5-fold with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membranes was determined to be >90% by electron microscope morphometry.

Spectrophotometric assay

NADH oxidase activity was determined from the disappearance of NADH measured at 340 nm with 430 nm as reference using an SLM Aminco DW-2000 spectrophotometer in the dual wavelength mode of operation. The reaction mixture contained in a final volume of 2.5 ml, 50 mM Tris-Mes buffer (pH 7.2), with or without 1 mM KCN, 100 to 200 µg plasma membrane protein, and 150 µM NADH. Assay was at 37 °C with constant stirring and with continuous recording over two or more 5 min intervals once a steady-state rate was reached. A millimolar extinction coefficient of 6.22 was used to determine specific activity of the NADH oxidase activity.

For those experiments where the reaction system was depleted of oxygen, the reaction mixture was first degassed en vacuo and then equilibrated with argon by bubbling argon through the solution for 30 min with assay with argon present in the sample chamber.

Fractionation of lower phase membranes

The lower phase was diluted 10-fold with 1 mM sodium bicarbonate and centrifuged for 30 min at 25,000 **g**. The supernatant, containing Golgi apparatus, was diluted 2-fold with 1 mM sodium bicarbonate and centrifuged for 45 min at 31,700 **g**. The Golgi apparatus were resuspended in 50 mM Tris-Mes buffer (pH 7.2).

Preparation of mitochondria

The mitochondrial pellet was resuspended in homogenization buffer containing 0.25 M sucrose, 0.01 M Tris HCl, and 0.1 mM EDTA (pH 7.4). Mitochondria were purified by differential centrifugation (Morré 1973).

Electron microscopy

Fixation was initiated by addition of 250 µl 2.5% glutaraldehyde in 0.1 M sodium phosphate, pH 7.2. After 10 min, the glutaraldehyde-containing culture medium was decanted and fresh 2.5% glutaraldehyde in 0.1 M sodium phosphate was added. The glutaraldehyde and the fixations were at room temperature. Growth of cells and all other manipulations were at 37 °C.

The pelleted cells were stored in glutaraldehyde at 4 °C for 1 to several days with fixation for 2 h in 1% osmium tetroxide in 0.1 M sodium phosphate, pH 7.0. Dehydration was through an acetone series with embedment in Epon (Luft 1961). Thin sections were viewed and photographed using a Philips EM200 electron microscope. Sections were photographed at a magnification of 14,000 and analyzed from photographic prints at final magnifications of 35,000.

Determination of marker enzyme and proteins

Mitochondrial activity was determined from the activity of succinate-tetrazolium reductase as described by Pennington (1961). Protein was determined by the bicinchoninic acid method (Smith et al. 1985).

Results

Growth

The growth of K-562 cells was inhibited in response to LY181984 (active antitumor sulfonylurea) but not in response to LY181985, a structurally related but

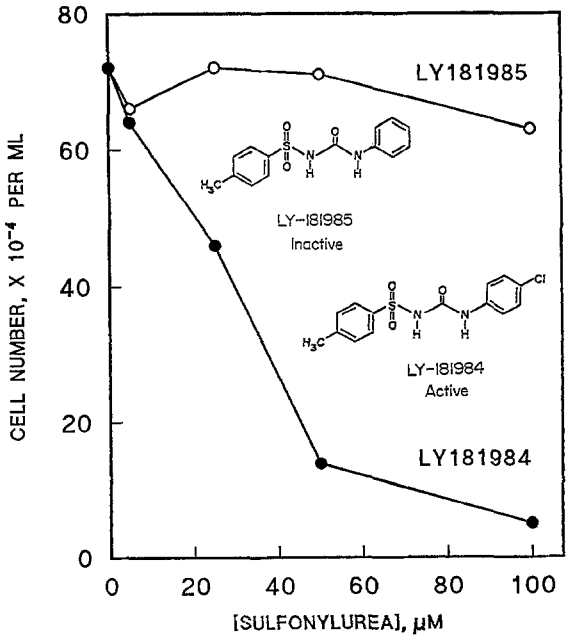


Fig. 1. Growth of K-562 cells as a function of the concentration of LY181984 (active) or LY181985 (inactive) after 72 h of drug treatment. The structures of LY181984 and LY181985 differ only in the absence of a chlorine from the B ring of LY181985 that is present in LY181984

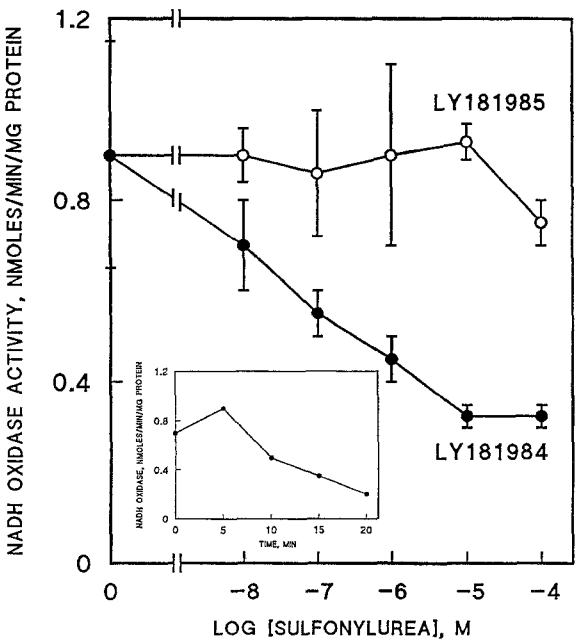


Fig. 2. NADH oxidation by plasma membrane vesicles isolated from K-562 cells by aqueous two-phase partition as a function of LY181984 (active) or LY181985 (inactive) concentration. As shown in the inset for 100 µM LY181984 the inhibition was time-dependent. The usual response of isolated plasma membrane vesicles was a transient stimulation during the first 5 min after LY181984 addition followed by inhibition. Results are averages of 3 experiments ± standard deviations. The inactive sulfonylurea LY181985 was without effect over 20 min of incubation

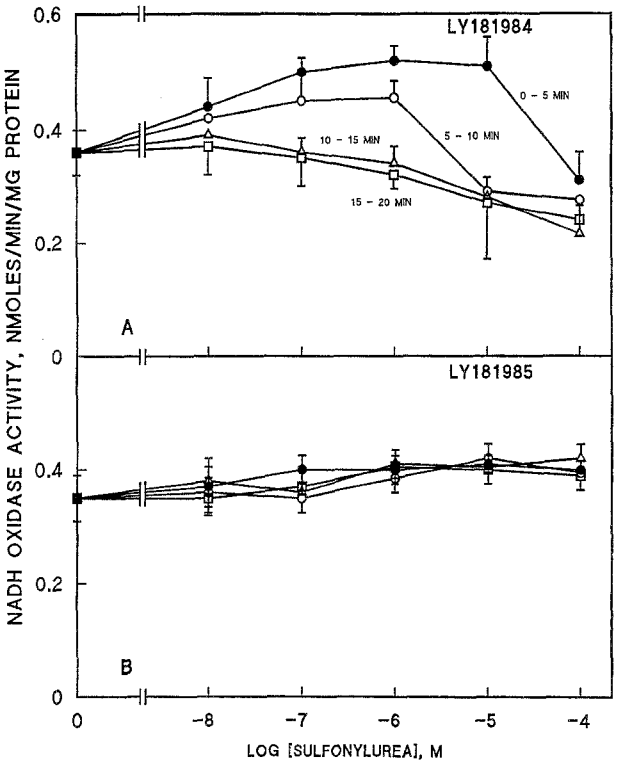


Fig. 3. Response of NADH oxidation by whole cells to sulfonylurea measured between 5 and 10 min of incubation. **A** The overall response was one of stimulation at 1 µM followed by inhibition. Both the stimulation at 1 µM and the inhibition at 10 µM were time-dependent. **B** The inactive sulfonylurea LY181985 was without effect. Results are averages of 3 experiments ± standard deviation. ● 0–5 min, ○ 5–10 min, △ 10–15 min, □ 15–20 min

inactive sulfonylurea analog (Fig. 1). Growth was inhibited by 50% at about 30 µM LY181984 and concentrations greater than 100 µM completely inhibited growth. LY181985 did not inhibit growth appreciably even at 100 µM (Fig. 1).

NADH oxidase activities of plasma membranes and whole cells

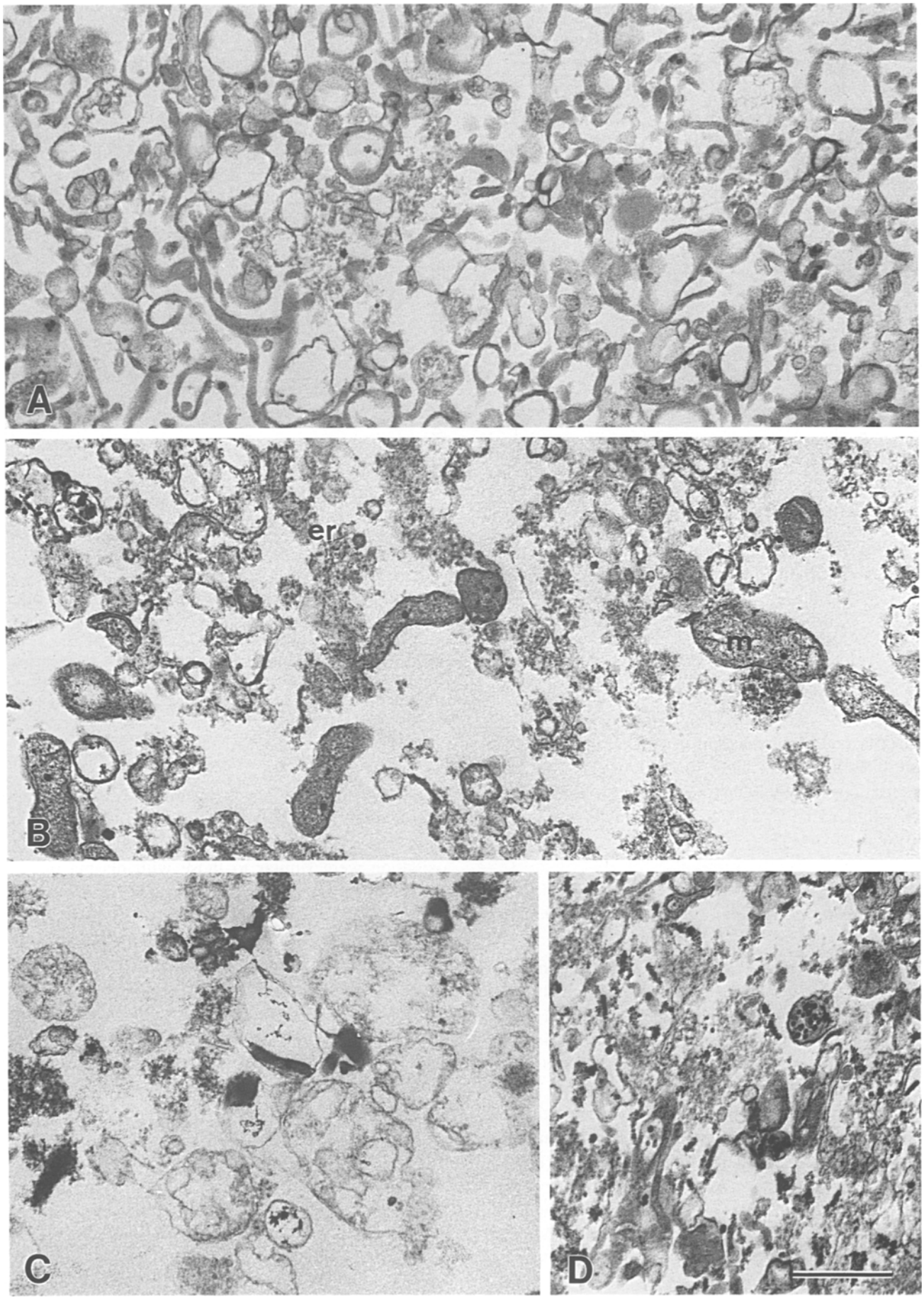
K-562 cells were fractionated by aqueous two-phase partition into a plasma membrane-enriched upper phase and a plasma membrane-depleted lower phase. Each was analyzed for activity of NADH oxidase in the presence and absence of both LY181984 (active) and LY181985 (inactive).

At micromolar and submicromolar concentrations of LY181984, NADH oxidation by plasma membranes was transiently stimulated but then inhibited (Fig. 2). The inhibition was time-dependent and occurred progressively over 20 min after sulfonylurea addition (Fig. 2 inset) with an ED_{50} of about 1 µM. The growth-inactive LY181985 was without effect on NADH oxidation by plasma membranes from K-562 cells (Fig. 2).

With whole cells, results with LY181984 on NADH oxidation were similar to those with plasma membranes (Fig. 3) except higher concentrations of LY181984 were required to inhibit and inhibition was never complete. The growth inactive LY181985 was without effect or stimulated slightly NADH oxidation by intact K-562 cells (Fig. 3 B). LY181984 stimulated NADH oxidation transiently over the first 5 min of assay. However, subsequently, NADH oxidation was inhibited and the inhibition again was observed to be time dependent as with isolated plasma membranes (Fig. 3).

NADH oxidase activities of lower phase membranes and mitochondria

Lower phase membranes (Fig. 4 B) depleted of plasma membranes (Fig. 4 A) also were inhibited by LY181984 between 0.1 and 10 µM but not by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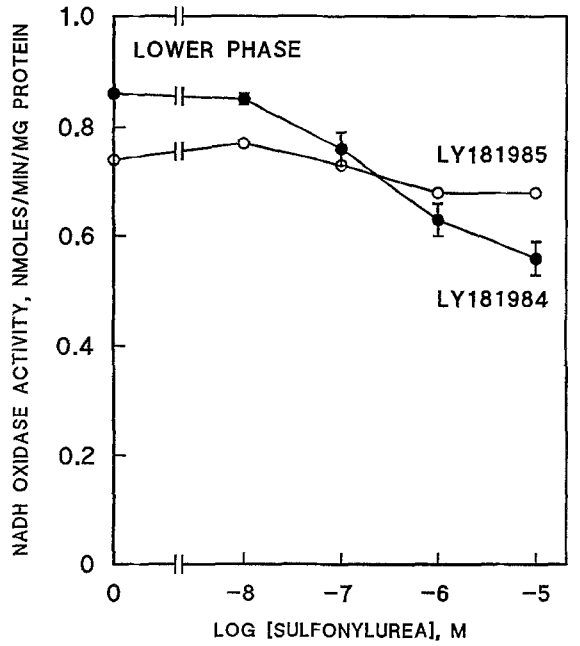

Fig. 5. Inhibition of NADH oxidase activity of the plasma membrane-depleted lower phase fraction of K-562 cells as a function of the concentration of LY181984 and comparison with LY181985. Assay was over 10 min in the presence of 1 mM cyanide. Results are from duplicate determinations for LY181984 ± mean average deviations and from a single experiment for LY181985

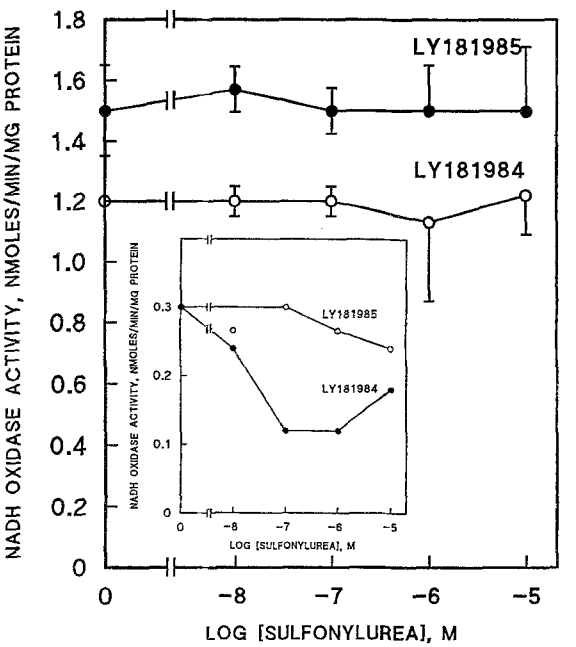

Fig. 6. NADH oxidation by a mitochondria-enriched fraction prepared from the plasma membrane-depleted lower phase of a two-phase separation of K-562 cells. Assay was over 10 min in the presence of 1 mM cyanide as a function of concentration of LY181984 and of LY181985. Results are from duplicate experiments ± mean average deviations. The inset shows LY181984 inhibition of NADH oxidase activity of the light membrane fraction recovered in the preparation of mitochondria also assayed in the presence of 1 mM cyanide

LY181985 at these same concentrations (Fig. 5). The lower phase fractions consisted of mitochondria, endoplasmic reticulum and internal membranes other than plasma membrane such as Golgi apparatus (Fig. 4 B–D).

The inhibition of lower phase activity appeared not to be due to inhibition of mitochondrial NADH oxidase activity. A mitochondria-enriched fraction depleted of other internal membranes was prepared (Fig. 4 C). The residual NADH oxidation by these mitochondria-enriched fractions remaining after addition of 0.1 mM cyanide was unaffected by either LY181984 or LY181985 (Fig. 6) nor was the NADH oxidase activity of the mitochondria-enriched fractions inhibited by LY181984 or LY181985 in the absence of cyanide (Fig. 7).

NADH oxidase activities of a light membrane and Golgi apparatus-enriched fractions

A by-product of the mitochondrial isolation was a light fraction entering the lower phase. This fraction retained the sulfonylurea-inhibited activity (Fig. 6 inset). When the lower phase material, after removal of the bulk of the mitochondria, was diluted and recentrifuged, a light fraction, which was inhibited by LY181984 but not by LY181985, was obtained (Fig. 8). This fraction appeared to be enriched in fragments of the Golgi apparatus (Fig. 4 D). The light fraction recovered during preparation of mitochondria was of similar appearance but not as well preserved morphologically (not illustrated).

Fig. 4. Electron microscope appearance of cell fractions prepared from K-562 cells. **A** Plasma membrane-enriched upper phase of an aqueous two-phase separation. **B** Plasma membrane-depleted lower phase from the aqueous two-phase separation enriched in mitochondria (*m*) and endoplasmic reticulum (*er*) fragments. **C** Mitochondria-enriched fraction. **D** A Golgi apparatus-enriched fraction prepared from the plasma membrane-depleted lower phase of an aqueous two-phase separation. Bar: 0.5 μm

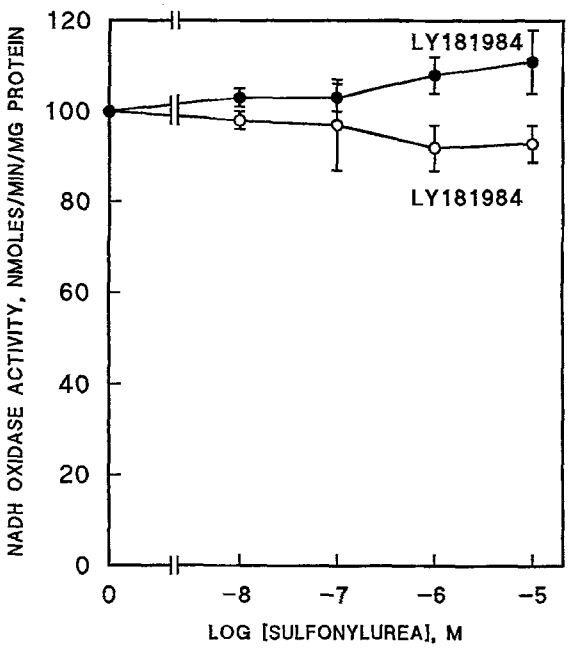

Fig. 7. NADH oxidation by a mitochondrial-enriched fraction from the plasma membrane-depleted lower phase of a two-phase separation of K-562 cells in the absence of cyanide as a function of concentration of LY181984 or of LY181985 in DMSO compared to DMSO alone (control). The final concentration of DMSO was 0.1%. LY181984 was without effect compared to DMSO alone and LY181985 was slightly stimulatory. Results are from duplicate determinations ± mean average deviations

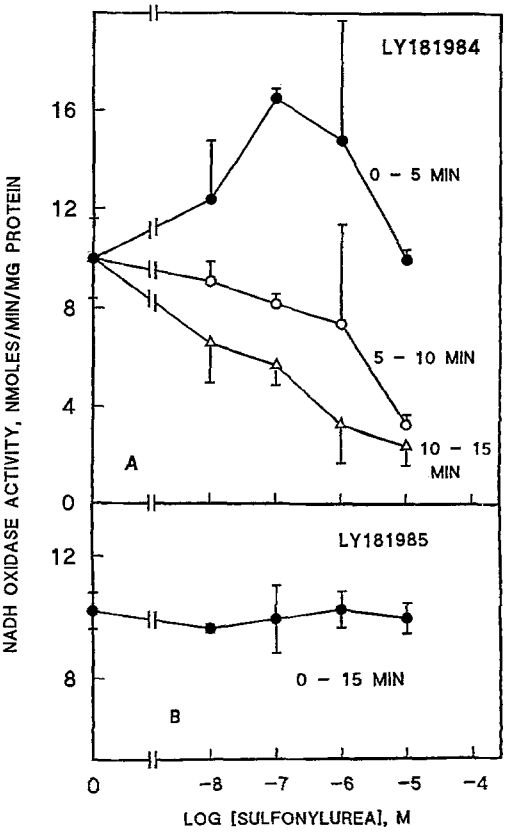

Fig. 8. NADH oxidation in the presence of cyanide of the Golgi apparatus-enriched fraction prepared from the plasma membrane-depleted lower phase of an aqueous two-phase separation of K-562 cells as a function of concentration of LY181984 and of LY181985. **A** With LY181984, specific activities were determined between 0 and 5, 5 and 10, and 10 and 15 min of sulfonylurea addition to show an initial stimulation of activity between 0 and 5 min and inhibition thereafter. Inhibition was near maximum after 20 min. **B** LY181985 was without effect over 20 min of incubation. Results for LY181984 are from two experiments ± mean average deviations and from three experiments ± standard deviations for LY181985

The inhibition of NADH oxidation of the Golgi apparatus-enriched fraction (Fig. 4 D) by LY181984 was markedly time-dependent (Fig. 8 A). Between 0 and 5 min of assay, NADH oxidase activity was stimulated by 0.01 to 1 µM LY181984 and progressively inhibited up to 15 min of incubation. Further incubation was largely without effect. The inactive LY181985 was without effect on NADH oxidation by the Golgi apparatus-enriched fraction at all incubation times and concentrations tested (Fig. 8 B).

Response to cyanide and/or an argon atmosphere

The oxidation of NADH by the plasma membranes and Golgi apparatus and that of whole cells was resistant to cyanide whereas the oxidation of NADH by the mitochondrial fractions was strongly inhibited by cyanide (Table 1). NADH oxidative activities of the plasma membranes, Golgi apparatus and whole cells were further distinguished from that of mitochondria by the ability of the plasma membranes and whole cell activities to take place under an argon atmosphere whereas NADH oxidation by mitochondria was strongly reduced in an oxygen-depleted system or an argon atmosphere (Table 1).

Electron microscopy

K-562 cells treated with LY181984 or with LY181985 were observed morphologically. Mitochondria were similar in appearance comparing no treatment (Fig. 9 A), treatment with 0.1% DMSO alone (Fig. 9 B), 100 µM LY181985 (inactive) in DMSO (0.1% final concentration) (Fig. 9 C) or 100 µM LY181984 (active) in DMSO (Fig. 9 D). Golgi apparatus, however, were observed to be smaller and with fewer, smaller, and more swollen cisternae per stack

Table 1. Effect of KCN (1 mM) and argon on NADH oxidase activities of whole K-562 cells and of cell fractions prepared from K-562 cells and succinate-INT-reductase activities of plasma membranes and mitochondria

Whole cells	Whole cells	Plasma membranes	Golgi apparatus	Mitochondria
NADH oxidase				
No treatment	0.45 ± 0.05	0.97 ± 0.09	10.0 ± 1.6	6.0 ± 0.2
+ argon	0.34 ± 0.05	0.9 ± 0.05	–	1.5 ± 0.15
+ KCN	0.36 ± 0.04	0.95 ± 0.1	10.8 ± 0.8	1.5 ± 0.15
+ KCN + argon	0.31 ± 0.04	0.94 ± 0.01	–	1.5 ± 0.1
Succinate-INT reductase	–	0.9	–	5.0

Values are nmoles/min/mg protein
Results are from duplicate (Golgi apparatus) or triplicate determinations ± mean average or standard deviations

with 100 µM LY181984 in 0.1% DMSO (Fig. 10 D) compared to no treatment (Fig. 10 A), 0.1% DMSO alone (Fig. 10 B) or 100 µM LY181985 in 0.1% DMSO (Fig. 10 C).

Discussion

The growth of K-562 cells was inhibited by the active antitumor sulfonylurea LY181984. The concentra-

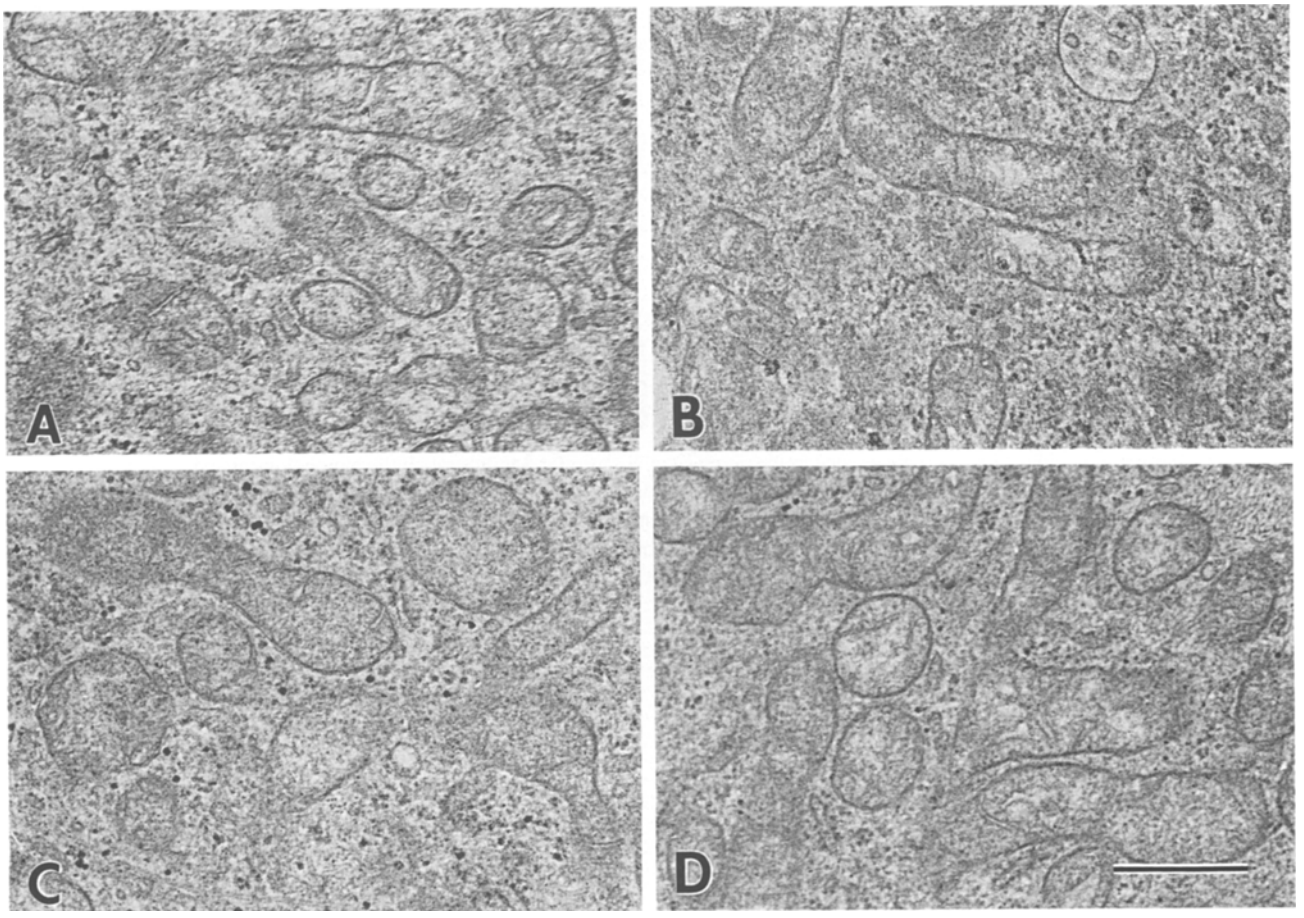

Fig. 9. Electron micrographs of K-562 cells treated in the presence or absence of sulfonylurea for 3 h illustrating the morphological appearance of the mitochondria. **A** No treatment. **B** DMSO alone. **C** 100 µM LY181985 (inactive) in DMSO. **D** 100 µM LY181984 (active) in DMSO. The final DMSO concentration throughout was 0.1%. Bar: 0.5 µm

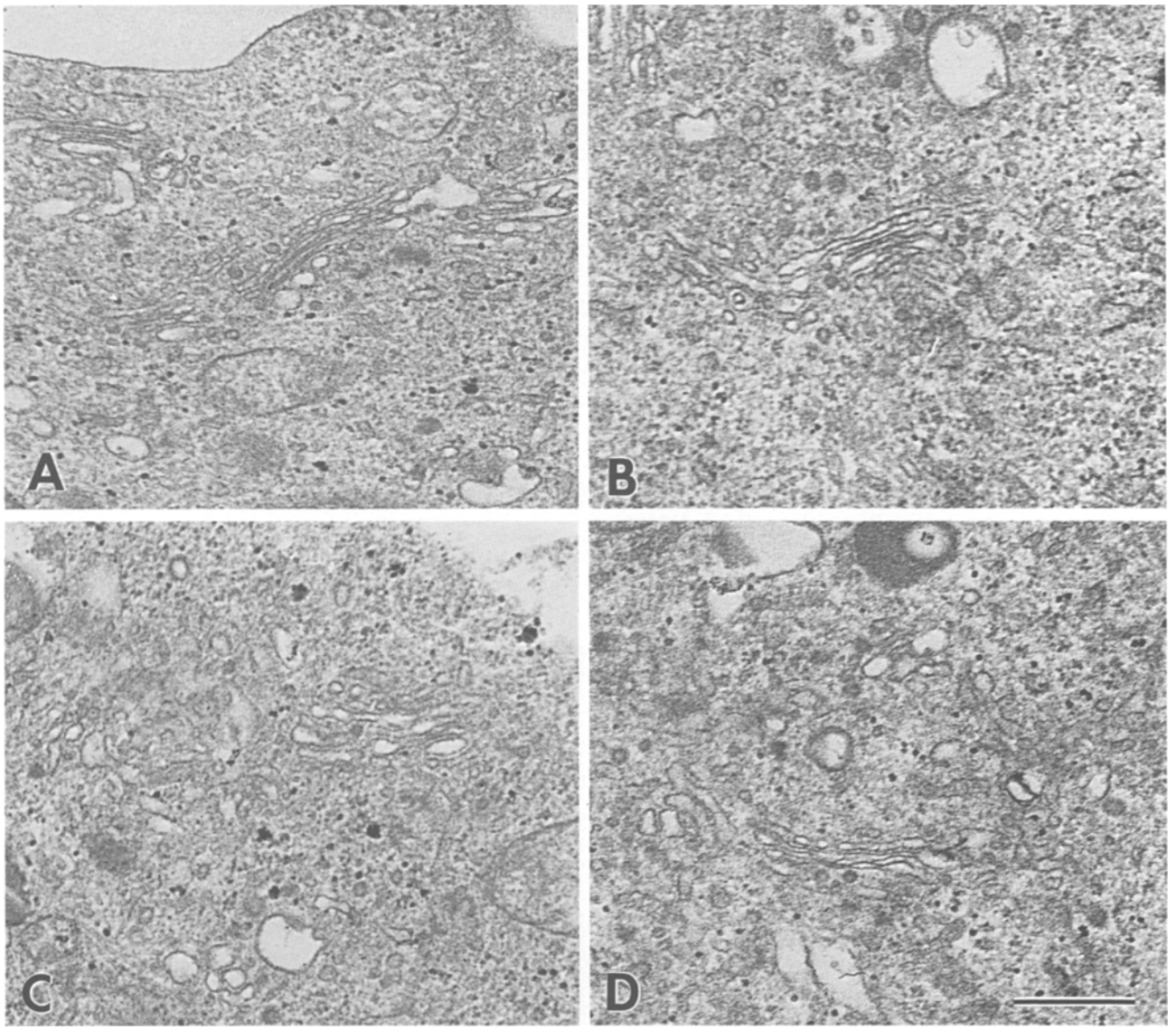

Fig. 10. Electron micrographs of K-562 cells treated in the presence or absence for 3 h illustrating the morphological appearance of the Golgi apparatus. **A** No treatment. **B** DMSO alone. **C** 100 µM LY181985 (inactive) in DMSO. **D** 100 µM LY181984 (active) in DMSO. The final DMSO concentration throughout was 0.1%. The Golgi apparatus appeared normal except for a tendency to appear smaller with fewer, shorter and more swollen cisternae per stack in the LY181984-treated cells. Bar: 0.5 µm

tion to achieve 50% inhibition of growth was about 30 µM. In contrast, the inactive LY181985 which differs from the active LY181984 only by a single chlorine atom on the B ring (right) of the sulfonylurea (Fig. 1) was nearly without effect on cell growth. Therefore, in subsequent enzymatic studies, comparisons of LY181984 were with LY181985 as a specificity control.

We had previously shown a correlation between inhibition of a plasma membrane NADH oxidase potentially involved in growth and growth control for HeLa (DM Morré et al. 1995) and transformed rat kidney and human colonic carcinoma lines (DJ Morré et al. 1995). With K-562 cells, a transient stimulation of the NADH oxidase activity of the plasma membrane by LY181984 was followed by a time-dependent inhibition of NADH oxidative activity (Fig. 2). Inhibition of plasma membrane oxidation by LY181984 was time-dependent both with whole cells and with the putative Golgi apparatus fractions.

Time-dependent inhibition has been considered a characteristic of quinone site inhibitors (Schloss et al.

1988). The sulfonylurea herbicides appear to interact with a vestigial quinone site of their target protein, acetolactate synthase, a key enzyme of branched chain amino acid biosynthesis (LaRossa and Schloss 1984). Whether or not a quinone site may be involved in the antitumor sulfonylurea inhibition of NADH oxidation in K-562 cell membranes is not known. Based on reduced NADH oxidase activity of isolated rat liver plasma membranes upon solvent extraction to remove quinones, restoration of activity by added coenzyme Q_{10} and effects of quinone inhibitors, a quinone site has been implicated for the plasma membrane NADH oxidase (Sun et al. 1992).

Despite a consistent inhibitory response to LY181984 not given by LY181985, maximum inhibition achieved was about 75% for either Golgi apparatus or plasma membranes and only about 50% for whole cells. Several explanations for a lack of complete inhibition of the NADH oxidase activity are possible. Certainly with Golgi apparatus and plasma membranes, the fractions were not pure. Based on assay of the marker enzyme succinate-INT-reductase, the preparations of plasma membranes appeared to contain perhaps as much as 25% by mitochondria with low levels of NADH oxidase activity resistant to cyanide and unresponsive to LY181984. An alternative explanation might be that plasma membranes and, perhaps Golgi apparatus as well, contained more than one NADH oxidase activity, some of which were inhibited by LY181984 and others of which were not. When a mitochondrial fraction was prepared from K-562 cells, NADH oxidation by this fraction was not inhibited by submillimolar concentrations of LY181984 as was the NADH oxidase activity of plasma membrane and Golgi apparatus. Rush et al. (1992) reported inhibition of mitochondrial oxidative phosphorylation by sulfonylureas. While activity was inhibited, however, it was inhibited nearly equally by both inactive and active sulfonylureas suggesting that some basis other than inhibition of mitochondrial activity was responsible for the differential growth inhibition by the active antitumor sulfonylureas. Our results with K-562 cells suggest that mitochondrial oxidation of NADH, either in the presence or absence of cyanide, was not inhibited by LY181984 over the range of concentrations sufficient to inhibit NADH oxidase activities of plasma membranes and Golgi apparatus and growth of cells.

A response of Golgi apparatus to LY181984 was the subject of a previous report (Morré et al. 1994 b). In cells and tissues susceptible to LY181984, membranes of the trans Golgi apparatus failed to swell to the same extent upon treatment with monensin when treated with LY181984 as compared to untreated cells. The inactive LY181985 was generally without effect. The inhibition of NADH oxidation by lower phase membranes from K-562 cells was traced to a light fraction containing structures resembling isolated Golgi apparatus cisternae. Inhibition of NADH oxidation in the fraction appeared to be as or more sensitive to inhibition by LY181984 than was that of the plasma membrane fraction.

The NADH oxidase activity inhibited by LY181984 in plasma membrane and Golgi apparatus was, in contrast to NADH oxidation by mitochondria, resistant to cyanide and took place in an argon atmosphere. These characteristics clearly distinguished the plasma membrane and Golgi apparatus activities from those of mitochondria. Because displacement of oxygen by an argon atmosphere had little or no effect on NADH oxidation by K-562 plasma membranes and Golgi apparatus fractions, some electron acceptor endogenous to the membrane preparations such as protein disulfides, must be the natural electron acceptor for the plasma membrane NADH oxidase inhibited by the antitumor sulfonylurea LY181984. The nature and identity of this natural electron acceptor is under investigation.

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