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Caffeine Enhancement of the Effect of Anticancer Agents on Human Sarcoma Cells

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It is necessary to find modifiers which enhance the effects of known anticancer agents in order to improve both survival rate and local curability of patients with high-grade sarcomas. In this study, the effect of anticancer agents combined with caffeine was examined on cultured sarcoma cells and fresh human sarcoma specimens, utilizing the human tumor clonogenic assay technique. The combination of cisplatin and caffeine showed a synergistic inhibition of the growth of two strains of cultured sarcoma cells tested, and 14 of 18 fresh human sarcoma specimens (77.8%). This synergistic effect of caffeine was also observed with cyclophosphamide (44.8%), mitomycin C (44.8%) and adriamycin (27.8%). The combination of vincristine or methotrexate with caffeine, however, did not exhibit a synergistic effect. Caffeine, therefore, enhanced the effect of four cytotoxic DNA damaging agents. No antagonistic effects were seen in our series. This study suggests that caffeine may be useful in enhancing the tumoricidal effect of anticancer drugs, especially DNA-damaging agents, and possibly may aid in overcoming natural drug resistance.

Key words: Anticancer agents — Caffeine — Synergism — Human tumor clonogenic assay

In recent years, the therapy of bone and soft-tissue sarcomas has markedly improved owing to the advent of multidisciplinary treatment and especially new chemotherapeutic agents.[1-3] However, there are still few anticancer drugs which are effective against sarcomas, and tumor cells frequently acquire resistance to these during treatment. While limb preservation surgery will improve the quality of life, this type of surgery has a limited probability of curing the tumor. It is therefore necessary to find new anticancer agents, or modifiers which enhance the effects of known agents in order to improve both survival rate and local curability.

There have been several reports that caffeine, which seems to have an inhibiting effect on DNA repair, enhances the cytocidal effects of DNA-damaging agents such as ultraviolet light and alkylating agents.[4-14] In the present work, we studied the effect of combinations of several anticancer agents with caffeine against specimens of human sarcomas and two cell culture preparations.

MATERIALS AND METHOD

Materials Cultured human osteosarcoma cells (OST strain[15]) produced by successive cultivation in our laboratory, and a cultured human fibrosarcoma strain (HT-1080[16]) were used. Human sarcoma specimens obtained at the time of surgery were also studied; there were 8 osteosarcoma specimens, 3 malignant fibrous histiocytoma specimens, 2 rhabdomyosarcoma specimens, and one specimen each of epithelioid sarcoma, mesenchymal chondrosarcoma, synovial sarcoma, leiomyosarcoma, and malignant giant cell tumor, making a total of 18 specimens.

Drugs used Anticancer agents used were cisplatin (CDDP), 4-hydroperoxycyclophosphamide (CPA) as an active form of cyclophosphamide, mitomycin C (MMC), adriamycin (ADM), vincristine (VCR) and methotrexate (MTX). The concentration of anticancer agents was chosen according to the method of von Hoff *et al.*[17] at 1/10th the peak plasma concentration when administered to man. In cultured sarcoma cells, the study used a drug concentration equivalent to 1/100th the peak plasma concentration. The concentration of caffeine was either 2 m*M* or 0.2 m*M*.

Method In the human tumor clonogenic assay (HTCA), the double soft agar method developed by Hamburger and Salmon[18] was used with some modification. When the number of suspended cells obtained from the human sarcoma specimens was small, the cells were grown in a single layer cultivation system before testing.[19, 20] The number of cells disseminated was 1×10^4 per dish for OST strain, 5×10^4 per dish for HT-1080 strain and 1–5 $\times10^5$ per dish for the fresh human sarcoma cells. The cells were cultured at 37°C and in 7–7.5% CO_2 for 10 days to 3 weeks. Cell clumps of 50 or more cells were counted as colonies, and the number of these colonies was computed with a microscope and an automatic counter (Handex CP-2000). We have already discussed the clinical efficacy of human tumor clonogenic assay using sarcoma specimens, and a 1-h exposure to anticancer agents at a concentration of 1/10th the peak

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Table I. Evaluation of the Combined Effect in Human Tumor Clonogenic Assay

SF(A)≦SF(B)≦1.0	
SF(A+B)<SF(A)×SF(B)	synergistic effect
SF(A+B)=SF(A)×SF(B)	additive effect
SF(A)×SF(B)<SF(A+B)≦SF(A)	sub-additive effect
SF(A)<SF(A+B)≦SF(B)	interferential effect
SF(B)<SF(A+B)	antagonistic effect

SF, survival fraction.
We modified the evaluation system of the combination therapy proposed by Valeriote and Lin.[22]
A or B, One experiment using a single agent.
A+B, The experiment with the combination of A and B.

Table II. Combined Effect of Anticancer Agents and Caffeine on Cultured Sarcoma Cells in Human Tumor Clonogenic Assay

Drugs	Colony inhibition rate (%)	
	OST	HT-1080
0.2 m*M* caffeine	9.2	12.5
2.0 m*M* caffeine	15.0	100.0
Adriamycin (0.04 μg/ml)	2.6	6.2
+0.2 m*M* caffeine	3.9 d	50.6 a**
+2.0 m*M* caffeine	14.2 d	100.0
Cisplatin (0.2 μg/ml)	11.8	0.0
+0.2 m*M* caffeine	61.2 a**	13.9 a**
+2.0 m*M* caffeine	82.6 a**	100.0
Cyclophosphamide (3.0 μg/ml)	14.2	8.5
+0.2 m*M* caffeine	27.8 a*	56.2 a**
+2.0 m*M* caffeine	57.1 a**	100.0
Mitomycin C (0.1 μg/ml)	7.2	50.4
+0.2 m*M* caffeine	8.2 d	80.4 a**
+2.0 m*M* caffeine	8.8 d	100.0
Vincristine (0.01 μg/ml)	2.8	92.9
+0.2 m*M* caffeine	6.4 d	95.6 a
+2.0 m*M* caffeine	12.1 d	100.0
Methotrexate (30 μg/ml)	9.3	18.3
+0.2 m*M* caffeine	9.1 d	20.4 c
+2.0 m*M* caffeine	17.9 c	100.0

a, Synergistic effect; c, sub-additive effect; d, interferential effect.
*, $P<0.05$; **, $P<0.01$ vs. without caffeine by ANOVA followed by Scheffé's multiple comparison.

plasma concentration suitably reflected the clinical response.[21] Therefore after an initial 1-h exposure of the tumor cell culture to the anticancer agent, caffeine was added to the top layer for the subsequent 10-day to 3-week combined exposure.

Evaluation The experiment was conducted in triplicate and specimens showing more than 30 colonies/dish were used for evaluation. In the assay utilizing cultured cells, we calculated the mean value from five separate experiments. When colony inhibition was 50% or more, sensitivity was rated as positive. In judging the existence of a synergistic effect, additive effect or subadditive effect, the definition of Valeriote and Lin[22] was used as a guide; the treatment was judged to have a synergistic effect when the survival fraction obtained by combined administration of drugs was smaller than the product of the survival fraction obtained by administration of each drug alone (Table I). The analysis of variance (ANOVA) followed by Scheffé's multiple comparison was used to test the statistical significance of differences between results with and without caffeine addition.

RESULTS

The effect of combined administration of an anticancer agent with caffeine in cultured sarcoma cells (Table II) The combination of CDDP (0.2 μg/ml) and caffeine showed a synergistic effect on the OST strain and the HT-1080 strain. Colony inhibition was 11.8% and 0%, respectively, with CDDP alone. It increased to 61.2% and 13.9%, respectively, with combined administration of CDDP and 0.2 m*M* caffeine, demonstrating a synergistic effect. With 2 m*M* caffeine used in combination in the OST strain, colony inhibition was even greater at 82.6%. When the concentration of CDDP was 1/10 th the concentration usually used in the HTCA (0.02 μg/ml), colony inhibition of 56.2% was observed with 2 m*M* caffeine in the OST strain, and this synergistic effect was observed even with 0.2 m*M* caffeine. The combined use of CPA (3.0 μg/ml) and caffeine showed a synergistic effect against the OST and HT-1080 strain. With administration of CPA alone, the colony inhibition was 14.2% and 8.5%, respectively. Addition of 0.2 m*M* caffeine showed a synergistic effect, with 27.8% and 56.2% inhibition, respectively. When 2 m*M* caffeine was used in combination, synergism was observed with colony inhibition of 57.1% in the OST strain. When the concentration of CPA was 1/10th the concentration used in the usual HTCA (0.3 μg/ml), the combined use of caffeine at either 0.2 m*M* or 2 m*M* concentration still showed synergism. The combination of MMC and caffeine showed synergism in the HT-1080 strain. With MMC (0.1 μg/ml) and 0.2 m*M* caffeine in combination, colony inhibition increased from 50.4% to 80.4%. When the concentration of MMC was 1/10th the concentration usually used in the HTCA (0.01 μg/ml), the addition of 0.2 m*M* showed synergism with colony inhibition of 72.4% in the HT-1080 strain. The combination of ADM (0.04 μg/ml) and caffeine also showed synergism with the HT-1080

Table III. The Combined Effect of Anticancer Agents and Caffeine on Fresh Human Tumor Specimens Using Human Tumor Clonogenic Assay

Case	Colony inhibition rate (%)												
	2 m*M* Caf	CDDP	CDDP +2m*M* Caf	CDDP +0.2m*M* Caf	CPA	CPA +2m*M* Caf	CPA +0.2m*M* Caf	MMC	MMC +2m*M* Caf	MMC +0.2m*M* Caf	ADM	ADM +2m*M* Caf	ADM +0.2m*M* Caf
1. Osteosarcoma	15.6	10.8	59.3 a**	25.0 b*	9.8	16.5 c	12.2 d	21.0	29.8 c	23.8 c	53.2	61.2 c	54.5 c
2. Osteosarcoma	18.5	0.0	61.2 a**	32.1 a**	25.4	69.7 a**	50.5 a**	13.2	22.0 c	15.0 d	11.0	22.0 c	20.7 c
3. Osteosarcoma	8.9	0.0	12.9 a	10.8 a	12.3	19.2 b	15.1 c	10.5	12.5 c	11.7 c	12.5	66.6 a**	32.2 a**
4. Osteosarcoma	16.4	51.5	82.8 a**	92.8 a	52.9	79.8 a**	65.2 c	5.2	53.8 a**	25.7 a**	7.5	17.5 a	15.2 c
5. Osteosarcoma	20.2	12.5	51.9 a**	31.5 b*	52.3	68.8 a*	55.5 c	7.6	19.2 d	17.6 d	59.2	76.2 a*	60.8 c
6. Osteosarcoma	15.6	61.8	70.2 a**	67.5 c	33.4	48.2 a*	34.6 c	39.5	52.4 a*	38.2 d	6.2	17.1 c	14.7 d
7. Osteosarcoma	22.8	55.2	71.5 a**	56.8 c	13.8	14.2 d	13.5 c	56.2	73.5 a**	58.6 c	11.0	25.2 c	19.6 d
8. Osteosarcoma	19.5	23.8	53.1 a**	22.6 d	16.8	50.4 a**	35.3 a	10.2	59.3 a**	28.6 b	7.6	19.8 c	16.3 d
9. MFH	32.4	11.2	63.7 a**	50.5 a**	10.5	32.2 d	30.8 d	12.1	18.5 d	17.2 d	59.5	65.2 c	60.0 c
10. MFH	22.1	6.5	49.5 a**	13.4 d	19.7	20.2 d	19.7 d	18.5	56.2 a**	30.6 c	51.8	89.5 a**	62.4 a*
11. MFH	15.2	49.1	68.3 a**	51.2 c	15.8	26.4 c	18.8 c	57.2	59.5 c	57.8 c	5.9	18.1 c	14.6 c
12. Rhabdomyosarcoma	12.8	21.2	23.5 c	21.0 d	28.2	51.8 a**	38.2 b	13.2	18.2 c	15.0 c	18.5	21.3 c	19.2 c
13. Rhabdomyosarcoma	0.0	25.5	26.2 a	25.9 a	56.9	57.2 a	55.9 d	60.4	79.2 a**	71.2 b	23.5	38.2 a	25.6 a
14. Mesenchymal chondrosarcoma	11.5	12.0	50.0 a**	24.8 b	10.5	16.4 c	12.7 c	54.6	55.0 c	54.8 c	31.2	39.8 a*	32.0 c
15. Synovial sarcoma	21.3	18.5	60.2 a**	25.3 c	13.2	55.8 a**	20.5 d	11.2	25.1 c	22.2 c	22.2	75.4 a**	47.6 b
16. Leiomyosarcoma	10.4	58.2	59.8 c	57.5 d	0.0	9.8 d	6.5 d	23.9	31.6 b	24.5 c	62.4	72.7 a*	65.3 c
17. Epithelioid sarcoma	32.2	56.4	73.8 a*	57.5 c	26.7	30.8 d	30.5 d	39.9	67.4 a**	51.2 a**	16.2	46.2 c	22.1 c
18. MGCT	48.2	11.5	89.6 a**	54.8 a*	63.6	87.5 a**	71.2 a*	5.4	62.7 a**	58.8 a**	11.5	49.5 c	30.1 c

MFH, Malignant fibrous histiocytoma; MGCT, malignant giant cell tumor; Caf, caffeine.
a, Synergistic effect; b, additive effect; c, sub-additive effect; d, interferential effect; e, antagonistic effect.
**, $P<0.01$; *, $P<0.05$ vs. without caffeine by ANOVA followed by Scheffé's multiple comparison.

strain. With addition of ADM alone, colony inhibition was 6.7% and with 0.2 m*M* caffeine used in combination, colony inhibition rose to 50.6%.

With administration of 0.2 m*M* caffeine alone, colony inhibition was 9.2% in the OST strain and 12.5% in the HT-1080 strain, while with administration of 2 m*M* caffeine alone, the colony inhibition was 15.0% in the OST strain and 100% in the HT-1080 strain. The 100% colony inhibition shown by addition of 2 m*M* caffeine alone made it impossible to evaluate the effect of combined administration of each anticancer agent with this concentration of caffeine.

The effect of combined addition of anticancer agents and caffeine in human sarcoma specimens With addition of CDDP plus 0.2 m*M* caffeine, only 6 out of 18 specimens showed synergism. With 2 m*M* caffeine, however, the synergistic effect was observed in 14 out of 18 specimens (77.8%), and these 14 specimens showed statistically significant synergism by ANOVA followed by Scheffé's multiple comparison analysis. Thirteen of these specimens showed a synergistic effect that resulted in positive sensitivity. A subadditive effect was observed in 2 specimens. Continuous exposure to 0.2 m*M* caffeine in combination with CDDP resulted in 3 cases of a significant synergistic effect, 2 cases of a significant additive effect and 3 cases of positive sensitivity. With CPA (3.0 μg/ml) administered alone, only 4 of 18 specimens showed positive sensitivity. With 2 m*M* caffeine exposure in combination, however, 8 of 18 specimens (44.4%) showed a significant synergistic effect; of these, 7 specimens showed positive sensitivity. An additive effect was observed in 1 specimen and a sub-additive effect in 3 specimens. Continuous exposure to 0.2 m*M* caffeine in combination resulted in a significantly synergistic effect in 2 specimens. With MMC (0.1 μg/ml) and 2 m*M* caffeine added in combination, 8 of 18 specimens (44.4%) showed a statistically synergistic effect. Combination with 0.2 m*M* caffeine showed a synergistic effect in three specimens. ADM (0.04 μg/ml) and 2 m*M* caffeine administered in combination showed a synergistic effect in 6 of 18 specimens (33.3%); 5 specimens has positive sensitivity. Addition of 0.2 m*M* caffeine in combination with ADM resulted in 2 cases of synergistic effect and 1 case of an additive effect.

Addition of caffeine alone to fresh human sarcoma specimens resulted in a colony inhibition rate of 0.0%–48.2% (mean, 19.1%) and there was no case with positive sensitivity. Furthermore, there has been no specimen in which the combined use of vincristine or methotrexate with caffeine showed a synergistic effect in the two kinds of cultured sarcoma cells or in fresh human sarcoma specimens, and there has been no example of an antagonistic effect (Table III).

DISCUSSION

We have utilized HTCA as a guide to the clinical administration of cancer chemotherapeutic drugs since 1982. In our experience, the number of effective anticancer drugs in this assay is quite small. Because of this, there is need to develop new, effective anticancer agents with a broad spectrum of tumor activity and to discover drug modifiers which may increase the efficacy of known anticancer agents.

In the present study, caffeine, which is said to inhibit DNA repair, was found to increase the effect of several anticancer agents. It increased the effect of DNA-damaging agents on two kinds of cultured sarcoma cells and 18 fresh human tumor specimens. CDDP and CPA in the OST strain of cultured sarcoma cells and CPA, ADM and MMC in the HT-1080 strain, when used in combination with caffeine, showed a marked synergistic effect that resulted in increased drug sensitivity. In human sarcoma specimens, the combination of caffeine and anticancer agent also produced a synergistic effect. The synergism was observed with three of the anticancer agents in 7 specimens, with two of the anticancer agents in 4 specimens and with one of the anticancer agents in 7 specimens. This finding suggests that caffeine will enhance the effect of such-DNA-damaging agents as CDDP, CPA, MMC and ADM. It appeared to show the greatest synergy with CDDP.

In 4 specimens with CDDP, 2 specimens with CPA, 2 specimens with MMC and 3 specimens with ADM, specimens which already showed positive sensitivity showed increased inhibition after caffeine addition. In 9 specimens with CDDP, 4 specimens with CPA, 6 specimens with MMC and 2 specimens with ADM, specimens which lacked sensitivity to these drugs showed positive sensitivity to the combination treatment with 2 m*M* caffeine. This suggests that caffeine will be useful in overcoming resistance to some anticancer agents. In this study we have not studied multiple drug combinations or tumors other than sarcomas. However, we would anticipate that multidrug additions of DNA-damaging agents in combination with caffeine will also show a marked synergistic effect and that this potentiation will be equally evident in carcinomas as in sarcomas.

Ishida *et al.*[23] suggested that caffeine itself damages DNA. Whether caffeine has an anticancer effect remains unknown. With addition of 2 m*M* caffeine alone in our experiments, colony inhibition was 8.9%–48.2% in fresh human sarcoma specimens, but there was no specimen that showed positive sensitivity. However, in the HT-1080 culture which is derived from a human fibrosarcoma, exposure to 2 m*M* caffeine alone caused 100% colony inhibition. This finding suggests that caffeine alone may exert a tumor-specific cytocidal effect. On the other hand, it has been reported by Kihlman[5] and Roberts[10, 11] that the activity of DNA-damaging agents is increased by caffeine. van den Berg *et al.*[13] mentioned that CDDP toxicity and chromosome breakage are increased by caffeine. Pardee *et al.*, using baby hamster kidney cells, reported that combined use of nitrogen mustard and caffeine showed marked synergism.[6, 8] Furthermore, Roberts and Kotsaki-Kovatsi, using Chinese hamster cells (V79-379A) reported that the action of sulfur mustard and CDDP is increased by caffeine.[12] The mechanism by which caffeine increases the effect of anticancer agents was discussed in these reports. Pardee and Lau[6] reported an increase in the cytocidal effect of nitrogen mustard when used in combination with caffeine. Cells with damaged DNA show a prolonged G2 phase to accomodate increased DNA repair. Caffeine is believed to shorten the G2 phase by accelerating DNA synthesis or by stimulating replicon initiation. This results in the cell entering the mitotic phase without adequate DNA repair, probably leading to an increase in cell death.[6, 8] Contrary to this hypothesis, Roberts and Kotsaki-Kovatsi have suggested that the increase in the cytocidal effect on combined use of sulfur mustard, CDDP and caffeine is due to the double strands of DNA being destroyed by inhibition of replicon initiation.[12] There have been reports that the cytocidal effect of ultraviolet light or MMC is increased when caffeine is present in the G2 phase.[4, 7] Many aspects of the mechanism by which the effect of anticancer agents is increased by caffeine have yet to be elucidated. It has been suggested that the mechanism of caffeine action differs from one anticancer agent to another. Iliakis and Lazer reported that ADM and caffeine showed antagonism with Chinese hamster cells,[24] but with the various sarcoma cells used in this study we found no antagonistic effect. In one report, when exposure to an anticancer agent and caffeine was only for one hour, no synergistic effect was observed.[25] In the clonogenic assay used in this study, prolonged contact with caffeine may have brought about the high percentage of synergistic effect. This suggests that the effect of caffeine may be time-dependent.

In this study, caffeine markedly increased the tumoricidal effect of DNA synthesis-inhibiting drugs,

particularly CDDP. Caffeine has already gained wide clinical use as a cardiotonic diuretic and central nervous system stimulant, and the pharmacology of caffeine has been well characterized after nontoxic po doses. However severe neurotoxicity, which may be counteracted by barbiturates, is encountered after high-dose administration of caffeine. If this side effect of caffeine could be overcome, caffeine could be a useful drug capable of overcoming drug resistance in sarcoma chemotherapy.

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REFERENCES

1) Gehan, E. A., Glover, F. N., Maurer, H. M., Sutow, W. W., Hays, D. W., Lawrence, W., Newton, W. A. and Soule, E. H. Prognostic factors in children with rhabdomyosarcoma. *Natl. Cancer Inst. Monogr.*, **56**, 83–92 (1981).
2) Link, M. P. Adjuvant therapy in the treatment of osteosarcoma. *In* "Important Advances in Oncology," 1st Ed., pp. 193–207(1986). Lippincott, Philadelphia.
3) Rosen, G. Current management of Ewing's sarcoma. *Prog. Clin. Cancer,* **8**, 267–282 (1982).
4) Hansson, K., Kihlman, B. A., Tanzarella, C. and Palitti, F. Influence of caffeine and 3-aminobenzamide in G2 on the frequency of chromosomal aberrations induced by thiotepa, mitomycin C and N-methyl-N-nitro-N-nitrosoguanidine in human lymphocytes. *Mutat. Res.*, **126**, 251–258 (1984).
5) Kihlman, B. A. "Caffeine and Chromosome" (1977). Elsevier, Amsterdam.
6) Lau, C. C. and Pardee, A. B. Mechanism by which caffeine potentiates lethality of nitrogen mustard. *Proc. Natl. Acad. Sci. USA,* **79**, 2942–2946 (1982).
7) Palitti, F., Tanzarella, C., Degrassi, F., De Salvia, R., Fiore, M. and Natarajan, A. Formation of chromatid-type aberration in G2 stage of the cell cycle. *Mutat. Res.*, **110**, 343–350 (1983).
8) Pardee, A. B., Boorstein, R. J. and Lau, C. C. Interference with DNA repair mechanisms of mammalian cells: cell cycle dependence. *In* "ADP-Ribosylation, DNA repair and Cancer," ed. M. Miwa, pp. 287–294 (1983). Japan Scientific Societies Press, Tokyo/VNU Science Press, Utrecht.
9) Rauth, A. M. Evidence for dark-reactivation of ultraviolet light damage in mouse L cells. *Radiat. Res.*, **31**, 121–138 (1967).
10) Roberts, J. J. Repair of DNA modified by cytotoxic, mutagenic and carcinogenic chemicals. *In* "Advances in Radiation Biology," ed. J. T. Lett and H. Adler, pp. 211–436 (1978). Academic Press, New York.
11) Roberts, J. J. Mechanism of potentiation by caffeine of genotoxic damage induced by physical and chemical agents. *In* "DNA Repair and its Inhibition," ed. A. Collins, C. S. Downs and R. T. Johnson, pp. 193–215 (1984). IRL Press, Oxford.
12) Roberts, J. J. and Kotsaki-Kovatsi, V. Potentiation of sulphur mustard or cisplatin-induced toxicity by caffeine in Chinese hamster cells correlates with formation of DNA double-strand breaks during replication on a damaged template. *Mutat. Res.*, **165**, 207–220 (1986).
13) van den Berg, H. W. and Roberts, J. J. Postreplication repair of DNA in Chinese hamster cells treated with cis-platinum(II) diammine dichloride; enhancement of toxicity and chromosome damage by caffeine. *Mutat. Res.*, **33**, 279–284 (1975).
14) Waldren, C. A. and Rasko, I. Caffeine enhancement of X-ray killing in cultured human and rodent cells. *Radiat. Res.*, **73**, 95–110 (1978).
15) Yamazaki, A. Establishment of cell strain of human osteogenic sarcoma cells and histological appearance of this strain in tissue culture. *J. Juzen Med. Soc.*, **71**, 1–15 (1964).
16) Rasheed, S., Nelson-Ress, W. A., Toth, E. M., Arnstein, P. and Gardner, M. B. Characterization of a newly derived human sarcoma cell line (HT-1080). *Cancer,* **33**, 1027–1033 (1974).
17) von Hoff, D. D., Casper, J., Bradley, E., Sandbach, J., Jones, D. and Makuch, R. Association between human tumor colony-forming assay result and response of an individual patient's tumor to chemotherapy. *Am. J. Med.*, **70**, 1027–1032 (1981).
18) Hamburger, A. W. and Salmon, S. E. Primary bioassay of human tumor stem cells. *Science,* **197**, 461–463 (1977).
19) Tomita, K., Yokogawa, A. and Nomura, S. Human tumor clonogenic assay in osteosarcoma for evaluation of clinical efficacy of anti-cancer drugs. *Cancer Chemother. Pharmacol.*, **18**, 133–136 (1986).
20) Yokogawa, A. and Tomita, K. Experimental study on *in vitro* colony assay of sarcoma in extremities. Recent advances in chemotherapy. *Proc. 14th Int. Congress of Chemotherapy,* 292–293 (1985).
21) Tomita, K. and Tsuchiya, H. A study on human tumor clonogenic assay (HTCA) for malignant bone and soft tissue tumor. *Orthop. Surg. Traumatol.*, **31**, 407–414 (1988).
22) Valeriote, F. and Lin, S. Synergistic interaction of anticancer agents. A cellular perspective. *Cancer Chem. Rep.*, **59**, 895–900 (1975).

23) Ishida, R., Kozaki, M. and Takahashi, T. Caffeine alone cauese DNA damage in Chinese hamster ovary cells. *Cell Struct. Funct.*, **10**, 405–409 (1985).
24) Iliakis, G. and Lazar, W. Reduction by caffeine of adriamycin-induced cell killing and DNA damage in Chinese hamster cells: correlation with modulation in intracellular adriamycin content. *Cancer Res.*, **47**, 2224–2229 (1987).
25) Tsuchiya, H. and Tomita, K. The combined effect of antitumor agents with caffeine or calcium channel blockers on sarcoma cells by clonogenic assay. *J. Jpn. Soc. Cancer Ther.*, **22**, 1003–1012 (1987).