[CANCER RESEARCH 53, 5676–5679, December 1, 1993]

Therapeutic Tumor-specific Cell Cycle Block Induced by Methionine Starvation *in Vivo*[1]

Huiyan Guo, Valeryi K. Lishko, Hector Herrera, Ami Groce, Tetsuro Kubota, and Robert M. Hoffman[2]

Anticancer, Inc., 7917 Ostrow Street, San Diego, California 92111 [H. G., V. K. L., H. H., A. G., R. M. H.]; Laboratory of Cancer Biology, University of California, San Diego, La Jolla, California 92093-0609 [R. M. H.]; and Department of Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku Ku, Tokyo 160, Japan [T. K.]

ABSTRACT

The ability to induce a specific cell cycle block selectively in the tumor could have many uses in chemotherapy. In the present study we have achieved this goal of inducing a tumor-specific cell cycle block *in vivo* by depriving Yoshida sarcoma-bearing nude mice of dietary methionine. Further, we demonstrate that methionine depletion also causes the tumor to eventually regress. The antitumor effect of methionine depletion resulted in the extended survival of the tumor-bearing mice. The mice on the methionine-deprived diets maintained their body weight for the time period studied, indicating that tumor regression was not a function of body weight loss. The data reported here support future experiments utilizing methionine depletion as a target for tumor-selective cell cycle-dependent therapy.

INTRODUCTION

There has been a continuing search for agents that can selectively arrest tumor cells, in particular with a specific cell cycle block occurring only in the tumor. Such a tumor-specific cell cycle block could possibly be exploited by additional cell cycle-specific chemotherapy.

In this light, *in vitro* studies have suggested that targeting the excessive methionine dependence of tumors may exert tumor-selective efficacy via a tumor-specific cell cycle block (1–13). Under conditions of a limiting methionine source *in vitro*, methionine-dependent tumor cell lines arrest in the late-S/G_2 phase in the cell cycle (8, 9), which we have termed the MDCCB.[3] *In vitro*, the combination of methionine starvation and cell cycle-specific chemotherapy used in cocultures of tumor and normal cells eliminated the tumor cells while allowing the normal cells to flourish (13).

We have recently demonstrated that fresh human tumors grown *in vitro* show methionine dependence by measuring the MDCCB (9). Normal cells and tissues tested are methionine independent and grow after methionine is replaced by homocysteine (1, 3, 8).

Methionine dependence may be due to overutilization of methionine for transmethylation reactions resulting in low free-methionine pools and low *S*-adenosylmethionine/*S*-adenosylhomocysteine ratios, thereby blocking cell division under conditions of a limiting methionine source (11–13).

A number of investigators have attempted to exploit the methionine dependence of tumors for therapeutic effects *in vivo*. Breillout *et al.* (14) found for the RMS-J1 rat rhabdomyosarcoma tumor that a methionine-depleted diet lowered the metastatic potential of the tumor while not having significant effects on local tumor growth in rats. Goseki *et al.* (15) found that a methionine-free TPN mixture for rats bearing the Yoshida sarcoma extended the survival of the rats and slowed tumor growth of the rats, especially with the use of doxorubicin. Kreis observed that methioninase slowed the growth of the W-256 rat carcino-sarcoma in rats (16).

We demonstrate in this report that the Yoshida tumor growing in nude mice can be induced by a methionine-free diet to have a MDCCB, indicating that a tumor-selective cell cycle block can indeed be achieved *in vivo*. We also report here that the Yoshida tumor in the nude mice can actually regress with prolonged dietary methionine starvation, resulting in an extended survival period of the mice.

MATERIALS AND METHODS

Mice. Four- to 5-week-old outbred *nu*+/*nu*+ mice were divided randomly into 2 groups. The mice were bred and housed in a high-efficiency particulate air filtered barrier room under NIH guidelines. A total of 21 mice were used in this study.

Cells. A suspension of Yoshida sarcoma containing 1×10^7 cells previously grown in Eagle's minimum essential medium with 10% fetal calf serum was injected in each mouse at the axillary and inguinal site.

Diets. Defined diets with and without methionine were obtained from Teklad (Madison, WI). The contents of the diets are listed in Table 1. The methionine-free diet was depleted of homocysteine and choline to allow extensive depletion of methionine in the animal. Mice in one group were fed a methionine-containing diet, while mice in the other group were fed a methionine-free diet. Mice were given equal measured amounts of each diet. The defined diets were started on day 2 after the injection of Yoshida sarcoma cells. The mice on the methionine-free diets were housed separately from mice on the methionine-containing diets. Both groups of animals ate all the food supplied to them.

Tumor Weight and Carcass Weight Measurements. Measurements were made at both injection sites, axillary and inguinal. The lengths of the major and minor axes were measured with calipers. The growth of the tumors was evaluated every 3 days. Estimated tumor weight (Table 2) was calculated by the equation of Goseki *et al.* (15). TETW (see Fig. 2*B*) was calculated by a modification of the equation of Goseki *et al.* (15), as follows:

$$\text{TETW (mg)} = \frac{(A + B)}{4}$$

where TETW (mg) is the combined tumor weight of both the axillary and inguinal site, A is (length of the axillary major axis)^2 × (length of the axillary minor axis), and B is (length of the inguinal major axis)^2 × (length of the inguinal minor axis). Carcass weight was calculated by subtracting the TETW from the measured body weight.

DNA Staining. Slides containing histological 3-μm sections of the s.c. grown tumor and normal colon and liver tissue taken immediately after death from animals 6–9 weeks old were incubated in preheated 1 N hydrochloric acid at 60°C for 20 min. The slides were then rinsed with distilled water 6 times to remove excess acid, stained with Schiff's reagent for 20 min at room temperature, treated with 2 successive changes of freshly prepared sulfurous acid rinse, and washed in running tap water for 5 min. After dehydration, slides were coverslipped for DNA analysis.

Cell Cycle Position Determination by DNA Measurement. A Cambridge Instruments Quantimet-520 system was used to determine the DNA content of cell nuclei by measuring integrated absorbance of each cell (9); 500 cells were measured per slide. The integrated absorbance was directly proportional to DNA content. The G_1 reference peak in the tissues of the control animals was determined as the first major peak in the histogram.

Statistical Analysis. Based on our recent report (9), the MDCCB value was calculated by the equation G_1/total cells (for methionine-containing diet)/G_1/

Received 7/12/93; accepted 9/27/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by National Cancer Institute SBIR Grant R44CA43444 and National Cancer Institute Grant R01CA27564.

[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: MDCCB, methionine-dependent cell cycle block; TPN, total parenteral nutrition; TETW, total estimated tumor weight.

Table 1 *Composition of methionine-containing diet versus methionine-free diet*

	Methionine-containing diet[a] (g/kg)	Methionine-free diet[b] (g/kg)
L-Alanine	3.5	3.5
L-Arginine HCl	12.1	12.1
L-Asparagine	6.0	6.0
L-Aspartic acid	3.5	3.5
L-Cysteine	3.5	3.5
L-Glutamic acid	40.0	40.0
Glycine	23.3	23.3
L-Histidine $HCl.H_2$	4.5	4.5
DL-Homocysteine	**None**	**None**
L-Isoleucine	8.2	8.2
L-Leucine	11.1	11.1
L-Lysine HCl	18.0	18.0
L-Methionine	**8.2**	**None**
L-Phenylaline	7.5	7.5
L-Proline	3.5	3.5
L-Serine	3.5	3.5
L-Threonine	8.2	8.2
L-Tryptophan	1.8	1.8
L-Tyrosine	5.0	5.0
L-Valine	8.2	8.2
Sucrose	490.8716	499.0716
Calcium phosphate, dibasic C_aHPO_4	4.5	4.5
Cellulose	30.0	30.0
Corn oil	100.0	100.0
Corn starch	150.0	150.0
Folic acid	0.0081	0.0081
Mineral mix AIN-76 (170915)	35.0	35.0
Vitamin mix Teklad (40060)	10	10
Vitamin B_{12} (0.1% trituration)	0.0203	0.0203

[a] Methionine-containing diet TD 93030 from Teklad.
[b] Methionine-free diet TD 92077 from Teklad.

Table 2 *Regression of the total estimated tumor weight of the Yoshida sarcoma on a methionine-free diet*

Tumors were grown in nude mice and weights were estimated as described in the text.

Mouse Site	Peak estimated tumor wt (mg)	Postregression estimated tumor wt (mg)
1 A[a]	1353.7	18.7
I	181.1	27.0
2 A	870.4	281.0
I	207.7	80.0
3 A	1053.0	252.0
I	273.6	70.3
Av.	656.6 ± 205.2[b, c]	121.5 ± 47.0[b, c]

[a] A, axillary site; I, inguinal site.
[b] Mean ± SE.
[c] $P < 0.05$, paired Student's t test.

total cells (for methionine-free diet). Methionine dependence was defined as a MDCCB value of ≤0.65 (9).

RESULTS

DNA Content Measurement by Image Analysis of Cells from Tumors Growing in Nude Mice on Methionine-containing and Methionine-free Diets. Cell cycle analysis demonstrated that cells from the tumors grown in mice on the methionine-depleted diet had a much greater DNA content than the cells from the tumors grown in mice on the methionine-containing diet (Fig. 1*A*). These results thus indicate that a MDCCB occurred in the Yoshida sarcoma grown in nude mice on the methionine-free diet. Fig. 1*B* demonstrates cell cycle analysis of normal colon tissue (control) from animals that were on methionine-containing and methionine-free diets. The MDCCB value for the Yoshida sarcoma was 0.11 and the MDCCB value for the normal tissues was 1.02. Normal liver cells in methionine-free diet-treated animals also did not have a lowered MDCCB (data not shown). These data suggest that the Yoshida sarcoma had a tumor-selective MDCCB *in vivo*, based on the cutoff of 0.65 established previously (9).

Yoshida Sarcoma Growth *in Vivo* in Nude Mice on Methionine-containing and Methionine-free Diets. As can be seen in Fig. 2*A*, the Yoshida tumor grows very rapidly in nude mice on the methionine-containing diet after s.c. injection of 10^7 cells in the axillary and inguinal site of the mouse. By day 10, the tumors in the mice on the methionine-containing diet reached a mean TETW of 845 mg. These animals were all dead by day 12 (see below). On the other hand, when the animals were on the methionine-free diet the tumors grew with a lag and then grew rapidly until attaining a mean TETW of 660 mg. At this point the tumors started to regress, with a subsequent 81% reduction of the peak mean TETW (see below). A significant difference was observed between the mean TETW after regression and the pre-death mean TETW ($P < 0.05$, paired Student's t test) (Table 2) for mice on the methionine-free diet. In 2 subsequent experiments containing 12 mice, the tumors initially grew and then regressed on average more than 50% on the methionine-free diet ($P < 0.05$), thereby repeating the initial result.

Body Weights of Yoshida Sarcoma-bearing Animals on Methionine-containing Diet Compared to Methionine-free Diet. As can be seen in Fig. 2*B*, although the carcass weights of the animals on the methionine-free diet were less than on the methionine-containing diet,

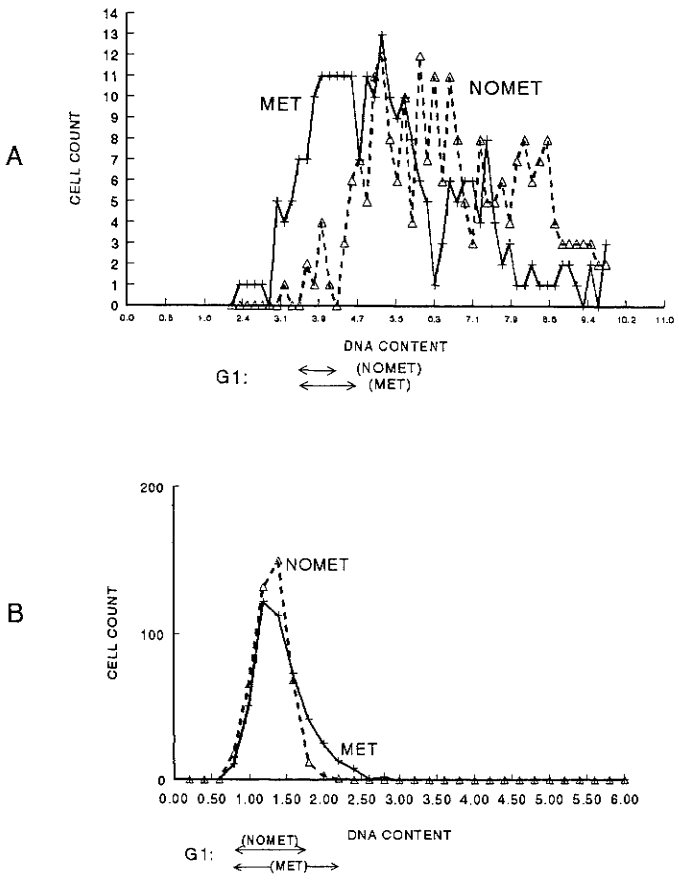

Fig. 1. Cell cycle distribution of the Yoshida sarcoma (*A*) and normal tissue (*B*) from nude mice on methionine-containing (MET) and methionine-free (NOMET) diets. The tissues were stained with Schiff's reagent and measured for DNA content, as described in the text. Note the accumulation of cells with high DNA content indicating a premitotic cell cycle block in the Yoshida sarcoma from mice on the methionine-free diet as opposed to the tumor from mice on the methionine-containing diet (*A*). Note also that even the untreated Yoshida sarcoma has a relatively high DNA content suggesting aneuploidy. Normal colon tissue, however, demonstrated no methionine-dependent cell cycle block (*B*).

there was no significant difference ($P > 0.05$, paired Student's t test) between the mean carcass weight of the mice on the 2 diets (taken on day 10, before any mice died). The animals' weights on the methionine-free diet were relatively constant for a long period, even during the time of tumor regression, indicating that tumor regression was not just a function of total body weight loss. The animals also maintained a normal performance status as determined by their ambulatory ability for approximately 30 days on the methionine-deficient diet, suggesting minimal relative toxicity (data not shown).

Survival of Nude Mice with Yoshida Sarcoma on Methionine-containing Diet Compared to Methionine-free Diet. As can be seen from Fig. 3, the nude mice bearing the Yoshida tumor on the methionine-containing diet were all dead by day 12, while the Yoshida tumor-bearing mice on the methionine-free diet were all alive at day 30, with the last animal dying at day 38. Two subsequent experiments also demonstrated extended survival of the Yoshida sarcoma-bearing mice on the methionine-free diet (data not shown). There was a significant difference between the mean survival time of the 2 groups, at $P < 0.05$ by paired Student's t test.

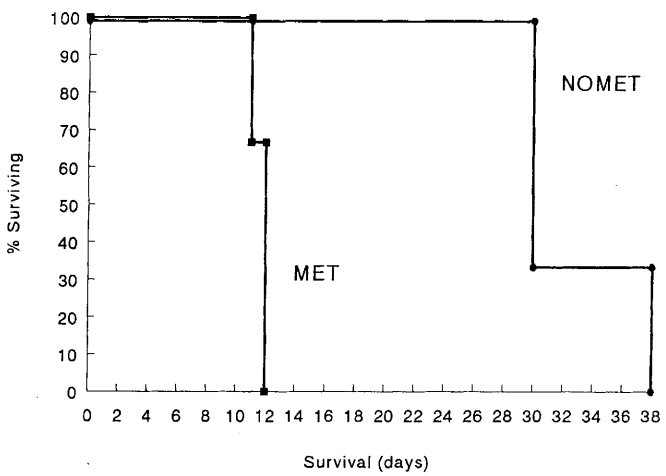

Fig. 3. Representative survival curve of 3 nude mice bearing the Yoshida sarcoma on the methionine-containing diet and 3 nude mice on the methionine-depleted diet. See "Materials and Methods" for details. Note that the animals on the methionine-free diets survived much longer ($P < 0.05$, paired Student's t test).

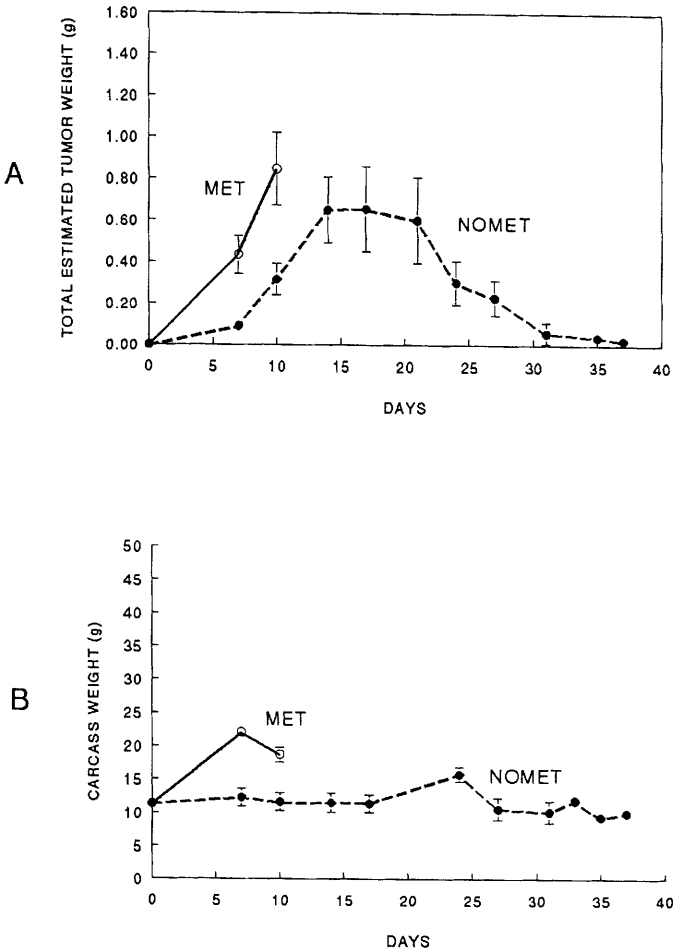

Fig. 2. *A*, growth curves of Yoshida sarcoma in 3 nude mice on the methionine-containing diet and 3 nude mice on the methionine-depleted diet. TETW was calculated from the combined axillary and inguinal sites. See "Materials and Methods" for details. Note a significant difference between the peak mean TETW and the postregression mean TETW ($P < 0.05$, Student's t test) (Table 2). *Bars*, SE. *B*, estimated carcass weights of nude mice bearing the Yoshida sarcoma on methionine-containing and methionine-depleted diets. The weights are the average of the TETW subtracted from the measured body weight for all mice alive at the time for each condition. There was no significant difference ($P > 0.05$, paired Student's t test) between the mean weights of the mice on the methionine-containing diet and the mice on the methionine-free diet (taken on day 10, before any mice died). *Bars*, SE.

DISCUSSION

The results presented here demonstrate that a tumor-selective cell cycle block (MDCCB) can be induced *in vivo*. The MDCCB value of 0.11 obtained from cell cycle analysis shows that the Yoshida sarcoma undergoes a cell cycle block when the host animal is on a methionine-free diet, as opposed to the normal tissue with a MDCCB value of approximately 1 under the same condition. The methionine-free diet lowers the serum methionine levels extensively from approximately 100 μM to approximately 30 μM.[4]

The results presented here also demonstrate that a methionine-free diet could cause tumor regression after a period of growth. The initial growth of the tumor on the methionine-depleted diet could be due to cellular and extracellular methionine stores. Earlier studies with this tumor by Goseki *et al.* (15) showed that treatment of Yoshida-sarcoma-bearing rats for a period of 8 days with TPN without methionine slowed tumor growth. However, the animals were not kept on the methionine-free TPN condition for a sufficient time to determine if the tumor would actually regress. The extended administration of the methionine-free diet in the study reported here seemed to be a key factor in demonstrating that tumor regression could occur (Fig. 2*A*). Apparently the tumor regression allowed the animals to have extended survival on the methionine-free diet (Fig. 3). However, it should be noted that the very long period on the strict diet deficient in methionine, homocysteine, and choline may have contributed to the eventual demise of animals. Future experiments will focus on dietary rescue of the animals.

It should be noted that during the period of tumor regression the carcass weight of the animals stayed relatively constant (Fig. 2*B*), demonstrating that weight loss was not a factor in the tumor regression. The maintenance of constant weight and the animals high performance status during prolonged periods of methionine starvation indicated that methionine-depleted diets may be relatively nontoxic in the period investigated.

Additional strategies may be necessary to fully cure the Yoshida sarcoma, as well as other tumors that may not be as methionine dependent, such as with the use of a methioninase to further lower circulating methionine levels (16). Another approach combines chemotherapy with methionine depletion. For example, Goseki *et al.* (15) found that doxorubicin enhanced survival and decreased growth of the

[4] O. Lishko and R. M. Hoffman, unpublished data.

Yoshida sarcoma in rats in combination with a methionine-free diet. In this light, we previously demonstrated *in vitro* that methionine-dependent tumors could be eliminated from a coculture with normal cells by first incubating the coculture in a methionine-free, homocysteine-containing (Met^-Hcy^+) medium and then shifting to a Met^+Hcy^- medium in the presence of an antimitotic drug (13). This strategy takes advantage of the reversible late-S/G_2 cell cycle block occurring over approximately 1 week caused by the Met^-Hcy^+ condition for methionine-dependent tumor cells (8). Such treatment eliminated the tumor cells and allowed the normal methionine-independent cells to grow.

Since Fig. 1 suggests that similar tumor-selective cell cycle arrest occurs *in vivo* under conditions of methionine depletion, methionine-dependent chemotherapy may also be effective *in vivo*. Indeed, preliminary experiments suggest that similar behavior occurs *in vivo* in the Yoshida sarcoma under these conditions.[5]

REFERENCES

1. Mecham, J. O., Rowitch, D., Wallace, C. D., Stern, P. H., and Hoffman, R. M. The metabolic defect of methionine dependence occurs frequently in human tumor cell lines. Biochem Biophys. Res. Commun., *117:* 429–434, 1983.
2. Chello, P., and Bertino, J. Dependence of 5-methyltetrahydrofolate utilization by L5178Y murine leukemia cells *in vitro* on the presence of hydroxycobalamin and transcobalin II. Cancer Res., *33:* 1898–1904, 1973.
3. Halpern, B. C., Clark, B. R., Hardy, D. N., Halpern, R. M., and Smith, R. A. The effect of replacement of methionine by homocysteine on survival of malignant and normal adult mammalian cells in culture. Proc. Natl. Acad. Sci. USA, *71:* 1133–1136, 1974.
4. Naylor, S. L., Busby, L. L., and Klebe, R. J. Biochemical selection systems for mammalian cells: the essential amino acids. Somat. Cell Genet., *2:* 93–111, 1976.
5. Kreis, W., and Goodenow, M. Methionine requirement and replacement by homocysteine in tissue cultures of selected rodent and human malignant and normal cells. Cancer Res., *38:* 2259–2262, 1978.
6. Kamely, D., Littlefield, J. W., and Erbe, R. Regulation of 5-methyltetrahydrofolate: homocysteine methyltransferase activity by methionine, vitamin B12, and folate in cultured baby hamster kidney cells. Proc. Natl. Acad. Sci. USA, *70:* 2585–2589, 1973.
7. Hoffman, R. M. Altered methionine metabolism, DNA methylation, and oncogene expression in carcinogenesis: a review and synthesis. Biochem. Biophys. Acta, *738:* 49–87, 1984.
8. Hoffman, R. M., and Jacobsen, S. J. Reversible growth arrest in SV40-transformed human fibroblasts. Proc. Natl. Acad. Sci. USA, *77:* 7306–7310, 1980.
9. Guo, H. Y., Herrera, H., Groce, A., and Hoffman, R. M. Expression of the biochemical defect of methionine dependence in fresh patient tumors in primary histoculture. Cancer Res., *53:* 2479–2483, 1993.
10. Coalson, D. W., Mecham, J. O., Stern, P. H., and Hoffman, R. M. Reduced availability of endogenously synthesized methionine for *S*-adenosylmethionine formation in methionine-dependent cancer cells. Proc. Natl. Acad. Sci. USA, *79:* 4248–4251, 1982.
11. Stern, R. H., Mecham, J. O., Wallace, C. D., and Hoffman, R. M. Reduced free-methionine in methionine-dependent SV40-transformed human fibroblasts synthesizing apparently normal amounts of methionine. J. Cell. Physiol., *117:* 9–14, 1983.
12. Stern, P. H., Wallace, C. D., and Hoffman, R. M. Altered methionine metabolism occurs in all members of a set of diverse human tumor cells. J. Cell. Physiol., *119:* 29–34, 1984.
13. Stern, P. H., and Hoffman, R. M. Enhanced *in vitro* selective toxicity of chemotherapeutic agents for human cancer cells based on a metabolic defect. J. Natl. Cancer Inst., *76:* 629–639, 1986.
14. Breillout, F., Hadida, F., Echinard-Darin, P., Lascaux, V., and Poupon, M-F. Decreased rat rhabdomyosarcoma pulmonary metastases in response to a low methionine diet. Anticancer Res., *7:* 861–867, 1987.
15. Goseki, N., Yamazaki, S., and Endo, M. Antitumor effect of methionine-depleting total parenteral nutrition with doxorubicin administration on Yoshida sarcoma-bearing rats. Cancer (Phila.), *69:* 1865–1872, 1992.
16. Kreis, W., and Hession, C. Biological effects of enzymatic deprivation of L-methionine in cell culture and an experimental tumor. Cancer Res., *33:* 1866–1869, 1973.

[5] H. Guo and R. M. Hoffman, unpublished data.

Therapeutic Tumor-specific Cell Cycle Block Induced by Methionine Starvation *in Vivo*

Huiyan Guo, Valeryi K. Lishko, Hector Herrera, et al.

Cancer Res 1993;53:5676-5679.