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A Circulating Form of NADH Oxidase Activity Responsive to the Antitumor Sulfonylurea *N*-4-(methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984) Specific to Sera from Cancer Patients

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Our laboratory has described a drug-responsive NADH oxidase activity of the external surface of the plasma membrane of HeLa and other cancer cells, but not from normal cells, that was shed into media conditioned by the growth of cancer cells such as HeLa. The shed form of the activity exhibited the same drug responsiveness as the plasma membrane-associated form. In this study, sera from tumor-bearing and control rats, cancer patients, normal volunteers, and patients with diseases other than cancer were collected and assayed for a cancer-specific form of NADH oxidase responsive to the antitumor sulfonylurea *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984). With sera from tumor-bearing rats and cancer patients, LY181984 added at a final concentration of 1 μM either inhibited or stimulated the activity. With sera from control rats, normal volunteers, or patients with disorders other than cancer, the drug was without effect on the NADH oxidase activity of the sera. The activity altered by the antitumor sulfonylurea was present both in freshly collected sera and in sera stored frozen. Inhibition was half maximal at about 30 nM LY181984. The sulfonylurea-altered activity was found in sera of nearly 200 cancer patients including patients with solid cancers (e.g., breast, prostate, lung, ovarian) and with leukemias and lymphomas. We postulate that the serum presence of the antitumor sulfonylurea-responsive NADH oxidase represents an origin due to shedding from the patient's cancer. If so, the antitumor-responsive NADH oxidase would represent the first reported cell surface change universally associated with all forms of human cancer.

KEY WORDS: Diarylsulfonylurea; sulfonylurea; antitumor drug; NADH oxidase; cancer; serum.

INTRODUCTION

Plasma membranes isolated from HeLa cells grown in culture bound with high affinity (K_d = 30 nM) the active antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea (LY181984)[2] (Morré *et al.*, 1995a). The antitumor sulfonylureas were identified as the result of a program of screening against *in vivo* murine solid tumors implanted subcutaneously (Grindey, 1988; Taylor *et al.*, 1989; Howbert *et al.*, 1990). Members of the series, including LY181984, were described with the potential of exhibiting a toxicity directed exclusively to transformed cells and tissues (Grindey, 1988; Taylor *et al.*, 1989; Howbert *et al.*, 1990).

Subsequently, a putative antitumor sulfonylurea binding protein with an approximate molecular weight of 34 kD was identified for the HeLa plasma membrane (Morré *et al.*, 1995b) and found to exhibit an NADH

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[2] Abreviations: LY181984, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl)urea; LY217447, *N*-(5-benzodioxolylsulfonyl)-*N'*-(4-chlorophenyl)urea.

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oxidase activity (Morré *et al.*, 1995c). This activity was inhibited by LY181984 with an EC_{50} of about 30 nM for plasma membranes of HeLa cells but not from rat liver (Morré *et al.*, 1995c) and by thiol reagents such as *N*-ethylmaleimide or *p*-chloromercuribenzoate (Morré and Morré, 1995). Also inhibited by antitumor-active but not -inactive sulfonylureas was proton release from HeLa cells and alkalinization of cytoplasm induced by diferric transferrin or ferricyanide (Sun *et al.*, 1995). The NADH oxidase activity of plasma membrane vesicles from HeLa cells was inhibited by the sulfonylurea only with NADH supplied to the external plasma membrane surface (Morré, 1995). These findings demonstrated that the sulfonylurea-inhibited activity was an ectoprotein of the plasma membrane (Morré, 1995). Subsequently, the activity was shown to be shed in soluble form from the cell surface of the HeLa cells (Morré *et al.*, 1996). The presence of a sulfonylurea-inhibited shed form of the NADH oxidase activity of media conditioned by growth of HeLa cells then prompted the present study to seek a comparable activity in sera of tumor-bearing rats and cancer patients.

The soluble shed form of the NADH oxidase activity inhibited by LY181984 and isolated from culture media conditioned by growth of HeLa S cells was similar to that associated with the outer surface of the plasma membrane (Morré *et al.*, 1996). The activity was absent from media in which cells had not been grown and was present in conditioned culture media from which cells had been removed by centrifugation both for serum-containing and serum-free media. The K_m with respect to NADH and the response to thiol reagents were similar to those of the corresponding activity of the plasma membrane of HeLa cells. The conditioned HeLa culture media bound [^{3}H]LY181984 with high affinity (Morré *et al.*, 1996). Both antitumor sulfonylurea-inhibited and -resistant forms of the NADH oxidase were isolated by preparative free-flow electrophoresis. The antitumor sulfonylurea-inhibited activity was purified to apparent homogeneity and was identified with a 33.5 (34) kD protein with an isoelectric point of about pH 4.5. The 33.5 kD protein from conditioned HeLa culture medium both bound [^{3}H]LY181984 and retained an LY181984-inhibited NADH oxidase activity.

This report describes the presence and distribution of a sulfonylurea-modulated form of the NADH oxidase from autochthonous tumors in sera of cancer patients. In contrast to the form from culture media conditioned by growth of HeLa cells which was sulfonylurea-inhibited, the serum form was either inhibited or stimulated by the antitumor sulfonylurea depending on individual patients. In either situation, the results demonstrate a shed form of the sulfonylurea-inhibited NADH oxidase potentially universally associated with malignancy.

MATERIALS AND METHODS

Animals and Rat Sera

Male Fischer 344 rats weighing 100–125 g were purchased from Harlan Animal Supply (Indianapolis, Indiana, U.S.A.). Hepatocellular carcinomas induced initially with 2-acetylaminofluorene (RLT-28) or diethylnitrosamine (RLT-N) were propagated *in vitro* in syngeneic recipients as described by Kloppel and Morré (1980). In brief, the tumors were harvested immediately after the animal was killed, cleaned in basic salt solution, and trimmed to remove any capsular or necrotic material. Approximately two pieces (1 mm × 1 mm × 7 mm) were subcutaneously injected with a cancer-implant needle (Popper and Sons, New Hyde Park, New York, U.S.A.) on the left midlateral surface. The animals were fed *ad libitum* and killed by decapitation after about 3–4 weeks and exsanguinated. After clotting for 1 to 2 h at room temperature, the blood from control and tumor-bearing animals was cleared of erythrocytes by centrifugation (1000 rpm) and serum was withdrawn and analyzed fresh. Unused serum was stored at −70°C for later analysis.

Patient Sera

Patient sera obtained from the patient populations from several sources, including the Arnett Clinic of Lafayette, Indiana, St. Elizabeth Hospital of Lafayette, Indiana, the Michiana Hematology-Oncology Polyclinic, South Bend, Indiana and the Hôpital Cantonale, Geneva, Switzerland. Sera from normal volunteers were collected by the Purdue University Health Center. Informed consent was obtained and confidentiality of medical records was assured by assigning a number to each serum sample. Patients were confirmed as having been diagnosed with active Stage III or Stage IV (metastatic) disease at the time of serum collection and cancers were identified only as to organ site. With the exception of those samples designated by "G" where sera were obtained at the time of diagnosis, all

patients had received standard radiation and/or chemotherapy appropriate to their disease conditions.

Sulfonylureas were from Lilly Research Laboratories, Indianapolis, Indiana and were dissolved in DMSO. The final concentration of DMSO in the assay was 0.1%. Blanks contained only DMSO.

Spectrophotometric NADH Oxidase Assay

NADH oxidase activity was determined at 37°C as the disappearance of NADH measured at 340 nm with 430 nm as reference using an SLM DW 2000 spectrophotometer in the dual wavelength mode of operation. The reaction mixture contained 25 mM Tris-Mes buffer (pH 7.2), 1 mM KCN, 150 μM NADH, and 200 μl serum. Stirring and recording were continuous over 5 min intervals. The activity was assayed for 5 min at 37° until a stable steady state was achieved after which 2.5 μl 1 mM LY181984 in DMSO was added (1 μM final concentration). The assay was then continued for an additional 5 min. A parallel sample with an equivalent amount of DMSO alone was assayed as a control. The rate in the absence of NADH (sera alone) was zero (<0.01 nmol/min/ml sera). The rate in the absence of sera was less than 0.1 that in the presence of sera (ca. 0.05 nmol/min). A millimolar extinction coefficient of 6.22 was used to calculate the rate of NADH disappearance.

RESULTS

Initial findings were with sera of rats. NADH oxidation rates of sera from more than 40 normal rats showed no response to the antitumor sulfonylurea LY181984 in DMSO compared to DMSO alone (mean 1.01 ± 0.32 nmol/min/ml serum for both 1 μM LY181984 in DMSO or DMSO alone comparing sera from 40 different animals). In contrast, sera from rats carrying transplantable hepatomas did respond to LY181984 (Table I). Of eleven animals carrying RLT-28 hepatomas, NADH oxidase activities of sera from three animals were inhibited, whereas with sera from the eight remaining tumor-bearing animals, the NADH oxidase activities were stimulated by 1 μM LY181984 (Table I). With two animals bearing RLT-N-hepatomas, NADH oxidase activity from sera from one of the animals was stimulated by LY181984 whereas NADH oxidase activity from sera of the other animal was inhibited. The inhibitions ranged from 10 to 50% whereas the stimulations ranged from 20 to 70%. Absolute rates of NADH oxidation of sera both from normal rats and sera of tumor-bearing animals varied from 0.35 to 3.8. With sera from a rat bearing a transplanted WJ jaw sarcoma, the activity was stimulated by LY181984 (not shown). Hemolyzed samples containing methemoglobin reductase exhibited high background rates unaffected by sulfonylureas. Even when these were excluded from consideration, the rate of NADH oxidation was about 20% greater for sera of tumor-bearing animals than for controls.

Table I. NADH Oxidase Activity of Sera from Rats Bearing Transplanted Hepatomas and Response to the Antitumor Sulfonylurea LY181984 in DMSO (+SU) Compared to DMSO Alone (−SU)

Tumor designation	NADH oxidase (nmol/min/ml serum) −SU	+SU	Ratio
1. RLT-28 hepatoma	1.1	1.3	1.2
2.	1.2	1.5	1.25
3.	0.3	0.5	1.7
4.	1.7	0.9	0.5
5.	2.4	1.7	0.7
6.	1.7	1.5	0.9
7.	1.1	1.3	1.2
8.	1.25	1.5	1.2
9.	1.0	1.25	1.25
10.	1.2	1.4	1.2
11.	0.7	1.0	1.4
Mean = 1.24 ± 0.55 (n = 11)			
1. RLT-N hepatoma	1.3	1.6	1.2
2.	3.3	2.4	0.7

Human sera also exhibited NADH oxidase activity measured spectrophotometrically as a decrease in A_{340} (Fig. 1). Activity was proportional to time and serum amount. The activity exhibited a K_m for NADH of 25 μM over the range 90–350 μM NADH. As with sera from tumor-bearing rats, LY181984 either promoted or inhibited NADH oxidase activity at a final concentration of 1 μM in DMSO compared to DMSO alone with sera from cancer patients. The response to LY181984 (inhibition or stimulation) was reproducible for a given serum sample. Additionally, both the inhibition or the stimulation was proportional to sulfonylurea concentration (Fig. 2). Inhibition or stimulation was maximal at 0.1 μM and inhibition was half maximal at about 30 nM. The NADH oxidase activity from sera of normal volunteers was unaffected by the LY181984.

To evaluate the generality of the responses of NADH oxidase activity of sera of cancer patients to the

active antitumor sulfonylurea, LY181984, individually collected sera were examined. Values are presented in the order the data were collected. No values were excluded.

With sera from 25 normal volunteers, the mean NADH oxidase activity was 0.38 ± 0.08 nmol/min/ml serum both in the presence or in the absence of 1 μM sulfonylurea in DMSO (not shown). Values were estimated to the nearest 0.02 nmol/min/ml serum. Within the variation of the measurement estimates (±5%) no change in rate, either inhibition or stimulation, could be observed in response to the sulfonylurea.

With sera from patients with breast cancer, absolute activities varied over a range of 50% or more (Table II) but were, on average, 60% higher than those of the laboratory volunteers. In sera from 19 of the 40

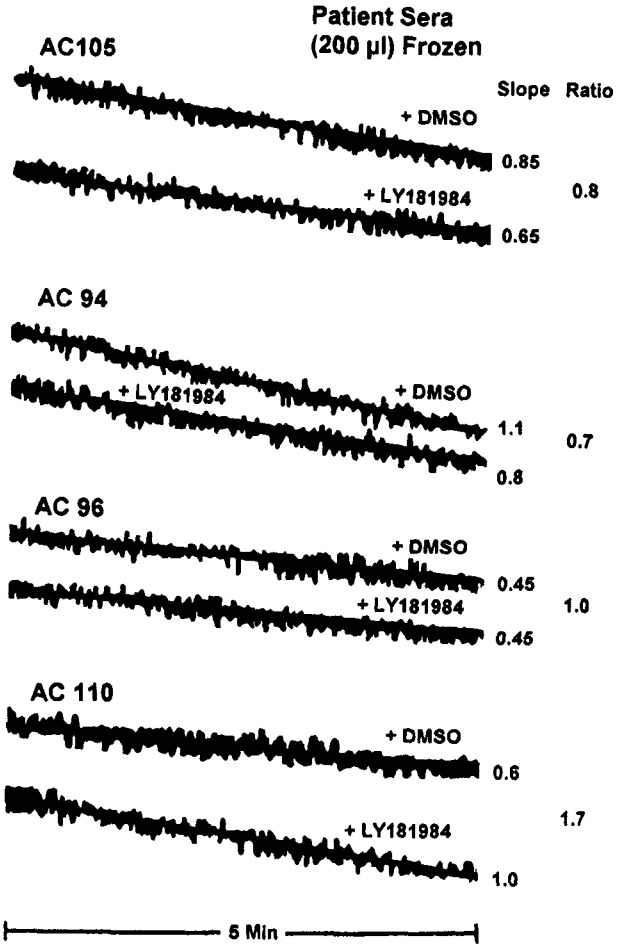

Fig. 1. Spectrophotometer traces of sera of human cancer patients showing inhibition by 1 μM antitumor sulfonylurea LY181984 in DMSO (AC-105 and AC-94), no response (AC-96), or stimulation (AC-110) compared to DMSO alone. The rates are nmol/min/ml serum.

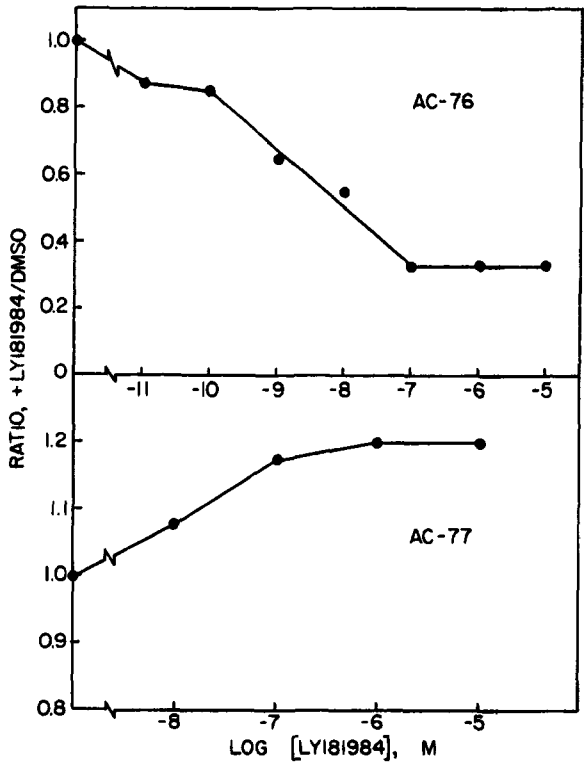

Fig. 2. Dose response to sulfonylurea of NADH oxidase of a serum sample (AC-76) where activity was inhibited and a serum sample (AC-77) where activity was stimulated. AC-76 was sera from a patient with metastatic colon cancer and AC-77 was sera from a patient with metastatic breast cancer.

patients, the sulfonylurea inhibited NADH oxidation between 20 and 60% and in sera from 16 of the 40 patients, NADH oxidation was stimulated 10 to 70%. Very similar results were obtained for sera of 23 patients with colon cancer (Table III). The mean specific activity was 0.65 ± 0.31 nmol/min/ml serum. Of these, NADH oxidase activity was inhibited with sera of 15 patients and stimulated with sera from 8 patients.

NADH oxidase activities of sera from 35 patients with lung cancer (0.71 ± 0.51 nmol/min/ml serum) also responded to sulfonylurea (Table IV). The response to sulfonylurea was a 10–50% reduction of activity in sera of 19 of the 35 patients, no response in one, and 10–40% stimulation in sera of the 15 remaining patients.

Values for NADH oxidase activity of sera from a total of 201 patients with active cancer, including patients with solid tumors arising from major organ sites, are summarized in Table V. The sulfonylurea was without effect with 8 of the 201 sera and inhibited the NADH oxidase activity in 105 and stimulated it

Table II. NADH Oxidase Activity of Sera from 40 Breast Cancer Patients with Active Disease and Response to 1 μM LY181984 in DMSO (+SU) Compared to DMSO Alone (−SU)

Designation	NADH oxidase (nmol/min/ml sera)			Designation	NADH oxidase (nmol/min/ml sera)		
	−SU	+SU	Ratio		−SU	+SU	Ratio
1. AB-5	2.0	2.7	1.35	21. SB-66	0.5	0.4	0.8
2. AB-6	1.1	0.55	0.5	22. SB-75	0.6	0.6	1.0
3. AB-7	0.6	1.0	1.7	23. SB-78	0.35	0.45	1.3
4. AB-8	0.9	1.0	1.1	24. SB-79	0.7	0.8	1.1
5. AC-15	1.05	0.75	0.7	25. SB-80	0.35	0.45	1.3
6. AC-66	0.7	0.4	0.6	26. SB-84	0.5	0.35	0.7
7. AC-70	0.3	0.2	0.7	27. SB-89	0.55	0.75	1.4
8. AC-73	0.55	0.45	0.8	28. G-12	1.3	1.5	1.2
9. AC-106	0.12	0.06	0.5	29. SB-91	0.75	0.5	0.7
10. AC-107	0.45	0.25	0.6	30. SB-93	0.65	0.75	1.2
11. SB-6	0.45	0.55	1.2	31. SB-97	0.5	0.3	0.6
12. SB-20	0.35	0.45	1.3	32. SB-100	1.6	1.8	1.1
13. SB-21	0.22	0.35	1.6	33. SB-103	0.6	0.75	1.25
14. SB-22	0.3	0.2	0.7	34. BS-104	0.45	0.35	0.8
15. SB-40	0.25	0.45	1.8	35. SB-120	0.6	0.65	1.1
16. SB-41	0.9	0.75	0.8	36. AC-108	0.55	0.35	0.6
17. SB-44	0.65	0.25	0.4	37. AC-111	0.35	0.25	0.7
18. SB-50	0.55	0.65	1.2	38. AC-118	0.55	0.45	0.8
19. SB-56	0.35	0.45	1.3	39. AC-120	0.45	0.3	0.7
20. SB-63	0.4	0.55	1.4	40. AC-122	0.35	0.5	1.4

Mean = 0.61 ± 0.37 (n = 40)

Table III. NADH Oxidase Activity of Sera from 23 Colon Cancer Patients with Active Disease and Response to 1 μM LY181984 in DMSO (+SU) Compared to DMSO Alone (−SU)

Designation	NADH oxidase (nmol/min/ml sera)			Designation	NADH oxidase (nmol/min/ml sera)		
	−SU	+SU	Ratio		−SU	+SU	Ratio
1. AB-1	0.65	1.0	1.5	13. SB-27	0.55	0.65	1.2
2. AB-2	1.15	1.6	1.4	14. SB-36	0.35	0.45	1.3
3. AB-3	1.3	1.6	1.2	15. SB-38	0.35	0.3	0.9
4. AB-4	1.45	1.8	1.2	16. SB-54	0.8	0.6	0.75
5. AC-13	0.75	0.7	0.9	17. SB-61	0.45	0.35	0.8
6. AC-27	0.45	0.4	0.9	18. SB-77	0.6	0.5	0.8
7. AC-41	0.5	0.45	0.9	19. G-3.	0.5	0.35	0.7
8. AC-46	0.8	0.6	0.75	20. G-21	0.7	0.6	0.9
9. SB-1	0.4	0.3	0.75	21. SB-102	0.75	0.65	0.9
10. SB-2	0.9	0.3	0.3	22. SB-106	0.45	0.35	0.8
11. SB-19	0.3	0.5	1.7	23. SB-119	0.3	0.45	1.5
12. SB-26	0.55	0.45	0.8				

Mean = 0.65 ± 0.31 (n = 23)

in 88. Overall the response pattern seen with breast (Table II), colon (Table III), and lung (Table IV) cancer extended to the other solid tumors (Table V).

Results with leukemias and lymphomas (Table V) were similar to those for the solid tumors (Table II–IV). Of 43 patient sera tested, the NADH oxidase activity was unchanged in 3, inhibited in 26, and stimulated in 14. The ranges of inhibitions (10–40%) and stimulations (10–50%) also was similar to those for solid tumors.

Table IV. NADH Oxidase Activity of Sera from 35 Patients with Lung Cancer and Response to 1 μM LY181984 in DMSO (+SU) Compared to DMSO Alone (−SU)[a]

Designation	NADH oxidase (nmol/min/ml sera) −SU	+SU	Ratio	Designation	NADH oxidase (nmol/min/ml sera) −SU	+SU	Ratio
1. AB-9	1.7	2.1	1.2	18. SB-73	0.45	0.5	1.1
2. AB-10	0.55	0.6	1.1	19. SB-74	0.7	0.6	0.9
3. AB-11	1.0	0.9	0.9	20. SB-81	0.55	0.6	1.1
4. AB-12	3.1	2.6	0.8	21. SB-82	0.45	0.55	1.2
5. AC-21	0.6	0.5	0.8	22. SB-85	1.15	1.0	0.9
6. AC-24	0.65	0.8	1.2	23. SB-86	0.85	0.75	0.9
7. AC-38	0.6	0.8	1.3	24. SB-87	0.65	0.5	0.8
8. AC-71	0.25	0.35	1.4	25. G-4	0.45	0.6	1.3
9. SB-5	0.4	0.2	0.5	26. G-16	0.8	1.0	1.25
10. SB-25	0.5	0.35	0.7	27. SB-99	0.35	0.6	1.7
11. SB-34	0.35	0.3	0.9	28. SB-107	0.7	0.8	1.1
12. SB-42	1.05	0.8	0.8	29. SB-108	0.45	0.65	1.4
13. SB-46	0.7	0.5	0.7	30. SB-110	0.55	0.6	1.1
14. SB-49	0.5	0.4	0.8	31. SB-113	0.35	0.3	0.9
15. SB-57	0.5	0.5	1.0	32. SB-117	0.3	0.35	1.2
16. SB-67	0.5	0.35	0.7	33. SB-121	1.2	1.05	0.9
17. SB-72	0.8	0.65	0.8	34. SB-126	0.6	0.5	0.8
				35. SB-130	0.7	0.6	0.9

Mean = 0.71 ± 0.51 (n = 35)

[a] Diagnoses represented by numbers 5, 10, 11, 14, and 19 were small cell lung cancer.

Table V. Summary of NADH Oxidase Activity of Patient Sera and Response to 1 μM LY181984 in DMSO (+SU) or DMSO Alone (−SU)

Cancer	Total number of patients	NADH oxidase (nmol/min/ml) ± S.D.	Ratio, number of patients 0.5–0.85	0.9–0.95	1.0	1.05–1.1	1.15–2.0
Breast	40	0.61 ± 0.31	19	0	1	4	16
Lung	35	0.71 ± 0.51	11	8	1	5	10
Leukemia	24	0.51 ± 0.24	10	2	0	1	11
Colon	23	0.65 ± 0.31	9	6	0	0	8
Myeloma	17	0.42 ± 0.21	8	2	0	0	7
Lymphoma (non-Hodgkin's)	16	0.50 ± 0.13	11	0	3	0	2
Ovarian	7	0.49 ± 0.13	3	1	1	1	1
Prostatic	6	0.50 ± 0.14	2	0	0	1	3
Bladder	5	0.35 ± 0.08	0	1	0	1	3
Pancreatic	5	0.50 ± 0.22	3	0	0	0	2
Rectal/anal	5	0.57 ± 0.20	2	1	0	0	2
Esophageal	4	0.44 ± 0.26	0	0	1	1	2
Hodgkin's lymphoma	3	0.57 ± 0.08	2	1	0	0	0
Renal cell	3	0.47 ± 0.16	0	0	0	0	3
Cholangiocarcinoma	1	0.35	0	0	1	0	0
Uterine	2	0.83	0	0	0	0	2
Hepatic	1	0.45	1	0	0	0	0
Melanoma	1	0.3	0	0	0	0	1
Mesothelioma	1	0.55	0	0	0	0	1
Stomach	1	0.8	0	1	0	0	0
Testicular	1	0.6	0	1	0	0	0
Total	201		81	24	8	14	74

Of the more than 200 patient sera examined, NADH oxidase activity of 96% responded to 1 μM LY181984 in DMSO. The responding sera were in two populations determined by either inhibition by LY181984 or stimulation by LY181984. Of 201 sera assayed, the NADH oxidase activities of 105 were inhibited by the sulfonylurea and the NADH oxidase activities of 88 were stimulated by the sulfonylurea (Table V).

Only 8 of the approximately 200 human sera analyzed did not appear to respond to the sulfonylurea. The basis for the lack of response was not obvious since all patients were confirmed as having active disease. When challenged with 1 μM concentrations of a second sulfonylurea, LY217447 (*N*-(5-benzodioxolylsulfonyl)-*N'*-(4-chlorophenyl) urea, however, SB-7 (ovarian), SB-75 (breast), SB-57 (lung), SB-65 and SB-71 (lymphomas), and SB-83 (esophageal) did respond whereas LY181984 evaluated in parallel still was without effect. The other two negatives (a cholangiocarcinoma and a lymphoma) could not be retested with the second sulfonylurea due to insufficient serum remaining after initial testing. LY217447 supplied in DMSO, like LY181984, did not alter the NADH oxidase activity assayed for sera of 25 normal volunteers compared to DMSO alone.

In addition, 100 sera selected at random from the outpatient population of St. Elizabeth Hospital in Lafayette, Indiana were analyzed. Of these, 91 were unresponsive to sulfonylurea, 5 were inhibited greater than 10%, and 1 was stimulated by about 10%. About 50 patients with diseases other than cancer were analyzed and NADH oxidase values were unchanged by sulfonylurea treatment.

DISCUSSION

In two previous reports (Morré, 1995; Morré *et al.*, 1996), the sulfonylurea-responsive NADH oxidase of the plasma membrane of HeLa cells was demonstrated to be an ectoprotein. While both right side-out and inside-out plasma membrane vesicles exhibited NADH oxidase activity, only the NADH oxidase activity of the right side-out vesicles was inhibited by the sulfonylurea LY181984 (Morré, 1995). The sulfonylurea did not inhibit with NADH supplied to inside-out vesicles. Being an impermeant substrate, NADH apparently was able to reach the sulfonylurea site only when supplied to right side-out vesicles, suggesting that the drug-inhibited NADH site was at the external plasma membrane surface.

As is characteristic of many ectoproteins of the mammalian cell surface, the sulfonylurea-responsive NADH oxidase was shed from the surface of HeLa cells and the shed form of the oxidase retained its sulfonylurea sensitivity (Morré *et al.*, 1996). Among the ectoproteins of the cell surface known to be shed into the media are galactosyltransferases (Podolsky and Weiser, 1979; Nishiwaki *et al.*, 1992), sialyltransferases (Bernacki and Kim, 1977; Ip and Dao, 1978), 5'-nucleotidase (Ip, 1978), and dipeptidylaminopeptidase IV (Hanski *et al.*, 1986; Fuyamada *et al.*, 1977). A sulfonylurea-inhibited shed form of the NADH oxidase activity was found in media conditioned by growth of HeLa cells (Morré *et al.*, 1996). The activity was characterized as a ca. 33.5 (34) kD peptide capable of binding [^{3}H]LY181984 (Morré *et al.*, 1996) and with NADH oxidase activity inhibited by LY181984 (Morré *et al.*, 1995c).

The molecular basis for the anticancer activity of the antitumor sulfonylureas is not known. There is no evidence for cell cycle specificity of the drugs (Sosinski *et al.*, 1993). There is no inhibition of DNA, RNA, or protein synthesis (Taylor *et al.*, 1989). The sulfonylureas exhibit few, if any, mechanistic parallels to other known antitumor agents. As a result, their mode of action may be expected to be unique. The drugs are membrane active and uncouple mitochondrial oxidative phosphorylation (Houghton *et al.*, 1990). The mitochondrial response is given, as well, by sulfonylureas inactive as antitumor agents (Rush *et al.*, 1992). Additionally, the active LY181984 but not the inactive LY181985 was shown to inhibit proton release from HeLa cells and alkalinization of cytoplasm induced by differic transferrin or ferricyanide (Sun *et al.*, 1995). The concentration dependence of the inhibition was similar to that for inhibition of NADH oxidation (Morré *et al.*, 1995c).

The existence of a shed or soluble form of the sulfonylurea-inhibited NADH oxidase in culture media conditioned by growth of HeLa cells, prompted a search for a circulating form of the drug-responsive activity. Activity was first demonstrated in rat sera and subsequently in patient sera.

The unexpected findings of both stimulation and inhibition of the soluble NADH oxidase of sera by antitumor sulfonylurea LY181984 were observed with both rat and patient sera over a range of tumors, both solid (e.g., carcinomas) and cellular (e.g., leukemias and lymphomas). The activity of sera from 25 normal

volunteers encompassing a broad spectrum of age groups (ages 21–60) was not affected by 1 μM LY181984 in DMSO compared to DMSO alone. However, with patient sera, a nearly equal number were stimulated by the drug as were inhibited.

The tendency for the LY181984 to stimulate or inhibit appeared to be a characteristic of the host and not of the tumor type as shown with the rat sera where a different response was seen even for syngeneic animals bearing a transplanted tumor of common origin. The response in the rat did not correlate with tumor burden nor was it a response to drug concentration. With both rat and human sera, the EC_{50} to LY181984 was about 30 nM both for inhibition and for stimulation.

Activity of hemolyzed serum samples was usually several times greater than sera where hemolysis was not obvious. The additional activity of the hemolyzed samples appeared to be due to an NADH methemoglobin reductase, a soluble activity of red cells (Kuma *et al.*, 1972). This activity was inhibited by LY181984 but at concentrations ca. 100-fold higher than those required to inhibit the NADH oxidase. The EC_{50} for the inhibition of the NADH oxidase was determined to be about 30 nM in serum, whereas that for the NADH methemagoblin reductase was determined to be > 5 μM. In the human studies which followed, hemolyzed serum samples were avoided.

In general, the specific activity of patient sera was higher and more variable than that of normal volunteers. A similar trend was seen with the experimental rat tumors. Results with serum freshly collected and then frozen and reassayed several days, weeks, or months later were similar. The activity appeared to be quite stable with sera stored at −70°C.

The degree of sulfonylurea responsiveness varied over the range 50% inhibition to 40% stimulation for rat sera. Human sera exhibited a similar range of response. A 10% change in rate of NADH oxidation could be detected with confidence in these studies and changes of 5% or less were recorded as unchanged from DMSO alone. With human sera, 23% of the responding sera were in the ±10% range whereas 40% were inhibited by 15 to 50%, and 37% were stimulated by 15% or more. With 100 random outpatient sera analyzed, exclusive of severely hemolyzed samples, only 3% were inhibited by 15% or more and only 2% were stimulated by 15% or more, a response rate of 5% in keeping with the expected proportion of outpatients with active cancer either diagnosed or undiagnosed.

Serum proteins, especially albumin, may bind sulfonylureas (Schultz *et al.*, 1992). Serum protein levels and compositions may vary. However, the LY181984 was added at 1 μM which is approximately one order of magnitude greater than the amount required to saturate the high-affinity sites of the circulating binding protein (Morré *et al.*, 1995a). Increasing the concentration of LY181984 to 10μM did not enhance the response (Fig. 2).

About 50 samples of sera from patients with disorders other than cancer were analyzed. Included were cardiac disorders (congestive heart failure, atrial fibrillation), respiratory disorders, e.g., bronchitis (inflammatory), respiratory failure and pneumonia (infection), and blood disorders (anemia, thrombocytopenia, and polycythemia). None exhibited NADH oxidase activities that responded to LY181984. The findings, taken together, demonstrate that the drug-responsive NADH oxidase form represents a potential pancancer marker present in sera of a wide range of patients with both solid and cellular forms of cancer.

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