J. Pharm. Pharmacol. 1998, 50: 1173–1177
Received March 5, 1998
Accepted June 12, 1998

Synergistic Effects of Some Pairs of Antioxidants and Related Agents on Mouse Leukaemia L5178Y Cell Growth In-vitro

TAKAHIRO SUZUKI, TORU EZURE AND MASARU ISHIDA

Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan

Abstract

The effects of simultaneous administration of some dyadic combinations of antioxidants or vitamins and related agents on cellular proliferation of mouse leukaemia L5178Y cells in-vitro have been examined experimentally.

The data were analysed on the basis of the concept of independence for evaluation of interactions between biologically active agents. An approach for evaluation of the synergism or antagonism of the action of two agents is proposed in which the types and extents of interactions are described by response-surface diagrams. The combinations phytol with *trans*-retinol, abscisic acid with *trans*-retinol, and menadione with sodium L-ascorbate were synergistic, whereas menadione with *trans*-retinol, and plumbagin with *trans*-retinol were antagonistic in the dose-range tested.

These results reveal that the interactions between two agents depend not only on the combinations of agents but also on the dose ranges or the ratios of agents under the experimental domain studied.

It has been observed that natural products such as vitamins A, C, E and K_3, or carotenoids from a wide variety of organisms and higher plants have antineoplastic activities (Mathews-Roth 1982; Schwartz & Shklar 1987; Combs et al 1989; Wu et al 1993). A promising approach to increasing the effectiveness of anticancer therapy is to seek combinations of agents for which the efficacy is greater than that expected from the effects of single agents. Synergistic effects of various antioxidants on prevention of cancer in man were addressed by Gey et al (1987). Stimulated antitumour activity by interleukin-2 and murine interferon-γ in non-adherent peritoneal cell cultures from tumour dormant mice has also been observed (Chen et al 1990), and potentiating effects of combined administration of sodium ascorbate (vitamin C) and menadione (vitamin K_3) on tumour cell-lines from man have been reported (Noto et al 1989; Venugopal et al 1996). However, the combined actions of such agents have not been well studied quantitatively.

We have previously reported the in-vitro cytotoxicity of crude extracts from five species of cyanobacteria (blue-green algae) against mouse leukaemia L5178Y cells (Suzuki et al 1993). The findings suggested that lipophilic antioxidants such as phycotene, an algae extract rich in carotenoids and lipophilic vitamins with known antineoplastic activity (Combs et al 1989), or β-carotene and vitamin E found in such algae inhibit the growth of this cell-line. In subsequent studies the sole and synergistic effects of 22 chemicals, including carotenoids and lipophilic vitamins, on cellular proliferation of the same cell-line were determined, and the relationship between molecular structure and antitumour activity modelled using linear and non-linear methods (Suzuki et al 1994; Suzuki & Ishida 1995). The most potent growth-inhibitory chemical against this cell-line was found to be plumbagin, with menadione a close second. The objective of the current study was to quantify the effects of dyadic combinations of some antioxidants, vitamins and related agents on tumour cell growth. The effects of these dyadic combinations were compared with that of sodium ascorbate + menadione against the same cell-line.

Correspondence: T. Suzuki, Research Laboratory of Resources Utilization, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8503, Japan.

Materials and Methods

Test chemicals

Menadione and abscisic acid were products of Tokyo Chemical Industry (Tokyo, Japan).

trans-Retinol (vitamin A) and plumbagin were obtained from Sigma (St Louis, MO), and phytol and sodium L-ascorbate from Wako (Osaka, Japan). All compounds were stored at −20°C, in tubes protected from light, as 1×10^{-2} M stock solutions in dimethylsulphoxide (DMSO) and added to culture media just before use. The concentration of DMSO in test cultures never exceeded the predetermined non-cytotoxic level of 1% (w/w). An equivalent amount of DMSO was added to all control cultures.

Cell-line

In-vitro studies were performed by tissue-culture methods. The mouse leukaemia L5178Y cell-line RCB0135 was obtained from the Riken Gene Bank (Ibaraki, Japan). Cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) calf serum (Dainippon Pharmaceutical, Osaka, Japan) in an incubator containing 5% CO_2 in humidified air at 37°C.

In-vitro cytotoxicity

L5178Y cells (initial cell concentration $6–9 \times 10^7$ viable cells L^{-1}) were incubated for five days after addition of test chemicals in a 96-well tissue-culture microtitre plate (Corning Costar, Cambridge, MA). Initial dose levels were set at one-log intervals in the range 1×10^{-3} to 1×10^{-6} M with two tubes per level, based on the NCI protocol. Numbers of viable cells were counted in a haemocytometer. Cell viability was monitored by the trypan blue-exclusion method.

Cytotoxicity was evaluated as percentage inhibition of tumour growth by use of the formula:

$$\text{Percentage growth inhibition} = [1 - (T/C)] \times 100 \quad (1)$$

where T and C are the mean numbers of viable cells in the test and control samples, respectively. IC values IC10, IC20, IC30 and IC50, corresponding to the molar concentrations of test chemicals required to inhibit growth by 10%, 20%, 30% and 50%, respectively, were determined from log probability plots.

Results and Discussion

Activity of single compounds

Experimental IC10, IC20, IC30 and IC50 values are reported in Table 1. The shapes of the dose–response curves were similar for all the compounds tested and the responses were linearly dependent on the logarithms of the doses of the compounds. The relative potencies of the six compounds tested (IC50) were: plumbagin > menadione > *trans*-retinol > phytol > abscisic acid > sodium ascorbate.

Comparison of antitumour activities of vitamin K congeners (K_1, K_2 and menadione) against tumour cells from man showed that the relative potencies of menadione compared with vitamin K_2 and K_1 were about 60- and 300-fold, respectively (Wu et al 1993). Such compounds with a quinone structure have been known to play a prominent role in cancer chemotherapy. The concentrations of the agents required for a cytotoxic effect differ from one cell-line to another, but the results obtained for vitamin K congeners might be comparable with our previous evaluation (Suzuki et al 1994) that the potency of menadione is between those of plumbagin and vitamin K_1.

The mechanisms underlying inhibition of tumour growth by the compounds under study are not well understood, but several hypotheses have been proposed. Cytotoxicity induced by sodium ascorbate seems to be mediated by hydrogen peroxide generated by its metabolically oxidized form (Noto et al 1989). Inhibitory activities of the quinones menadione and plumbagin might be attributable to the generation of active oxygen species and consequent cell damage (Rotilio et al 1985). Another factor contributing to the antineoplastic action of sodium ascorbate and quinones seems to be reactivation of nucleases in tumour cells and consequent DNA destruction (Taper 1981).

Less information is available about the mechanisms of action of *trans*-retinol, phytol and abscisic acid, although their antioxidant properties are probably important in inhibiting the proliferation of tumour cells (Wheater 1991). A recent study of retinoides suggests that they act by binding to nuclear retinoid receptors such as RAR β (Moriwaki et al 1994).

Activity of pairs of compounds

Because the mechanisms of action of the growth-inhibitory substances used in this study are not well understood, their interactions were assessed by use of the synergistic index (SI) rather than the more common isobole method or other approaches (Berenbaum 1989; Sühnel 1990; Martinez-Irujo et al 1996):

$$SI = \{1 - (T/C)\}_{i+j} / [\{1 - (T/C)\}_i + \{1 - (T/C)\}_j - \{1 - (T/C)\}_i\{1 - (T/C)\}_j] \quad (2)$$

where $\{1 - (T/C)\}_{i+j}$ is the combined cytotoxic activity of agents i and j, and $\{1 - (T/C)\}_i$ and $\{1 - (T/C)\}_j$ are the individual cytotoxic activities of agents i and j, respectively. The SI index is based on the concept of independence (Webb 1963). Drug

Table 1. Cytotoxic activity of test chemicals against mouse leukaemia L5178Y cells in-vitro.

Compound	Inhibiting concentration (M)*			
	IC10	IC20	IC30	IC50
trans-Retinol	$7{\cdot}4\times10^{-6}$	$1{\cdot}2\times10^{-5}$	$1{\cdot}6\times10^{-5}$	$3{\cdot}0\times10^{-5}$
Phytol	$4{\cdot}1\times10^{-5}$	$4{\cdot}7\times10^{-5}$	$5{\cdot}0\times10^{-5}$	$6{\cdot}0\times10^{-5}$
Abscisic acid	$6{\cdot}0\times10^{-5}$	$7{\cdot}8\times10^{-5}$	$9{\cdot}2\times10^{-5}$	$1{\cdot}3\times10^{-4}$
Menadione	$2{\cdot}0\times10^{-6}$	$2{\cdot}4\times10^{-6}$	$2{\cdot}8\times10^{-6}$	$3{\cdot}7\times10^{-6}$
Plumbagin	$1{\cdot}7\times10^{-7}$	$2{\cdot}6\times10^{-7}$	$3{\cdot}5\times10^{-7}$	$6{\cdot}6\times10^{-7}$
Sodium L-ascorbate	$5{\cdot}6\times10^{-4}$	$6{\cdot}1\times10^{-4}$	$6{\cdot}5\times10^{-4}$	$7{\cdot}5\times10^{-4}$

* Mean values of results from triplicate runs.

interactions can be classified into three types (Sühnel 1990) according to the SI value:

$SI > 1$ for synergism or positive interaction (3)

$SI = 1$ for zero interaction or independent (4)

$SI < 1$ for antagonism or negative interaction (5)

Experiments to determine the interaction between two agents were based on a 3^2 (2-factor-3-level) factorial design (Carlson 1992). A comparatively low concentration, e.g. IC10, IC20 and IC30, for each agent, as shown in Table 1, was adopted in this study, because the range of doses evaluated must be valid for the evaluation of synergism up to high levels of activity, i.e. 90% growth inhibition. Five pairs of agents were tested by referring to our preliminary study on synergistic effects of pairs of 20 compounds (Suzuki et al 1994): retinol–plumbagin, retinol–menadione, retinol–abscisic acid, retinol–phytol and sodium ascorbate–menadione.

Because the resulting SI values will depend on experimental factors, it is assumed that the response SI is a function of the doses of two agents:

$$SI = f(IC_1, IC_2) \quad (6)$$

where IC_1 and IC_2 are IC values (%) of agents 1 and 2, respectively. The nature of the function defining SI is usually unknown, but is probably continuous and multiply differentiable with respect to the experimental variables, in which case it is possible to approximate SI in the experimental domain of interest by a second-degree Taylor expansion. Thus for two variables IC_1 and IC_2 the model reduces to a quadratic polynomial:

$$SI = a_0 + a_1IC_1 + a_2IC_2 + a_{12}IC_1IC_2 + a_{11}IC_1^2 + a_{22}IC_2^2 \quad (7)$$

where a_n denotes coefficients characterizing linear (a_1, a_2), quadratic (a_{11}, a_{22}) and interaction (a_{12}) effects. These coefficients can be determined by multiple linear regression to fit a polynomial to observed experimental data. When estimates of all these coefficients have been obtained, response-surface models can be constructed showing SI as functions of IC_1 and IC_2 (Carlson 1992).

The resulting contour plots are shown in Figure 1, where the doses (IC) of the two agents are given on the x- and y-axes, and SI values are described by contour lines. This diagram directly indicates regions of synergism, antagonism or zero interaction within the dose range under study. Visual interpretation of the projections shown in Figure 1 indicates that within the range of doses studied phytol + retinol, abscisic acid + retinol and sodium ascorbate + menadione are synergistic combinations whereas menadinone + retinol and plumbagin + retinol are antagonistic combinations.

The agents could be grouped into two categories on the basis of similarities in the mode of cytotoxic action—group 1 consisting of plumbagin, menadione and sodium ascorbate, and group 2 of retinol, phytol and abscisic acid, as was mentioned above. Combinations of two agents in the same group resulted in synergism and combinations of two agents with different modes of action resulted in antagonism in this study. This observation might suggest the site of interaction and some physical or chemical interferences. Synergistic inhibition of oxidation by vitamin E + sodium ascorbate has long been known (Golumbic & Mattill 1941), and it has been found that sodium ascorbate regenerates vitamin E by reacting with the vitamin E radical during oxidation of phosphatidylcholine in aqueous-dispersed liposomes (Niki et al 1985).

According to the SI indices synergy increases as the doses of both agents decrease for phytol + retinol and for menadione + sodium ascorbate,

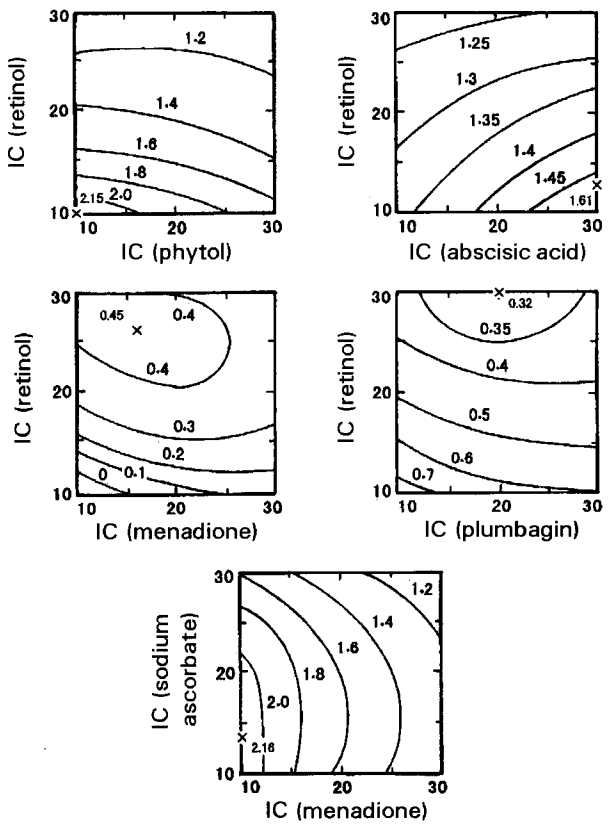

Figure 1. Contour plots of the synergistic index (SI) for dyadic combinations of agents against mouse leukaemia L5178Y cells in-vitro. SI values equal to, greater than, or smaller than unity are interpreted as representative of zero interaction, synergism, or antagonism, respectively. The points marked x show maximum or minimum SI values.

whereas for abscisic acid + retinol maximum synergy occurs at a combination of increased dose of abscisic acid and reduced dose of retinol. It is interesting to note that the pattern of the contour plot of phytol + retinol is similar to that of menadione + sodium ascorbate.

Combined administration of menadione and sodium ascorbate has been tested against tumour cell-lines from man by Noto et al (1989) and Venugopal et al (1996). Although their studies were performed at a fixed menadione/sodium ascorbate ratio of 1 : 100, higher potentiation of antitumour activity was observed at lower doses. Treatment of MCF-7 (breast carcinoma) cells with the combined agents resulted in 74% inhibition (Noto et al 1989). Because the individual doses of menadione and sodium ascorbate are IC2 and IC28, respectively, the value of SI = 2·51 is available. Similarly, SI values of 4·2 and 3·6 respectively were obtained for the action of lower-dose combinations of the two agents on the cell-lines KB (oral epidermoid carcinoma) and AN3-CA (endometrial adenocarcinoma).

Venugopal et al (1996) employed the FIC (fractional inhibitory concentration) index (Elion et al 1954) for quantification of synergism in their combination experiments. Because the FIC index is for criteria at iso-effective dose levels (dose-combinations having the same effect) and so constitutes nothing more than the isobole method (Sühnel 1990), their data are expressed in terms of doses of the two agents used alone and in combination that result in 50% cytotoxicity. The interpretation of FIC index is that values < 1·0 indicate synergism. FIC values in the range 0·136–0·301 were obtained for eight urologic tumour cell-lines from man. The results correspond to four- to sixfold reductions in the IC50 values for sodium ascorbate and 10–21-fold reductions in the IC50 values for menadione. Unfortunately, evaluation of their data using the SI index is not possible, because of a lack of individual dose–response curves for pairs of agents.

Their experimental points which showed higher synergistic effects are outside the domain explored in the current study, although the existence of a domain in which synergism is stronger can be expected from the diagram in Figure 1. The response-surface model predicts a maximum SI value of 2·16 at a dose combination ($IC_1 = 10$, $IC_2 = 13$) shown by the marked point (x) in this study. For other synergistic combinations, SI values of 2·15 at ($IC_1 = 10$, $IC_2 = 10$) for the combination phytol + retinol and 1·61 at ($IC_1 = 30$, $IC_2 = 12{\cdot}5$) for the combination abscisic acid + retinol are predicted, as shown in Figure 1.

With regard to antagonistic combinations, the antagonism of menadione + retinol is minimal within the domain tested, as shown by the marked point (x). The estimated maximum SI value is 0·45 at ($IC_1 = 16$, $IC_2 = 26$). In contrast, the antagonism observed for plumbagin + retinol decreases as the doses of both agents are reduced; for this combination a region of synergy is suggested at lower doses than IC10 of both agents. The minimum SI value of 0·32 is predicted at ($IC_1 = 20$, $IC_2 = 30$).

As pointed out elsewhere (Sühnel 1990; Martinez-Irujo et al 1996), the isobole method has some disadvantages. Finding different combinations of agents that produce the effect expected from the effects of the single agents can be a very laborious task. In addition, isobolograms are only valid for assessing interactions with a given effect. It is important to note that the type and extent of the interaction might depend not only on the combination of agents but also on the dose ranges. That combinations of two agents might act synergistically in one dose range and antagonistically in another is suggested by the results shown in Figure 1. In comparison with the conventional isobole

method, the current approach requires fewer experimental points to evaluate interactions between two agents in the first explored domain. From these experiments, it is possible to determine in which direction a higher synergistic effect or an optimum experimental domain is to be expected.

Conclusions

The cytotoxicities of six compounds and selected dyadic combinations thereof towards mouse leukaemia L5178Y cells were determined in-vitro. To quantify the type and extent of interactions of drug combinations an index was defined and response-surface modelling techniques were employed. The approach provides a basis for finding combinations and dose ratios of drugs with greater antitumour activity as a first series of experiments with a view to finding a better experimental domain to be explored.

Acknowledgements

The authors acknowledge Dr S. Shapiro (Zentrum für Zahn-, Mund- und Kieferheilkunde der Universität Zürich, Switzerland) and Professor Kazuhisa Ohtaguchi (Department of Chemical Engineering, Tokyo Institute of Technology, Japan) for their helpful suggestions. This work was supported by a Grant-in-Aid for Scientific Research (Project No.08650905) from the Ministry of Education, Science, Sports and Culture of Japan.

References

Berenbaum, M. C. (1989) What is synergy? Pharmacol. Rev. 41: 93–141

Carlson, R. (1992) Design and optimization in organic synthesis. Elsevier, Amsterdam, pp 249–324

Chen, L., Suzuki, Y., Liu, C.-M., Wheelock, E. F. (1990) Maintenance and cure of the L5178Y murine tumor-dormant state by interleukin 2: dependence of interleukin 2 on induced interferon-γ and on tumor necrosis factor for its antitumor effects. Cancer Res. 50: 1368–1374

Combs, W., Sonis, S. T., Fitzgerald, J., Tracy, C., Wilson, R. (1989) In vivo and in vitro effects of β-carotene and algae extracts in murine tumor models. Nutr. Cancer 12: 371–380

Elion, G. B., Singer, S., Hitchings, G. H. (1954) Antagonists of nucleic acid derivatives. Synergism in combinations of biochemically related antimetabolites. J. Biol. Chem. 208: 477–488

Gey, K. F., Brubacher, G. B., Stähelin, H. B. (1987) Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. Am. J. Clin. Nutr. 45: 1368–1377

Golumbic, C., Mattill, H. A. (1941) Antioxidants and the autoxidation of fats. XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroquinones and related compounds. J. Am. Chem. Soc. 63: 1279–1280

Martinez-Irujo, J. J., Villahermosa, M. L., Alberdi, E., Santiago, E. (1996) A checkerboard method to evaluate interactions between drugs. Biochem. Pharmacol. 51: 635–644

Mathews-Roth, M. M. (1982) Antitumor activity of β-carotene, canthaxanthin and phytoene. Oncology 39: 33–37

Moriwaki, H., Muto, Y., Shidoji, Y., Okuno, M. (1994) Cancer prevention and treatment by retinoids. Vitamins 67: 387–396

Niki, E., Kawakami, A., Yamamoto, Y., Kamiya, Y. (1985) Oxidation of lipids. VIII. Synergistic inhibition of oxidation of phosphatidylcholine liposome in aqueous dispersion by vitamin E and vitamin C. Bull. Chem. Soc. Jpn 58: 1971–1975

Noto, V., Taper, H. S., Jiang, Y.-H., Janssens, J., Bonte, J., De Loecker, W. (1989) Effects of sodium ascorbate (vitamin C) and 2-methyl-1,4-naphthoquinone (vitamin K_3) treatment on human tumor cell growth in vitro. I. Synergism of combined vitamin C and K_3 action. Cancer 63: 901–906

Rotilio, G., Mavelli, I., Rossi, L., Ciriolo, M. (1985) Biochemical mechanisms of oxidative damage by redox cycling drugs. Environ. Health Perspect. 64: 259–264

Schwartz, J., Shklar, G. (1987) Regression of experimental hamster cancer by beta carotene and algae extracts. J. Oral Maxilofac. Surg. 45: 510–515

Sühnel, J. (1990) Evaluation of synergism or antagonism for the combined action of antiviral agents. Antivir. Res. 13: 23–39

Suzuki, T., Ishida, M. (1995) Application of neural networks in quantitative structure-activity relationships of cytotoxicity of antioxidants. Pharm. Sci. 1: 297–300

Suzuki, T., Ohtaguchi, K., Koide, K. (1993) Cell growth inhibition in mouse leukaemia L5178Y cells in vitro by crude extracts from freshwater blue-green algae. J. Chem. Eng. Japan 26: 463–464

Suzuki, T., Ohtaguchi, K., Koide, K. (1994) Modelling the cytotoxicity of antioxidants to mouse leukaemia L5178Y cells in vitro. Pharm. Sci. Commun. 4: 171–181

Taper, H. S. (1981) Reversibility of acid and alkaline deoxyribonuclease deficiency in malignant tumor cells. J. Histochem. Cytochem. 29: 1053–1060

Venugopal, M., Jamison, J. M., Gilloteaux, J., Koch, J. A., Summers, M., Giammar, D., Sowick C., Summers, J. L. (1996) Synergistic antitumor activity of vitamins C and K_3 on human urologic tumor cell-lines. Life Sci. 59: 1389–1400

Webb, J. L. (1963) Effects of more than one inhibitor. In: Webb, J. L. (ed.) Enzyme and Metabolic Inhibitors. Academic Press, New York, pp 487–515

Wheater, C. (1991) Beta Carotene. Thorsons, London, Chapter 8

Wu, F. Y.-H., Liao, W.-C., Chang, H.-M. (1993) Comparison of antitumor activity of vitamins K_1, K_2, and K_3 on human tumor cells by two (MTT and SRB) cell viability assays. Life Sci. 52: 1797–1804