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Inhibition of Tumor Cell Growth by A Specific 6-Phosphofructo-2-kinase Inhibitor, *N*-Bromoacetylethanolamine Phosphate, and Its Analogues

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The high rate of glycolysis despite the presence of oxygen and mitochondria in tumor cells implies an important role for this process in cell division. The rate of glycolysis is assumed to be dependent on the cellular concentration of fructose 2,6-bisphosphate, the concentration of which in turn depends on a bifunctional enzyme and the ratio of this enzyme's 6-phosphofructo-2-kinase *versus* its fructose 2,6-bisphosphatase activities. To prove the hypothesis that inhibition of glycolysis in tumor cells by 6-phosphofructo-2-kinase inhibitors would cause inhibition of tumor cell proliferation, ten *N*-bromoacetylethanolamine phosphate analogues were designed, synthesized, and tested. They were screened for their activities against various human tumor cell lines to study the effects of inhibition of glycolysis on cell proliferation. The relationship between the structure of these compounds and their inhibitory activity on cell proliferation was also discussed. It was found that the activity of *N*-(2-methoxyethyl)-bromoacetamide, *N*-(2-ethoxyethyl)-bromoacetamide, and *N*-(3-methoxypropyl)-bromoacetamide was comparable to that of the positive control AraC. These three inhibitors showed *in vivo* anticancer effects in P388 transplant BDF_1 mice.

Key words: 6-Phosphofructo-2-kinase; tumor cell growth; enzyme inhibitor; glycolysis; *N*-bromoacetylethanolamine phosphate

The high rate of glycolysis despite the presence of oxygen and mitochondria in tumor cells, known as the Warburg effect,[1)] implies an important role for this process in cell division. The activity of phosphofructokinase, a key regulatory enzyme of glycolysis, depends on the cellular concentration of fructose 2,6-bisphosphate, which is the most powerful activator of phosphofructokinase.[2)] As the synthesis and hydrolysis of fructose 2,6-bisphosphate is catalyzed by a bifunctional enzyme, 6-phosphofructo-2-kinase [EC 2.7.1.105] / fructose-2,6-bisphosphatase [EC 3.1.3.46] (PFK-2/F2,6BPase), the rate of glycolysis is assumed to be dependent on the ratio of the activity of PFK-2 *versus* F2,6BPase (K/P ratio). This bifunctional enzyme is a homodimeric protein that has two distinct catalytic domains on each subunit, one for kinase activity and the other for phosphatase activity.

In mammals, several isozymes of PFK-2/F2,6BPase have been identified. Based on the differences in their molecular mass, immunogenecity, their response to phosphorylation by protein kinases, and their identified tissue origin, they are classified as liver, skeletal muscle, heart, and testis isozymes (reviewed in 3, 4). In addition to these isozymes, we cloned a new cDNA of an isozyme from human placenta,[5)] named placenta-type PFK-2/F2,6BPase, and expressed and characterized this enzyme.[6,7)] We believe that the study of the placenta-type PFK-2/F2,6BPase is significant, because the PFK-2 activity is higher than those of other isozymes, but the F2,6BPase activity is extremely low, and the K/P ratio is the highest among the known isozymes. Thus, the placenta-type PFK-2/F2,6BPase favors maintenance of an elevated F2,6BP level, resulting in a

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Abbreviations: BrAcNHEtOP, N-Bromoacetylethanolamine phosphate; PFK-2/F2,6BPase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

high glycolytic rate in cells containing placenta-type PFK-2/F2,6BPase.

These data led to the hypothesis that the placenta-type PFK-2/F2,6BPase may be active in cancer cells. This hypothesis, that the expression of the placenta-type PFK-2/F2,6BPase occurs in various human tumor cell lines, has been proven at the transcriptional and translational levels by our previous study.[8] These results suggest that proliferation of tumor cells could be inhibited by inhibiting PFK-2 activity. In this study, using an irreversible specific inhibitor of PFK-2, *N*-bromoacetylethanolamine phosphate,[9,10] we first examined if the inhibition of PFK-2 activity in tumor cells inhibited their proliferation. Next, we synthesized various derivatives of *N*-bromoacetylethanolamine phosphate to develope more effective PFK-2 inhibitors as tumor cell growth inhibitors.

Materials and Methods

Synthesis of N-bromoacethylethanolamine phosphate and Its derivatives. *N*-Bromoacetylethanolamine phosphate (BrAcNHEtOP, compound #0), a specific PFK-2 inhibitor,[9,10] was synthesized by the method of Hartman *et al.*[11] BrAcNHEtOP derivatives (#1 to #11) synthesized in this study are shown in Table 1. The following outlines the procedure for the synthesis of *N*-(3-methoxypropyl)-bromoacetamide (#3); all other derivatives (#1–2, #4–11) were obtained by a similar procedure. To a solution of 3-methoxypropylamine (1.1 g, 12 mmol) and trimethylamine (1.4 g, 14 mmol) in $CHCl_3$ (40 ml), bromoacetyl bromide (2.4 g, 12 mmol) in $CHCl_3$ (10 ml) was added dropwise at 0°C. The reaction mixture was stirred at room temperature for 5 h and then quenched with saturated NH_4Cl solution. The organic layer was separated, washed with 5% $NaHCO_3$ solution, and dried over $MgSO_4$. The solvent was removed to give a residual oil, which was distilled to give pure *N*-(3-methoxypropyl)-bromoacetamide (#3, 1.9 g, 75% yield), bp 180°C/0.5 mmHg. ^{1}H-NMR $CDCl_3$ δ 1.76–1.88 (m, 2H), 3.35–3.55 (m, 4H), 3.37 (s, 3H), 3.88 (s, 2H), 7.20-7.30 (bs, 1H). Analogue (#1), *N*-(2-Methoxyethyl)-bromoacetamide of 75% yield was prepared by the condensation of bromoacetyl bromide and 2-methoxyethylamine in the manner described above. Bp 150°C/0.5 mmHg. ^{1}H-NMR $CDCl_3$ δ 3.40 (s, 3H), 3.48–3.50 (m, 4H), 3.89 (s, 2H), 6.80–6.90 (bs, 1H). Analogue (#2), *N*-(2-Ethoxyethyl)-bromoacetamide of 70% yield was prepared by the condensation of bromoacetyl bromide and 2-ethoxyethylamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 1.22 (t, J=7.0 Hz, 3H), 3.48–3.58 (m, 6H), 3.89 (s, 2H), 6.80–7.00 (bs, 1H). Analogue (#4), *N*-(2-Methoxyethyl)-chloroacetamide of 75% yield was prepared by the condensation of chloroacetyl chloride and 2-methoxyethylamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 3.39 (s, 3H), 3.48–3.51 (m, 4H), 4.06 (s, 2H), 6.85–7.05 (bs, 1H). Analogue (#5), *N*-(2-Ethoxyethyl)-chloroacetamide of 70% yield was prepared by the condensation of chloroacetyl chloride and 2-ethoxyethylamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 1.22 (t, J=7.1 Hz, 3H), 3.40–3.60 (m, 6H), 4.06 (s, 2H), 6.90-7.05 (bs, 1H). Analogue (# 6), *N*-(3-Methoxypropyl)-chloroacetamide of 85% yield was prepared by the condensation of chloroacetyl chloride and 2-methoxypropylamine as described for the preparation of #3. Bp 130°C/0.5 mmHg. ^{1}H-NMR $CDCl_3$ δ 1.76–1.88 (m, 2H), 3.37 (s, 3H), 3.37–3.55 (m, 4H), 3.87 (s, 2H), 7.10–7.30 (bs, 1H). Analogue (#7), *N*-(2-Methoxyethyl)-2-bromo-2-methylacetamide of 40% yield was prepared by the condensation of 2-bromopropionyl bromide and 2-methoxyethylamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 1.88 (d, J= 7.1 Hz, 3H), 3.38 (s, 3H), 3.47–3.55 (m, 4H), 4.41 (q, J=7.1 Hz, 1H), 6.60–6.80 (bs, 1H). Analogue (#8), *N*-(2-Ethoxyethyl)-2-bromo-2-methylacetamide of 40% yield was prepared by the condensation of 2-bromopropionyl bromide and 2-ethoxyethylamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 1.22 (t, J=7.0 Hz, 3H), 1.88 (d, J=7.1 Hz, 3H), 3.42–3.58 (m, 6H), 4.41 (q, J=7.1 Hz, 1H), 6.65–6.85 (bs, 1H). Analogue (#9), *N*-(3-Methoxypropyl)-2-bromo-2-methylacetamide of 40% yield was prepared by the condensation of 2-bromopropionyl bromide and 2-methoxypropylamine as described for the preparation of #3. Bp 150°C/0.5 mmHg. ^{1}H-NMR $CDCl_3$ δ 1.75–1.90 (m, 2H), 1.88 (d, J=7.1 Hz, 3H), 3.33–3.54 (m, 4H), 3.37 (s, 3H), 4.40 (q, J=7.1 Hz, 1H), 7.00–7.20 (bs, 1H). Analogue (#10), *N*-(2-Hydroxyethyl)-chloroacetamide of 40% yield was prepared by the condensation of chloroacetyl chloride and ethanolamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 3.30–3.50 (bs, 1H), 3.40–3.55 (m, 2H), 3.70–3.80 (m, 2H), 4. 09 (s, 2H), 7.20–7.35 (bs, 1H). Analogue (#11), *N*-{ 2-(*O*-Chloroacetyl)-hydroxyethyl} -chloroacetamide of 40% yield prepared by the condensation of 2 eq. of chloroacetyl chloride and ethanolamine as described for the preparation of #3. ^{1}H-NMR $CDCl_3$ δ 3.60–3.70 (m, 2H), 4.09 (s, 2H), 4.11 (s, 2H), 4.30–4.40 (m, 2H), 6.80–7.00 (bs, 1H).

Cells and cell culture. Histiocytic leukemia U-937 cells, monocytic THP-1 cells, and T cell leukemia CCRF-HSB-2 cells of human origin were cultured in RPMI 1640 with 10% fetal calf serum. Oral epidermoid carcinoma KB cells and embryonic lung fibroblast HEL cells (normal diploid-cells), also of human origin, were cultured in MEM with 10% fetal calf serum. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air.

Assessment of inhibition of proliferation. Cells

were seeded at a density of approximately 1×10^5/well in 12-well tissue culture plates. These cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing either vehicle (water) or appropriate concentrations of each compound. After three days of culturing, viable cell numbers were counted by trypan blue exclusion or MTT test. IC_{50} for each compound was measured by calculating the rate of inhibition of proliferation using the following equation: Rate of inhibition of proliferation = $[1-(Tx-Co) / (Cx-Co)] \times 100$, where Tx is cell number after incubation with compounds, Cx is cell number after incubation with vehicle, and Co is cell number of before incubation.

Cytotoxicity. HEL cells were seeded at a density of approximately 1×10^4/well in 96-well tissue culture plates. After the cells attached, the medium was replaced with fresh medium containing vehicle (water) or appropriate concentrations of each compound. After the culturing for three and six days, cell viability was measured by MTT assay, and the LD_{50} of each compound was calculated.

Anticancer effect. P388 mouse leukemia cells (5×10^6 cells) were planted intraperitoneally into five-week-old female BDF_1 mice. From the day following planting, vehicle (saline containing 0.5% carboxymethylcellulose) or compounds dissolved in vehicle was injected intraperitoneally into BDF_1 mice (6 mice per a group), once a day for five days. Mice were observed daily for survival, and median survival time (MST) and increasing in life span (ILS) of each group was calculated.

Micellanea. PFK-2 activity was assayed by the method of Tominaga *et al.*[12] One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of F2,6BP per min. Lactate in cells and medium were extracted with 0.6 M perchloric acid (1:1 w/v). The extracts were neutralized with 1 M KOH and then centrifuged at 5,000 $\times g$ for 10 min. The level of lactate in the supernatants was a measured by the method of Noll.[13] AraC (1-β-D-Arabinofuranosylcystein) was commercial product of Yamasa Corporation.

Results

Inhibition of PFK-2 activity in U-937 cells by BrAcNHEtOP

BrAcNHEtOP is reported to be an irreversible, specific inhibitor of PFK-2 *in vitro*.[9,10] To find if BrAcNHEtOP inhibits PFK-2 at the cell level, U-937 cells were cultured in the presence of various concentrations of BrAcNHEtOP, and PFK-2 activity in the cell extracts was measured. As shown in Fig. 1, PFK-2 activity of U-937 cells was inhibited by BrAcNHEtOP in a dose-dependent manner.

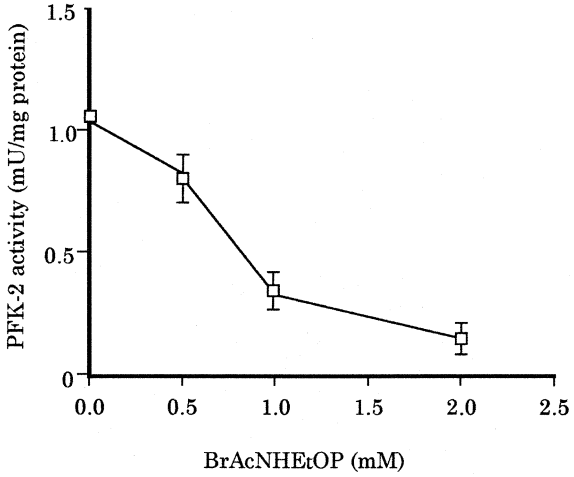

Fig. 1. Effects of BrAcNHEtOP on PFK-2 Activity in U-937 Cells.

Cells (2×10^7 cells) were seeded into 75 cm^2 tissue culture flasks with medium supplemented with 10% fetal calf serum. Cells were cultured with appropriated concentrations of BrAcNHEtOP at 37°C in a humidified atmosphere of 5% CO_2 in air. After 3 h of incubation, cells were harvested and PFK-2 activity was measured. The data represent mean values from four independent measurements.

Effects of BrAcNHEtOP and its analogues on the growth and production of lactate by various tumor cells

To discover the effects of BrAcNHEtOP and its analogues on proliferation and the inhibition of glycolysis in tumor cells, various tumor cells were cultured with the specified concentration of each compound for three days, and cell viability and lactate production were measured. The positive control AraC, an effective drug for acute leukemia that inhibits cellular DNA synthesis, and each compound tested inhibited cell proliferation in a dose-dependent fashion (data not shown). The IC_{50} values (Table 1) obtained indicated that the inhibiting activity of *N*-(2-methoxyethyl)-bromoacetamide (#1), *N*-(2-ethoxyethyl)-bromoacetamide (#2) and *N*-(3-methoxypropyl)-bromoacetamide (#3) was stronger than that of the parent compound BrAcNHEtOP and comparable to that of AraC. These compounds (#1, 2, and 3) also showed significant inhibition of lactate production by cells (Table 2).

Cytotoxicity of BrAcNHEtOP and its analogues

Cytotoxicity of BrAcNHEtOP and its analogues against normal diploid HEL cells was measured and compared with the positive control AraC. Analogue #1 has strong and #9 and #10 have weak inhibition of cell proliferation activities. All compounds tested were more cytotoxic than AraC, but the LD_{50} value of #1 was one order larger than its IC_{50} value (Table 3).

Table 1. IC_{50} Values of BrAcNHEtOP and Its Analogues against the Growth of Various Tumor Cells

Compound	IC_{50} (μg/ml)			
	CCRF-HSB-2	KB	U-937	THP-1
#0 $BrCH_2CONHCH_2CH_2OPO_3$	4.8	5.6	5.0	4.4
#1 $BrCH_2CONHCH_2CH_2OCH_3$	1.2	1.4	0.64	0.56
#2 $BrCH_2CONHCH_2CH_2OCH_2CH_3$	1.2	1.4	0.89	0.76
#3 $BrCH_2CONHCH_2CH_2CH_2OCH_3$	0.66	1.2	0.51	0.41
#4 $ClCH_2CONHCH_2CH_2OCH_3$	20	19	12	20
#5 $ClCH_2CONHCH_2CH_2OCH_2CH_3$	22	20	16	16
#6 $ClCH_2CONHCH_2CH_2CH_2OCH_3$	18	16	15	16
#7 $BrCH(CH_3)CONHCH_2CH_2OCH_3$	48	39	32	46
#8 $BrCH(CH_3)CONHCH_2CH_2OCH_2CH_3$	45	35	24	34
#9 $BrCH(CH_3)CONHCH_2CH_2CH_2OCH_3$	31	33	28	28
#10 $ClCH_2CONHCH_2CH_2OH$	33	20	ND	ND
#11 $ClCH_2CONHCH_2CH_2OCOCH_2Cl$	20	11	ND	ND
AraC	0.23	0.53	ND	ND

AraC was used as a positive control compound. ND, not determined.

Table 2. Inhibition of Lactate Production in U-937 Cells by BrAcNHEtOP and Its Analogues

Compound	Inhibition (%)		
	10 μg/ml	100 μg/ml	1 mg/ml
#0 $BrCH_2CONHCH_2CH_2OPO_3$	9.0	33.5	71.6
#1 $BrCH_2CONHCH_2CH_2OCH_3$	39.2	100	ND
#2 $BrCH_2CONHCH_2CH_2OCH_2CH_3$	19.8	95.9	ND
#3 $BrCH_2CONHCH_2CH_2CH_2OCH_3$	12.2	58.1	ND
#5 $ClCH_2CONHCH_2CH_2OCH_2CH_3$	14.3	35.9	62.3
#8 $BrCH(CH_3)CONHCH_2CH_2OCH_2CH_3$	0	22	54.7

Cells (1×10^6 cells) were seeded into 12-well plates with medium supplemented with 10% fetal calf serum and cultured with indicated concentrations of each compounds at 37°C in a humidified atmosphere of 5% CO_2 in air. After 3 h of incubation, lactate concentrations in cell suspension (cells and medium) was measured. The data represent as percent inhibition of lactate production. ND, not determined.

Table 3. Effects of BrAcNHEtOP and Its Analogues on Viability of HEL Cells

Compound	Incubation time (day)	Viability (% of control)			LD_{50} (μg/ml)
		4 μg/ml	20 μg/ml	100 μg/ml	
#0 $BrCH_2CONHCH_2CH_2OPO_3$	3	88.6	54.6	10.2	23.6
	6	89.7	55.4	5.4	23.8
#1 $BrCH_2CONHCH_2CH_2OCH_3$	3	78.4	26.2	7.9	9.6
	6	83.1	13.7	6.5	8.6
#9 $BrCH(CH_3)CONHCH_2CH_2CH_2OCH_3$	3	96.3	88.9	53.9	>100
	6	99.5	83.5	13.5	43.2
#10 $ClCH_2CONHCH_2CH_2OH$	3	93.4	66.6	9.1	31.8
	6	95.7	45.7	2.6	17.4
AraC	3	84.4	84.6	86.2	>100
	6	78.2	82.0	77.9	>100

Cells were seeded into 96-well microplates. After cells were attached, various concentrations of each compound were added. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Viability of HEL cells was measured by MTT assay after incubation for 3 and 6 days and LD_{50} values were calculated.

Anticancer effect of BrACNHEtOP analogues

P388 transplant BDF_1 mice were administered compounds or positive control AraC to measure the *in vivo* anticancer effect, median survival time (MST), and increasing in life span (ILS). As shown in Table 4, compounds #1, 2, and 3 showed slight *in vivo* anticancer effects compared to that of AraC.

Discussion

The high rate of aerobic glycolysis in tumor cells imply an important role for this process in cell division and proliferation. Earlier we reported that this high rate of glycolysis is due to the presence of the placenta-type bifunctional enzyme PFK-2/F2,6BPase in tumor cells,[8] because the placenta-type

Table 4. MST and ILS Values of P388 Transplant BDF_1 Mice Administered BrAcNHEtOP Analogues and AraC

Compound	MST (days)	ILS (%)
Control (vehicle)	10.5±2.1*	0
#1 $BrCH_2CONHCH_2CH_2OCH_3$	12±1.1	14
#2 $BrCH_2CONHCH_2CH_2OCH_2CH_3$	12±0.7	14
#3 $BrCH_2CONHCH_2CH_2CH_2OCH_3$	12±1.3	14
AraC	17±2.6	62

BrAcNHEtOP analogues (50 mg/day) and AraC (10 mg/day) were administered to mice for 5 days. *: Standard deviation.

PFK-2/F2,6BPase synthesis F2,6BP, and is the only isozyme the PFK-2 activity of which is dominant,[6] suggesting that tumor cell growth could be inhibited by PFK-2 inhibitors. *N*-Bromoacetylethanolamine phosphate (BrAcNHEtOP), originally developed as a synthetic substrate analog for fructose-1,6-bisphosphate aldolase,[11] is a specific active site-directed inactivator of PFK-2 *in vitro*.[9] This compound is a competitive inhibitor of PFK-2 with respect to fructose-6-phosphate without affecting F2,6BPase. The mechanism underlying the inhibition may involve alkylation of an active site histidine or lysine.[10] At the beginning of this study, we demonstrated that BrAcNHEtOP inhibits PFK-2 activity in tumor cells using a tumor cell line, U-937 (Fig. 1). Additionally we found that proliferation of several other tumor cells was also inhibited with IC_{50} values of approximately 5 μg/ml (Table 1). Next, we synthesized various analogues of BrAcNHEtOP for developing more effective inhibitors of tumor cell proliferation, because the IC_{50} value of BrAcNHEtOP was high compared to that of AraC, used as a positive control. IC_{50} values of three out of the 10 synthesized analogues, *N*-(2-methoxyethyl)-bromoacetamide (#1), *N*-(2-ethoxyethyl)-bromoacetamide (#2), and *N*-(3-methoxypropyl)-bromoacetamide (#3), were comparable to that of AraC. The increased activity may be caused by changing the phosphate group present on the parent inhibitor BrAcNHEtOP to an alkoxy group, such as methoxy (#1) and ethoxy (#2), or the carbon chain to a propyl group (#3), because perhaps the phosphate group is less stable than the alkoxy group in an aqueous solution. Also, after an aqueous solution of BrAcNHEtOP was allowed to stand at 4°C for 2 weeks, there was a 20-fold decrease in inhibitory activity. However, in the case of the alkoxy analogue under similar conditions, no decrease in activity was found. Furthermore, chlorine substituents (#4–6) instead of bromine (#1–3, #7–9) reduced inhibitory activity perhaps due to higher reactivity of bromine than chlorine as a leaving group. With respect to steric hindrance, the inhibitory effect of primary alkylbromides (#1–6) were more potent than secondary alkylbromides (#7–9).

In summary, this study demonstrates the relationships of inhibition of glycolysis by PFK-2 inhibitors such as compounds #1, 2, and 3 and their inhibitory effects on tumor cell growth. Also there was an increase in survival rate of mice with cancer (P388 transplant BDF_1 mice) due to the effect of these compounds, although this effect was weak compared to that of the positive control AraC. Furthermore, tumor cells (proliferating cells) were found to be more sensitive to these compounds than normal cells (nonproliferating cells) as seen by the IC_{50} and LD_{50} values measured in this study. Both energy and substrates of nucleic acid synthesis provided by glycolysis are essential for cell proliferation. Brand reported that the glycolytic rate of proliferating rat thymocytes is 19-fold higher than that of resting cells, however the rate of complete glucose oxidation is almost the same, and at the same time the proliferating thymocytes have lactate production, glucose use, and glycolytic enzyme activities that are characteristic of a variety of tumor cells.[14] Thus the proliferation of tumor cells is supposed to be dependent on the glycolysis. Taken together, we conclude that the development of PFK-2 inhibitors, especially placenta type PFK-2 specific inhibitors, could become appropriate molecular agents for tumor chemotherapy, because placenta-type PFK-2/F2,6BPase is an important regulator of glycolysis in tumor cells that in turn may be responsible for keeping high glycolytic rates in proliferating tumor cells.

References

1) Warburg, O., On the origine of cancer cells. *Science*, **123**, 309–314 (1956).
2) Uyeda, K., Furuya, E., Richard, C. S., and Yokoyama, M., Fructose-2,$6P_2$, chemistry and biological function. *Mol. Cell. Biochem.*, **48**, 97–120 (1982).
3) Uyeda, K. in "CRC Review: A study of enzymes" Vol. II, ed. by S. A. Kuby, CRC Press, Boston, pp. 445–456 (1991).
4) Kurland, I. J. and Pilkis, S. J., Covalent control of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase: insights into autoregulation of a bifunctional enzyme. *Protein Sci.*, **4**, 1023–1037 (1995).
5) Sakai, A., Kato, M., Fukasawa, M., Ishiguro, M., Furuya, E., and Sakakibara, R. Cloning of cDNA encoding for a novel isozyme of fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase from human placenta. *J. Biochem.*, **119**, 506–511 (1996).
6) Sakakibara, R., Kato, M., Okamura, N., Nakagawa, T., Komada, Y., Tominaga, N., Shimojo, M., and Fukasawa, M., Characterization of a human placental fructose-6-phosphate,2-kinase / fructose-2,6-bisphosphatase. *J. Biochem.*, **122**, 122–128 (1997).
7) Okamura, N. and Sakakibara, R., A common phosphorylation site for cyclic AMP-dependent protein kinase and protein kinase C in human placental 6 - phosphofructo - 2 - kinase/fructose - 2,6 - bisphos-

phatase. *Biosci. Biotechnol. Biochem.*, **62**, 2039–2042 (1998).
8) Hirata, T., Kato, M., Okamura, N., Fukasawa, M., and Sakakibara, R., Expression of human placental-type 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase in various cells and cell lines. *Biochem. Biophys. Res. Commun.*, **242**, 680–684 (1998).
9) Sakakibara, R., Kitajima, S., Hartman, F. C., and Uyeda, K., Hexose phosphate binding sites of fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.*, **259**, 14023–14028 (1984).
10) Kitamura, K., Uyeda, K., Hartman, F. C., Kangawa, K., and Matsuo, H., Catalytic site of rat liver and bovine heart fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase: Identification of fructose 6-phosphate binding site. *J. Biol. Chem.*, **264**, 6344–6348 (1989).
11) Hartman, F. C., Suh, B., Welch, M. H., and Barker, R., Inactivation of class I fructose diphosphate aldolase by the substrate analog *N*-bromoacetylethanolamine phosphate. *J. Biol. Chem.*, **248**, 8233–8239 (1973).
12) Tominaga, N., Minami, Y., Sakakibara, R., and Uyeda, K., Significance of the amino terminus of rat testis fructose-6-phosphate,2-kinase:fructose-2,6-bisphosphatase. *J. Biol. Chem.*, **268**, 15951–15957 (1993).
13) Noll, F. in "Methods of Enzymatic Analysis" Third ed., by H. U. Bergmwyer, Academic Press, New York and London, Vol. 6, pp. 582–588 (1984).
14) Brand, K., Aerobic glycolysis by proliferating cells: Protection against oxidative stress at the expense of energy yield. *J. Bioenerg. Biomem.*, **29**, 355–363 (1997).