

Methylthioadenosine Phosphorylase Deficiency in Human Non-Small Cell Lung Cancers¹

Tsutomu Nobori,² Istvan Szinai, Diane Amox, Barbara Parker, Olufunmilayo I. Olopade, Dorothy L. Buchhagen, and Dennis A. Carson

Department of Medicine and the Sam and Rose Stein Institute for Research on Aging, University of California, San Diego, La Jolla, California 92093-0663 [T. N., I. S., D. A., D. A. C.]; Cancer Center, University of California, San Diego, La Jolla, California 92093-0658 [B. P.]; Section of Hematology/Oncology, Department of Medicine, University of Chicago, Chicago, Illinois 60637 [O. I. O.]; National Cancer Institute-Navy Oncology Branch, National Cancer Institute, Bethesda, Maryland 20814 [D. L. B.]

ABSTRACT

Methylthioadenosine (MeSAdo) phosphorylase, a purine metabolic enzyme, is present in all normal mammalian tissues. A deficiency of this enzyme has been reported in some human leukemias and lymphomas and in a few solid tumors. In the present study, a specific immunoassay was used to assess the enzyme levels in human non-small cell lung cancer cell lines and primary tumors. We also tested the effects of MeSAdo phosphorylase-selective chemotherapy on the *in vitro* growth of enzyme-positive and enzyme-negative lung cancer cell lines. Of 29 non-small cell lung cancers, 9 (6 cell lines and 3 primary tumors, 31%) lacked detectable immunoreactive enzyme protein. Both 5,10-dideazatetrahydrofolate, an inhibitor of *de novo* purine synthesis, and methionine depletion, combined with MeSAdo, prevented the growth of the enzyme-negative non-small cell lung cancer cells under conditions in which enzyme-positive cells utilized MeSAdo to endogenously synthesize purine nucleotides and methionine. Our data suggest that MeSAdo phosphorylase deficiency is frequently found in non-small cell lung cancers and can be exploited in designing enzyme-selective chemotherapy.

INTRODUCTION

MeSAdo³ phosphorylase (methylthioadenosine: orthophosphate methylthioribosyltransferase) is involved in the metabolism of polyamines and purines. This enzyme is abundant in all normal tissues and in cell lines derived from normal cells (1) but is deficient in some cell lines established from leukemias, lymphomas, and solid tumors such as melanoma, breast cancer, lung squamous carcinoma, and rectal adenocarcinoma (1, 2). However, because the assay for MeSAdo phosphorylase catalytic activity requires commercially unavailable radiochemical substrates, because the enzyme is catalytically labile, and because contaminating normal cells (especially in the case of solid tumors) may give erroneous results with the enzyme assay, it has been difficult to determine the true incidence of the deficiency in human primary tumors. In an earlier report, we established the validity of an immunoassay in which antibodies against MeSAdo phosphorylase were used, and we quantitated the immunoreactive MeSAdo phosphorylase protein in primary human gliomas (3).

In mammalian cells, MeSAdo, the substrate for MeSAdo phosphorylase, is produced during synthesis of polyamines from decarboxylated *S*-adenosylmethionine (Fig. 1). MeSAdo does not accumulate in normal tissues but is cleaved rapidly to adenine and MTR-1-P by

MeSAdo phosphorylase (4). The adenine presumably is recycled to purine nucleotides via adenine phosphoribosyltransferase (5). The loss of MeSAdo phosphorylase, by decreasing adenine formation, would be expected to interfere with this salvage pathway. On the other hand, MTR-1-P is converted to methionine (6-9), which may also be synthesized from homocysteine by methionine synthase (10) and betaine-homocysteine methyltransferase (11). In the enzyme-negative malignant cells, however, methionine is synthesized solely from homocysteine. Accordingly, MeSAdo phosphorylase-deficient malignant cells might become more dependent than normal cells on an exogenous supply of methionine.

Thus, MeSAdo phosphorylase deficiency in human malignancy may permit the development of enzyme-selective chemotherapy, in which enzyme-negative cancer cells will be killed with drugs causing the depletion of purine nucleotides or methionine, under conditions in which enzyme-positive normal cells can be rescued by giving MeSAdo as a source of purines or methionine.

In this study, we used an immunoblotting method to screen human NSCLC cell lines and primary tumors for MeSAdo phosphorylase deficiency. The ability of a selective inhibitor of *de novo* purine synthesis, DDATHF (12, 13), in combination with MeSAdo to selectively prevent the growth of MeSAdo phosphorylase-negative NSCLC cells was also tested. Finally, we determined whether enzyme-negative NSCLC cells could proliferate in methionine-depleted medium supplemented with MeSAdo. The results indicate that 31% (9 of 29) of NSCLC cell lines and primary tumors lacked detectable enzyme protein. Moreover, only enzyme-negative NSCLC cells were prevented from growing by DDATHF, or by methionine depletion, in the presence of MeSAdo. Thus, MeSAdo phosphorylase deficiency distinguishes many NSCLCs from normal cells, and this tumor-specific metabolic abnormality may be exploited to selectively treat NSCLCs.

MATERIALS AND METHODS

DDATHF was kindly provided by Lilly Research Laboratories (Indianapolis, IN). Solutions of DDATHF were made in 0.1 N NaOH and were adjusted to pH 7.4 with phosphate-buffered saline (14). Dialyzed horse serum and D-MEM containing or lacking methionine were obtained from GIBCO BRL (Life Technologies, Inc., Gaithersburg, MD). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Tumor Tissue Specimens. The NSCLC cell lines listed in Table 1 either came from the American Type Culture Collection (Rockville, MD) or were established at the National Cancer Institute. Primary tumor specimens were obtained from the Frozen Tissue Bank at the Cancer Center of University of California, San Diego, and from the San Diego Veterans Administration Medical Center. All tested tumor specimens were estimated to contain <10% normal cells.

MeSAdo Phosphorylase Assay. Enzyme activity was measured by the radiochemical method of Pegg and Williams-Ashman (4), using [*methyl*-³H]-MeSAdo as the substrate, exactly as described earlier (5). The protein concentrations were determined by the method of Bradford (15) with bovine serum albumin as the standard.

Immunoblot Analysis. Enzyme protein was detected by a semiquantitative immunoblotting procedure, as described earlier (3). Briefly, crude extracts

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² To whom requests for reprints should be addressed, at Department of Medicine, University of California, San Diego, La Jolla, CA 92093-0663.

³ The abbreviations used are: MeSAdo, methylthioadenosine; MTR-1-P, 5'-methylthioribose 1-phosphate; NSCLC, non-small cell lung cancer; DDATHF, 5,10-dideazatetrahydrofolate; Dulbecco's modified Eagle's medium (D-MEM); BBS, buffered borate saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; kDa, kilodalton.

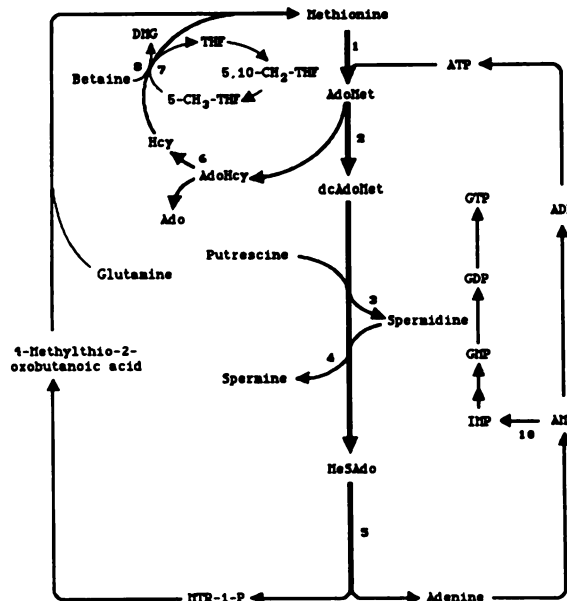


Fig. 1. Metabolic map of polyamine synthesis and MeSAdo reutilization. AdoMet, S-adenosylmethionine; dcAdoMet, decarboxylated AdoMet; MeSAdo, methylthioadenosine; AdoHcy, S-adenosylhomocysteine; Hcy, homocysteine; THF, tetrahydrofolate; Ado, adenosine; DMG, dimethylglycine. The enzymes involved are: 1, methionine adenosyltransferase; 2, AdoMet decarboxylase; 3, spermidine synthase; 4, spermine synthase; 5, MeSAdo phosphorylase; 6, AdoHcy hydrolase; 7, methionine synthase; 8, betaine-homocysteine methyltransferase; 9, adenine phosphoribosyltransferase; 10, AMP deaminase.

were separated by electrophoresis in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (16). After electrotransfer to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA), nonspecific binding sites were blocked with 1% powdered milk in BBS (0.2 M sodium borate-0.15 M NaCl, pH 8.5) containing 0.1% gelatin. The proteins were then probed for 1 h at room temperature with rabbit antiserum to MeSAdo phosphorylase, diluted 1:200 in BBS containing 1% powdered milk. After the proteins were washed with BBS containing 0.05% Tween 20, reactive bands were detected by the binding of ¹²⁵I-protein-A (1 mCi/ml; ICN Radiochemicals, Irvine, CA) for 1 h. After washing, the membranes were exposed to Kodak XAR-5 film at -80°C.

Growth Inhibition of MeSAdo Phosphorylase-negative Cells. Enzyme-positive (SK-MES-1) and -negative (A-549) cells were cultured in 96-well plates (0.2 ml/well; 5 × 10⁴ cells/ml) with various concentrations of DDATHF in D-MEM medium containing 10% dialyzed horse serum with or without MeSAdo. Alternatively, the cells were cultured in methionine-depleted medium supplemented with 10% dialyzed horse serum in the presence or absence of 16 μM MeSAdo. The growth of the cultures was measured spectrophotometrically with MTT after 4 days of incubation as described previously (17).

RESULTS

Incidence of MeSAdo Phosphorylase Deficiency in NSCLC. Having verified the validity of the immunoassay to quantitate MeSAdo phosphorylase in human gliomas (3), we used the antibodies to analyze 19 human NSCLC cell lines (10 adenocarcinomas, 4 squamous cell carcinomas, 3 large cell carcinomas, and 2 bronchoalveolar carcinomas). Immunoblot analysis demonstrated that the 32-kDa band corresponding to the homotrimeric subunit of MeSAdo phosphorylase was present in 13 cell lines, whereas 6 cell lines (3 adenocarcinomas, 2 large cell carcinomas, one bronchoalveolar carcinoma) were entirely deficient in immunoreactive enzyme protein (Table 1). Fig. 2 illustrates the appearance of representative immunoblots. In previous studies, the results with the immunoassay were found to correlate perfectly with measurements of MeSAdo phosphorylase catalytic activities (3). The lack of MeSAdo phosphorylase activity in some of the NSCLC cell lines that were deficient in immunoreactive enzyme protein was

also confirmed by direct enzyme assay (Table 1). We then analyzed 10 specimens from primary NSCLCs with different histological subtypes (Table 2). In immunoblots, the 32-kDa band was not detected in cases 1, 6, and 9 (Fig. 2B, lanes 2, 7, and 10). These three enzyme-negative tumors had the histological characteristics of adenocarcinoma.

Selective Chemotherapy of Enzyme-deficient NSCLC. We tested two chemotherapeutic regimens for the exploitation of MeSAdo phosphorylase deficiency in the NSCLC cells. When either enzyme-positive (SK-MES-1) or -negative (A-549) cells were cultured in a medium containing DDATHF, the growth of both cell lines was markedly inhibited. The concentration of DDATHF for 50% growth inhibition was 240 or 300 nM for SK-MES-1 or A-549, respectively. Why the MeSAdo phosphorylase-positive cell line was more sensitive to DDATHF is not clear. However, if MeSAdo was added to the same medium, only the SK-MES-1 cells containing MeSAdo phosphorylase were able to proliferate (Fig. 3). These data indicate that the combination of DDATHF and MeSAdo can selectively inhibit the growth of the enzyme-negative NSCLC cells.

In normal mammalian cells, methionine is synthesized from homocysteine and from MTR-1-P (6-11). MeSAdo phosphorylase-neg-

Table 1 MeSAdo phosphorylase deficiency in non-small cell lung cancer cell lines

Enzyme activities were measured radiochemically. Each value is the mean of at least two determinations and is expressed as nmol of product formed/min/mg of protein. The immunoreactive enzyme protein was determined by Western immunoblotting.

Cell line	Histology	MeSAdo phosphorylase	
		Activity	Protein ^a
A-427	Adenocarcinoma	0.283	+
A-549	Adenocarcinoma	<0.01	-
CALU-1	Squamous cell carcinoma	0.223	+
CALU-3	Adenocarcinoma	0.454	+
CALU-6	Adenocarcinoma	0.219	+
SK-LU-1	Adenocarcinoma	<0.01	-
SK-MES-1	Squamous cell carcinoma	0.261	+
Hs 242T	Adenocarcinoma	0.373	+
H322	Bronchoalveolar	<0.01	-
H441	Bronchoalveolar	0.119	+
H460	Large cell carcinoma	0.194	+
H520	Squamous cell carcinoma	N.D. ^b	+
H552	Adenocarcinoma	0.194	+
H676	Adenocarcinoma	N.D.	+
H1264	Squamous cell carcinoma	N.D.	+
H1334	Large cell carcinoma	N.D.	-
H1437	Adenocarcinoma	N.D.	-
H1581	Large cell carcinoma	N.D.	-
H1819	Adenocarcinoma	N.D.	+

^a +, present; -, absent.

^b N.D., not determined.

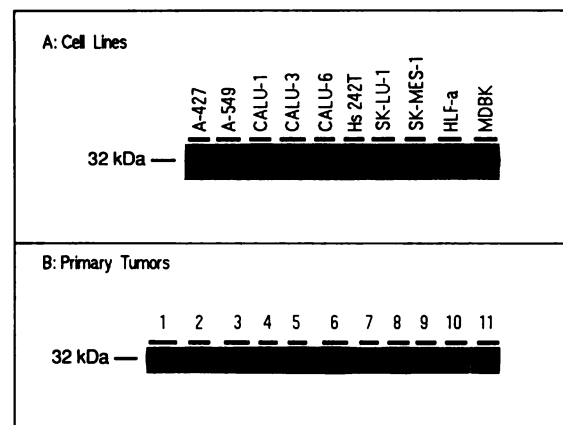


Fig. 2. Immunoblot analysis of representative cell lines (A) and primary tumors (B) from human non-small cell lung cancers. Crude extracts (150 and 200 μg per lane in A and B, respectively) were separated electrophoretically, transferred to polyvinylidene difluoride membranes, and probed with an antibody against MeSAdo phosphorylase. The bovine kidney MDBK cell line (20 μg) and the human lung fibroblast line HLF-a (150 μg) were used as positive controls. B, lane 1, MDBK cells; lanes 2-11, cases 1-10 in Table 2.

Table 2 Clinical characteristics of primary lung cancers

Case	Age (yr)/Sex	Tumor type ^a	Stage ^b	MeSAdo phosphorylase ^c
1	45/F	ADC	IIIA	-
2	^d /F	ADC	I	+
3	79/F	LCC	IV	+
4	^d /M	LCC	I	+
5	32/F	ADC	IV	+
6	^d	ADC	^d	-
7	71/M	ADC	I	+
8	52/M	ADC	I	+
9	59/M	ADC	I	-
10	65/M	SQC	IIIA	+

^a ADC, adenocarcinoma; LCC, large cell carcinoma; SQC, squamous cell carcinoma.

^b According to the classification of Mountain (31).

^c +, present; -, absent.

^d No further information available.

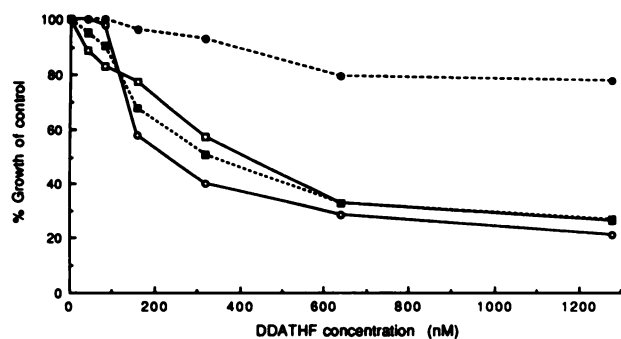


Fig. 3. Reversal of DDATHF growth inhibition of non-small cell lung cancer cells by MeSAdo. Enzyme-positive (SK-MES-1) and enzyme-negative (A-549) cell lines were incubated for 4 days with the indicated concentrations of DDATHF in D-MEM medium supplemented with 10% dialyzed horse serum in the presence or absence of 16 μ M MeSAdo. Viable cell numbers were compared with controls by the MTT colorimetric assay. The results are the average of triplicate experiments. \circ , SK-MES-1 incubated with DDATHF alone; \bullet , SK-MES-1 incubated with DDATHF and MeSAdo; \square , A-549 incubated with DDATHF alone; \blacksquare , A-549 incubated with DDATHF and MeSAdo.

ative malignant cells might become more dependent on an exogenous supply of methionine because of no methionine synthesis from MTR-1-P. We carried out experiments to determine whether the proliferation of the enzyme-negative cells could be selectively prevented in methionine-depleted medium supplemented with MeSAdo. The enzyme-positive (SK-MES-1) and -negative (A-549) cell lines were cultured for 4 days in (a) methionine-containing medium supplemented with 10% dialyzed horse serum, (b) methionine-depleted medium supplemented with 10% dialyzed horse serum, and (c) methionine-depleted medium supplemented with 10% dialyzed horse serum and 16 μ M MeSAdo. The proliferation of both cell lines, especially of the enzyme-negative A-549 cells, was markedly retarded in medium lacking methionine (27 and 3.3% growth of control for SK-MES-1 and A-549 cells, respectively). When MeSAdo was added to the same medium, it augmented the growth of enzyme-positive SK-MES-1 cells (77% growth of control). However, the proliferation of enzyme-negative A-549 cells was not enhanced in the presence of MeSAdo (4.3% growth of control) (Table 3). These data indicate that the growth of the MeSAdo phosphorylase-negative cells may be blocked selectively in methionine-depleted, MeSAdo-supplemented medium.

DISCUSSION

The absence of MeSAdo phosphorylase was first found in four murine leukemic cell lines (18). Among established human malignant tumor cell lines, 7 (23%) of 31 cell lines analyzed lacked detectable MeSAdo phosphorylase (1). In contrast, all 16 cell lines of nonmalignant origin, derived from lymphoblasts, fibroblasts, and epithelial cells, contained substantial enzyme activity (1). The enzyme defi-

ciency is not confined to tissue culture cells. Several human leukemias, as well as a few melanomas, one lung squamous carcinoma, and a rectal adenocarcinoma, have been reported to lack MeSAdo phosphorylase catalytic activity (2, 19). To facilitate screening for MeSAdo phosphorylase deficiency, we have generated antibodies to the enzyme and have developed a simple, semiquantitative immunoblot assay (3). Recently, this assay was successfully used to demonstrate that 75% of human glioma cell lines and primary malignant gliomas lack MeSAdo phosphorylase (3).

We have now used the immunoassay to quantitate MeSAdo phosphorylase in NSCLC cell lines and primary tumors. Nine (31%) of 29 NSCLC cell lines and primary tumors were completely MeSAdo phosphorylase deficient. The enzyme deficiency was most frequent in adenocarcinoma (6 of 10 enzyme-negative NSCLCs). All normal human tissues, including lung, are known to contain MeSAdo phosphorylase (1). Moreover, other studies have shown that RBCs from patients with MeSAdo phosphorylase-deficient neoplasms, including lung squamous cell carcinoma, have normal enzyme activity (2). Thus, MeSAdo phosphorylase deficiency in human NSCLCs is related to their malignant phenotype.

Lung cancer is the leading cause of death from cancer in men and the second leading cause in women. There are about 150,000 cases diagnosed each year in the United States, with almost 90% of the patients dying from the disease within 2 years of diagnosis (20). Therefore, it is of critical importance to develop procedures for its early detection that are coupled to effective therapeutic strategies. As depicted in Fig. 1, MeSAdo phosphorylase is related to the polyamine biosynthetic pathway and also to a pathway for the recycling of the stoichiometric products, adenine and MTR-1-P, to adenine nucleotides and methionine, respectively. Based on this metabolic difference between some malignant cells and normal cells, drugs such as azaserine and methotrexate have been used previously for the selective growth inhibition of MeSAdo phosphorylase-deficient leukemia cells (1). Neither enzyme-positive nor -negative cells proliferated in a medium containing azaserine alone or containing methotrexate supplemented with uridine and thymidine. But if MeSAdo was added to the same media, only cells containing MeSAdo phosphorylase divided, whereas enzyme-negative cells did not. However, because thymidylate synthesis is also inhibited by methotrexate and because azaserine is too toxic for clinical use, these strategies have not been tested *in vivo*. Recently, a new folate analogue, DDATHF, was found to inhibit glycinamide ribonucleotide transformylase, one of the key folate-dependent enzymes in *de novo* purine synthesis (13). The cytotoxic effects of DDATHF are reversed by purines (12). Our data indicate that DDATHF combined with MeSAdo can selectively prevent the proliferation of MeSAdo phosphorylase-negative cells in medium depleted of exogenous purines by dialysis. *In vivo*, purines can be obtained from dietary sources. However, in phase I studies, DDATHF has been found to have significant antitumor activity against various murine

Table 3 Selective killing of MeSAdo phosphorylase-negative cells

Cells were seeded at a density of 5×10^4 cells/ml in methionine-depleted medium supplemented with 10% dialyzed horse serum in the presence or absence of 16 μ M MeSAdo. Control cell cultures contained methionine-containing medium supplemented with 10% dialyzed horse serum. After 4 days, viable cell numbers were determined by MTT assay. The results are the means \pm SEM ($n = 10$).

Cell line	Enzyme status ^a	Growth (% of control) ^b	
		Methionine free	
		Without MeSAdo	With MeSAdo
SK-MES-1	+	27 \pm 2.6	77 \pm 4.7
A-549	-	3.3 \pm 0.6	4.3 \pm 1.1

^a +, present; -, absent.

^b Percentage of control growth = $100 \times$ (cell growth in methionine-depleted medium with or without MeSAdo)/(cell growth in methionine-containing medium).

and human solid tumors (21, 22). This suggests that diet alone cannot supply sufficient purines to support the proliferation of the malignant cells. Furthermore, folic acid and folic acid decreased the toxicity, but not the antitumor activity, of DDATHF (21, 22). The increased tumor specificity of DDATHF coadministered with folic acid or folic acid is likely due to the competitive interaction of these compounds with membrane-associated folate-binding proteins (23). The specificity of DDATHF plus MeSAdo for MeSAdo phosphorylase is based on an alternative biochemical rationale. Therefore, it is conceivable that one can use DDATHF combined with folic acid/folic acid and MeSAdo to prevent the growth of enzyme-negative malignant cells more specifically than with DDATHF combined with either folic acid/folic acid or MeSAdo.

Methionine dependency is a well-known characteristic of some malignant cells (24, 25), although its metabolic basis is unclear. Since MTR-1-P, one of the products of the MeSAdo phosphorylase reaction, is recycled to methionine, the loss of the enzyme will interfere with methionine salvage from MTR-1-P. Our data indicate that only enzyme-negative NSCLC cells fail to grow *in vitro* in methionine-depleted, MeSAdo-supplemented medium. However, we did not achieve two full logs of cell kill during the period of exposure. Although this might theoretically limit the clinical effectiveness of the chemotherapeutic protocol, Kreis et al. (26–29) have shown that purified L-methioninase (L-methionine- α -deamino- γ -mercaptomethanelyase), an L-methionine-degrading enzyme, effectively lowered plasma methionine levels and significantly inhibited the growth of Walker 256 carcinoma in rats, with negligible toxicity (25). Taken together with our data, these results suggest that chemotherapy with L-methioninase and MeSAdo may be selective for MeSAdo phosphorylase-negative NSCLC cells *in vivo*, under conditions in which normal cells can utilize MeSAdo to supplement methionine through the recycling pathway of MTR-1-P, the product of MeSAdo cleavage. Since L-methioninase, in the presence of pyridoxal 5'-phosphate, also catalyzes the α,γ elimination of L-homocysteine (30), the antitumor activity of L-methioninase and MeSAdo against enzyme-negative NSCLCs should not be prevented by the reconversion of homocysteine to methionine.

Thus, the common presence of MeSAdo phosphorylase deficiency in human NSCLC may facilitate the selective chemotherapy of this common malignancy.

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