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Anti-tumor Promoting Activity of Canventol and Its Synthetic Analogs through Inhibition of Protein Isoprenylation

Atsumasa Komori,[1] Sachiko Okabe,[1] Masami Suganuma,[1] Michael A. Kerr,[2] Jakob Busch-Petersen,[2] Lynette M. Oh,[2] Jincong Zhuo,[2] G. S. Kamali Kannangara,[2] Xianglong Zou,[2] Marcus A. Tius[2] and Hirota Fujiki[1,3]

[1]*Saitama Cancer Center Research Institute, 818 Komuro, Ina, Kitaadachi-gun, Saitama 362 and* [2]*Department of Chemistry, University of Hawaii at Manoa, Honolulu, Hawaii 96822, USA*

Canventol, a synthetic compound, is a new inhibitor of tumor promotion on mouse skin by okadaic acid. We previously reported that canventol acts by inhibiting both protein isoprenylation and tumor necrosis factor-α (TNF-α) release. In this study we examined the potencies of 10 newly synthesized canventol analogs through their effect on mevalonate metabolism, and then examined 3 representative analogs for inhibition of protein isoprenylation. Since canventol *in vitro* did not directly inhibit farnesyl protein transferase or geranylgeranyl protein transferase-I, the effects of canventol and its synthetic analogs on the fate of [^{3}H]mevalonate in cells were studied. Canventol at 500 μM changed the ratio of newly synthesized sterols (cholesterol and lathosterol) to ubiquinones from 0.7 to 8.2 in NIH/3T3 cells which had previously been labeled with [^{3}H]mevalonate, suggesting that the altered pattern of mevalonate metabolism is associated with inhibition of protein isoprenylation in the cells. We named this ratio the inhibition of protein isoprenylation index (IPI index). The 10 analogs showed a wide range of IPI indices. Two analogs, S3 and S9 had effects similar to, or stronger than, canventol. Three analogs, C44, C46 and C47, with lower IPI indices, inhibited tumor promotion on mouse skin slightly less than canventol itself did. This study shows that inhibition of protein isoprenylation in the cells, indicated by an increase in the IPI index, is a new biomarker for estimating inhibition of tumor promotion.

Key words: Canventol — Protein isoprenylation — Mevalonate metabolism

Evaluation of anticarcinogenic activity of cancer chemopreventive agents *in vitro* is of vital importance. If the inhibitory mechanisms of action of a compound are directly related to the carcinogenic process, such evaluation may be essential to the eventual development of cancer chemopreventive agents.[1)] We have reported previously that the synthetic compound canventol inhibited tumor promotion by okadaic acid in a two-stage carcinogenesis experiment on mouse skin, apparently through inhibiting both protein isoprenylation and TNF-α release.[2)] Inhibition of protein isoprenylation in cells by canventol may open a new approach to inhibiting cancer development,[3)] because most human cancer cells contain activated *ras* genes[4)] and the *ras* gene products are isoprenylated.[5)] Thus, we first studied how canventol inhibits protein isoprenylation in cells. We found that canventol and ten analogs that we have recently synthesized, modulate the ratio of newly synthesized sterols (cholesterol and lathosterol) to ubiquinones in NIH/3T3 cells, suggesting that a higher ratio of sterols to ubiquinones is closely associated with inhibition of protein isoprenylation in cells. Thus, we named this IPI index.

Three synthetic analogs, C44, C46 and C47, were tested for inhibition of tumor promotion on mouse skin by okadaic acid, and they all were found to be slightly weaker than canventol. Use of the IPI index may allow us to predict the inhibitory activity of such agents *in vivo* and further, should be helpful in the development of cancer preventive agents.

MATERIALS AND METHODS

Canventol and its analogs Canventol was synthesized as previously reported,[2)] and ten analogs of canventol, C44, C46, C47, C48, C49, C50, S3, S6, S9 and S11 (Fig. 1), were also synthesized. The preparation of all new synthetic analogs is described in Fig. 2.[6–12)] All these compounds are racemic, and S9 is a mixture of diastereomers.

Chemicals *d,l*-[5-^{3}H]Mevalonolactone (1.22 TBq/mmol), *d,l*-[2-^{14}C]mevalonolactone (1.85 GBq/mmol), [1-^{3}H(N)]FPP (555 GBq/mmol), and [1-^{3}H(N)]GGPP

[3] To whom correspondence should be addressed.

The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; FPP, farnesyl pyrophosphate; FPTase, farnesyl protein transferase; GGPTase-I, geranylgeranyl protein transferase-I; GGPP, geranylgeranyl pyrophosphate; IPI, inhibition of protein isoprenylation; TNF-α, tumor necrosis factor-α; KTSCVIM, Lys-Thr-Ser-Cys-Val-Ile-Met; YGGQNGCINCCKVL, Tyr-Gly-Gly-Gln-Asn-Gly-Cys-Ile-Asn-Cys-Cys-Lys-Val-Leu.

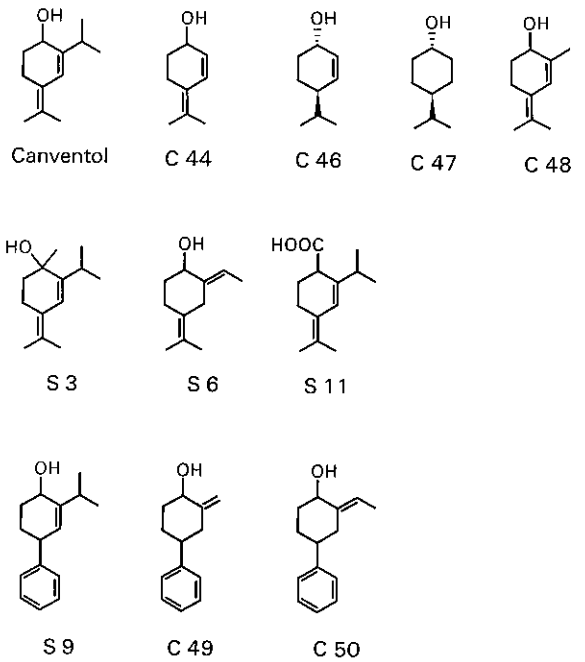

Fig. 1. Structures of canventol and its analogs.

(714 GBq/mmol) were purchased from Dupont-NEN, Boston, MA. A synthetic analog of lovastatin, L-654,969, was a gift from Banyu Pharmaceuticals Co., Ltd., Tokyo. FPP, GGPP, and biotinylated peptides were gifts from Mitsubishi Chemical Co., Research Center, Yokohama. Okadaic acid was isolated from the black sponge, *Halichondria okadai*.[13] DMBA was purchased from Sigma Chemical Co., St. Louis, MO.

Cells NIH/3T3 cells, which were kindly provided by Dr. T. Sekiya at the National Cancer Center Research Institute, were grown in Dulbecco's modified Eagle's medium containing 10% calf serum and were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Animals Female CD-1 mice, 7 weeks old, were obtained from Charles River Japan Inc. (Kanagawa).

***In vitro* assay of protein isoprenyl transferases** FPTase and GGPTase-I were partially purified from rat brain as described previously.[14] The assays were performed as described by Reiss *et al.*[15] using biotinylated-KTSCVIM (biotinylated-K-*ras*) and biotinylated-YGGQNGCINCCKVL (biotinylated-*rho*) as substrates for FPTase and GGPTase-I, respectively. FPTase and GGPTase-I assays contained the following components; for FPTase 50 m*M* Tris-HCl (pH 7.5), 20 m*M* KCl, 50 μM $ZnCl_2$, 1 m*M* DTT, 4.3 μM biotinylated-K-*ras*, 66 pmol of all-*trans*-[^{3}H]FPP, 9 μg of enzyme and various concentrations of canventol in a final volume of 100 μl; for GGPTase-I 50 m*M* Tris-HCl (pH 7.7), 5 m*M* $MgCl_2$, 5 μM $ZnCl_2$, 2 m*M* DTT, 1 μM biotinylated-*rho*, 100 pmol of all-*trans*-[^{3}H]GGPP, 15 μg of enzyme and canventol in 50 μl. After incubation for 20 min at 37°C, the reaction was stopped and [^{3}H]isoprenylated peptides were collected using streptoavidin-agarose beads, followed by scintillation counting.

Ratio of newly synthesized sterols to ubiquinones in cellular lipids NIH/3T3 cells were plated at a density of 4×10^5/60 mm dish. After 24 h, the cells were incubated in fresh medium containing *d,l*-[5-^{3}H]mevalonolactone (925 kBq/ml), 10 μM L-659,969, and a test compound for 18 h. Cells were washed with ice-cold phosphate-buffered saline three times, and then suspended in 110 μl of 0.15 *M* NaCl. The lipids were extracted from the cell lysates with chloroform/methanol 2/1 (v/v), using a modification of the method of Faust *et al.*, as reported previously.[16] The lipids in the lower phase were developed on silica gel plates (MERCK, 60 F 254) in hexane/diethyl ether/acetic acid (70/30/1.5).[17] The incorporation of [5-^{3}H]mevalonate into various lipids was measured with a Radio TLC analyzer, RITA 90 System (Raytest, Straubenhardt, Germany). Cholesterol, lathosterol (5α-cholest-7-en-3β-ol), squalene, dolichol and ubiquinone (Coenzyme Q_{10}) were used as standards and [^{3}H]labeled lipids were identified by comigration with authentic standards in several one- and two-dimensional TLC systems. Two repeated experiments resulted in similar values.

Inhibition of protein isoprenylation in NIH/3T3 cells NIH/3T3 cells were plated at a density of 4×10^5/60 mm dish. After 24 h, the cells were treated with 5 μM L-654,969 for 24 h and then incubated in fresh medium containing *d,l*-[2-^{14}C]mevalonolactone (555 kBq/ml), 10 μM L-654,969, and an appropriate concentration of test compound, for 4 h, as reported previously.[2] Cells were washed with ice-cold phosphate-buffered saline three times, and directly dissolved in electrophoresis sample buffer. The same amounts (200 μg protein) of the cell lysates were subjected to 12% SDS-PAGE, followed by measurement of radioactivity on a BAS-2000 Image Analyser (Fuji Film Co., Tokyo). Two repeated experiments gave similar results.

Inhibition of tumor promotion on CD-1 mouse skin Inhibition of tumor promotion in a two-stage carcinogenesis experiment on mouse skin was conducted as described previously.[18] Briefly, initiation was achieved by a single application of 100 μg of DMBA. Tumor promotion was conducted by repeated applications of okadaic acid (1.0 μg, 1.2 nmol per application). Canventol and its analogs (C44, C46 and C47) (12 nmol each per application) were applied topically 15 min before each application of okadaic acid. Inhibition of tumor promotion was estimated in terms of the decrease in the percentage of tumor-bearing mice.

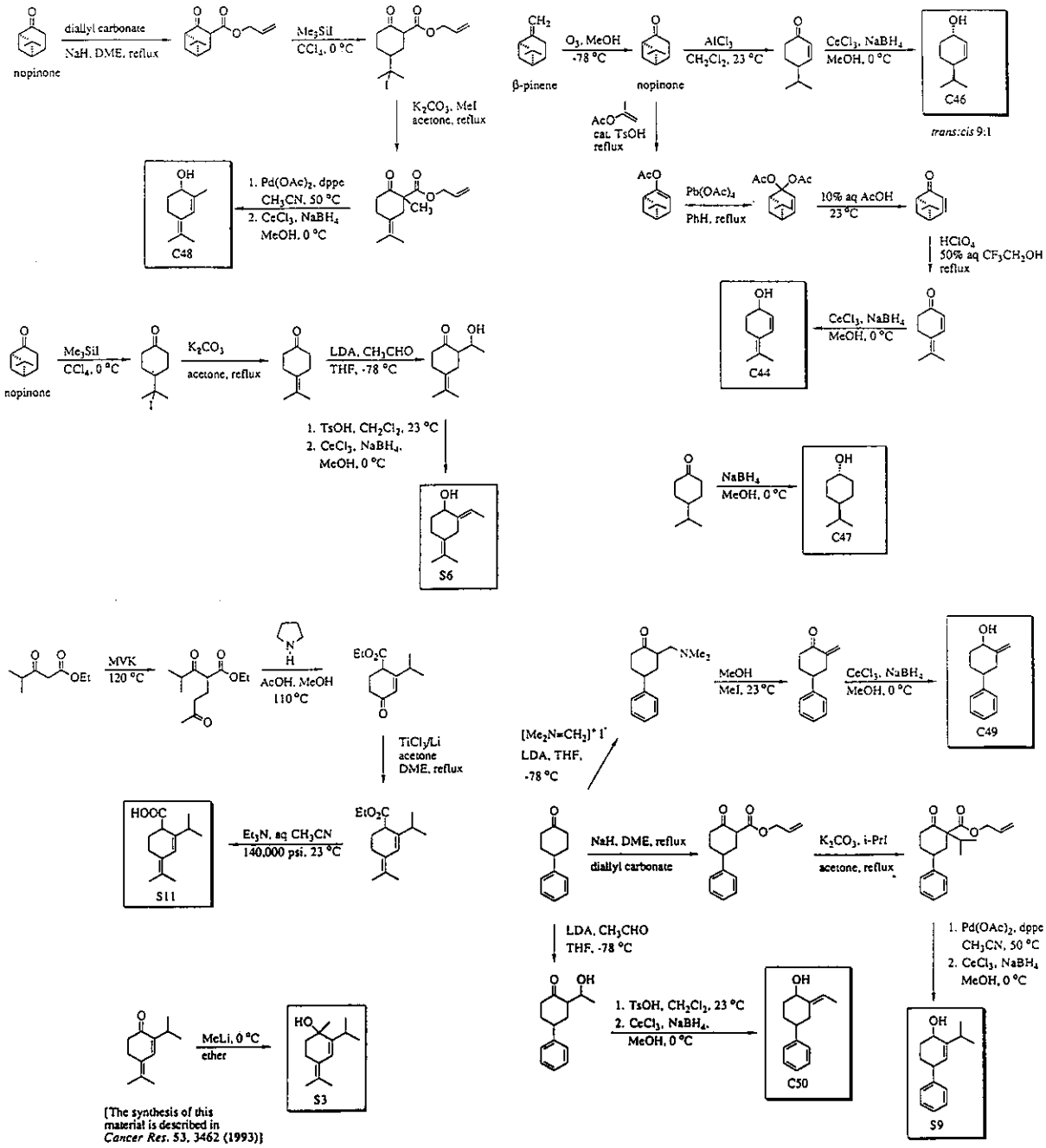

Fig. 2. Synthesis of canventol analogs.

RESULTS

Absence of canventol for inhibition of protein isoprenyl transferase activity *in vitro* In our standard enzyme assays, 10.6 pmol of [^{3}H]farnesyl group was transferred to biotinylated K-*ras* and 2.8 pmol of [^{3}H]geranylgeranyl group was transferred to biotinylated-*rho*. Canventol up to 500 μM did not inhibit the incorporation of radioactivity into either peptide; with 500 μM canventol, 12.2 pmol of [^{3}H]farnesyl was transferred to biotinylated K-*ras*, and 2.4 pmol of [^{3}H]geranylgeranyl was transferred to biotinylated-*rho*.

Canventol also did not inhibit FPTase when recombinant p21 Ha-*ras* was used as the substrate (data not shown). We concluded that canventol is not a direct inhibitor of either FPTase or GGPTase-I *in vitro*. Since the substrates for FPTase and GGPTase-I, that is, FPP and GGPP, are derived from mevalonate, we have postulated that canventol causes modulation of FPP and GGPP contents in the pool, and this was reflected in the inhibition of protein isoprenylation in cells.

Ratio of newly synthesized sterols to ubiquinones in cellular lipids [^{3}H]Mevalonolactone incorporated into cellular lipids was mainly separated into three fractions on TLC: sterols (including cholesterol and its precursor lathosterol), ubiquinones, and the remainder, consisting of polar materials. Treatment with canventol dose-dependently increased the radioactivity in sterols and suppressed that in ubiquinones (Fig. 3), suggesting that net sterol synthesis is increased and ubiquinone synthesis is suppressed. The radioactivity in the remainder was not changed significantly (data not shown). Thus, canventol altered the pattern of [^{3}H]mevalonate incorporation into cellular lipids, as revealed by the increased ratio of 8.2 of sterols to ubiquinones (Δsterols/Δubiquinones), whereas the ratio for the control was 0.7 (Fig. 3). The ratios of [^{3}H]sterols to [^{3}H]ubiquinones after treatment with each canventol analog at 500 μM could be roughly classified into three groups (Fig. 4): a group having no effect on mevalonate metabolism, that is, with a ratio of less than 1.0 (C48, C49 and S11) (Class 1), in which ubiquinone synthesis predominated over cholesterol synthesis; a group having weaker effects than canventol with a ratio less than 5.0 (C44, C46, C47, C50 and S6) (Class 2); and a group having effects similar to, or stronger than, canventol with a ratio of more than 5.0 (S3 and S9) (Class 3). Next, we studied the relation between modulation of mevalonate incorporation into cellular lipids and inhibition of protein isoprenylation.

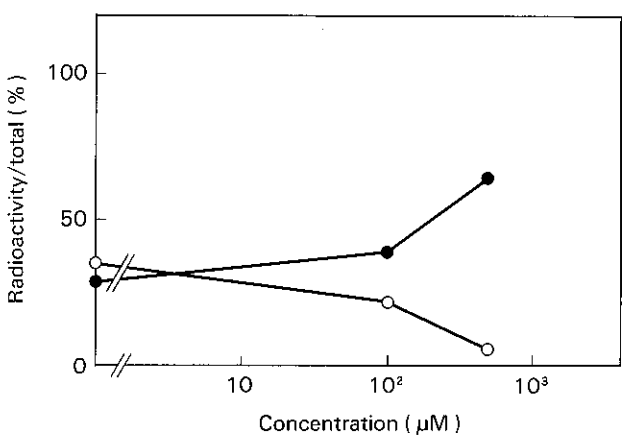

Fig. 3. Modulation of [^{3}H]mevalonolactone incorporation into cellular lipids by canventol. NIH/3T3 cells were treated with 925 kBq/ml [5-^{3}H]mevalonolactone, 10 μM L-654,969, and canventol (100 μM or 500 μM) for 18 h. Extracted cellular lipids were separated on a thin layer plate, followed by development in hexane/diethyl ether/acetic acid (70/30/1.5). The percentages of radioactivity co-migrating with sterols (●), which included cholesterol and lathosterol, and with ubiquinones (○) were measured and plotted (each point is the mean of two separate experiments), as reported in "Materials and Methods."

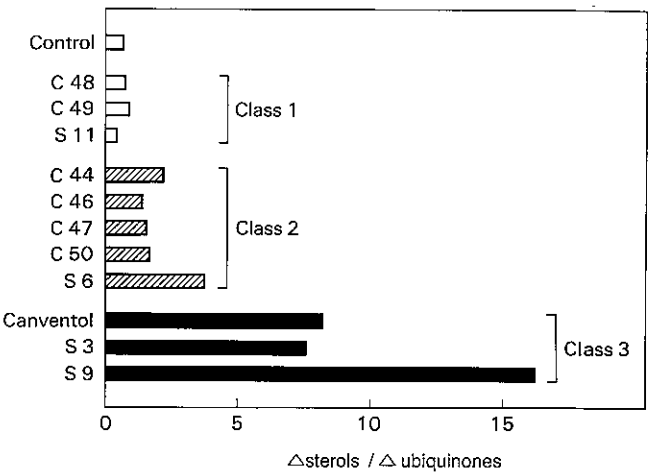

Fig. 4. Classification of canventol and its analogs in terms of the ratio of newly synthesized sterols to ubiquinones (Δsterols/Δubiquinones). NIH/3T3 cells were treated with [5-^{3}H]mevalonolactone 925 kBq/ml, 10 μM L-654,969, and 500 μM test compound. Extracted cellular lipids were analyzed as described in the legend to Fig. 3.

Inhibition of protein isoprenylation in NIH/3T3 cells [2-^{14}C]Mevalonolactone was incorporated into proteins with various molecular weights indicating that proteins are isoprenylated in the cells (Fig. 5). Representatives of each of the 3 classes were examined for their inhibitory activities. One of the class 1 compounds, S11, which was inactive on modulation of mevalonate incorporation into cellular lipids, did not significantly inhibit protein isoprenylation. C46 in class 2 showed weaker inhibition of protein isoprenylation than canventol did. S9 of class 3, which showed a higher ratio of sterols to ubiquinones than did canventol, suppressed the radioactivity of various proteins with almost the same potency as canventol at equimolar concentrations. Each analog at 1 mM suppressed radioactivity of various proteins; for the 28–26 kDa bands which were reported to be geranylgeranylated small GTP binding proteins,[19] treatment with the four compounds, S11, C46, canventol, and S9, changed the radioactivity from 100 to 87.0, 68.5, 39.2, and 46.0%, respectively (mean values of two separate experiments).

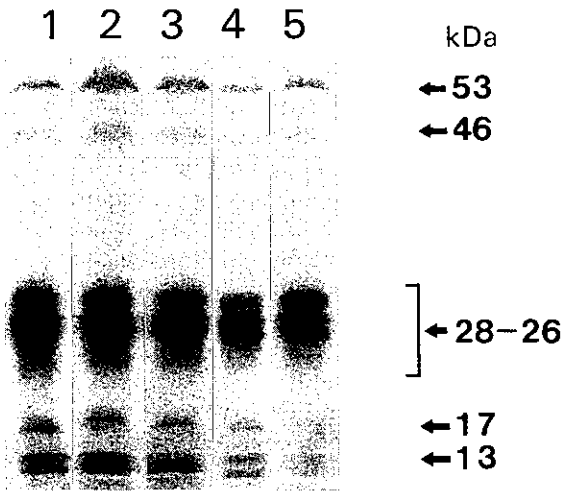

Fig. 5. Inhibition of protein isoprenylation by canventol and some of its analogs. NIH/3T3 cells were treated with 5 μM L-654,969 for 24 h and then incubated in fresh medium containing [2-^{14}C]mevalonolactone 555 kBq/ml, 10 μM L-654,969, and the test compound at 1 mM for another 4 h. Lane 1, control; Lane 2, S11; Lane 3, C46; Lane 4, canventol; Lane 5, S9; whole cell lysates were subjected to 12% SDS-PAGE followed by measurement of radioactivity as described in "Materials and Methods." Representative results are shown from two independent experiments.

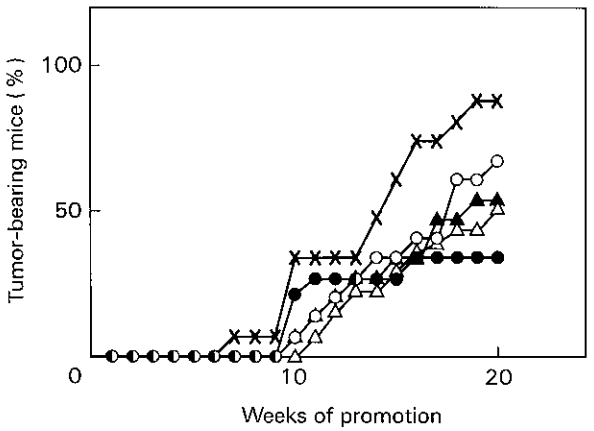

Fig. 6. Inhibition of tumor promotion by canventol and its three analogs. Percentages of tumor-bearing mice of the groups treated with DMBA and okadaic acid (×), DMBA and okadaic acid plus C44 (○), DMBA and okadaic acid plus C46 (▲), DMBA and okadaic acid plus C47 (△) and DMBA and okadaic acid plus canventol (●) are shown. Experimental groups each consisted of 15 mice. Each of these compounds was dissolved in 0.1 ml of acetone.

On the other hand, for 53 kDa farnesylated protein,[19] S11, C46, canventol, and S9 changed the radioactivity from 100 to 100.0, 110.0, 41.8, and 42.5%, respectively. Thus, inhibition of protein isoprenylation in the cells correlated well with the increased ratio of sterols to ubiquinones. This ratio is thus an indirect, but useful biomarker for evaluating the inhibitory activity of these agents on protein isoprenylation. We therefore named this ratio the IPI index. We may consider that an increase in the ratio of sterols to ubiquinone accompanies an increase in inhibition of protein isoprenylation. Alternatively, compounds with higher IPI index can be defined as "agents which can modulate mevalonate metabolism significantly so as to inhibit protein isoprenylation in cells."

Inhibition of tumor promotion on CD-1 mouse skin Tumor promotion in the control group was achieved by DMBA plus okadaic acid, which resulted in 86.7% tumor-bearing mice. Treatment with three compounds, C44, C46, and C47, and canventol as a control reduced the percentages of tumor-bearing mice from 86.7 to 66.7, 53.3, 50.0 and 33.3%, respectively, in week 20 (Fig. 6). In accordance with their lower IPI indices, C44, C46 and C47 showed less inhibitory activity than did canventol, suggesting that inhibition of tumor promotion in mouse skin is well represented by the IPI index. In a separate experiment, a class 1 analog, S11, had much less inhibitory activity than canventol on tumor promotion (from 76.6% to 66.7% tumor-bearing mice in week 20). The control groups treated with DMBA alone or with okadaic acid alone did not produce any tumors (data not shown).

DISCUSSION

We have confirmed that canventol is a new inhibitor of tumor promotion on mouse skin by okadaic acid. To elucidate the mechanism of action, we studied inhibition of protein isoprenylation in cells, as represented by the IPI index, using ten analogs of canventol. We found that C44, C46 and C47 are new inhibitors of tumor promotion on mouse skin. Thus, the IPI index should be a useful biomarker for screening new cancer inhibitors.

Inhibition of protein isoprenylation in cells by canventol is the result of indirect inhibition of protein isoprenylation caused by modulation of mevalonate incorporation into cellular lipids. Inhibition of protein isoprenylation should be reflected in an increase in the IPI index. Canventol and S9 did not inhibit either FPTase and GGPTase-I directly *in vitro*, but inhibited protein isoprenylation at the protein level, with an increase in the IPI index. These results suggested that canventol shifted utilization of FPP more to net sterol synthesis than to ubiquinone synthesis, resulting in inhibition of protein

isoprenylation in cells, because FPP is a common precursor.[20–22] Alternatively, it is possible that an increase of some metabolites of cholesterol, such as oxygenated derivatives of cholesterol, might inhibit protein isoprenylation and ubiquinone synthesis in cells. Gould and Ren previously reported that the monoterpene perillyl alcohol, an inhibitor of protein isoprenylation, inhibits ubiquinone synthesis and blocks conversion of lathosterol to cholesterol.[23] They concluded that terpenes modulate the mevalonate metabolism pathway at several enzyme reaction points.[23] Our standpoint in this paper is based on the modulation of mevalonate incorporation into cellular lipids. Although compounds having a higher IPI index are mechanistically neither direct nor specific FPTase inhibitors, as peptide mimetics of the C terminus of $p21^{ras}$ are,[19, 24, 25] we regard this as an advantage because: 1) modulation of mevalonate incorporation into cellular lipids, expressed as the IPI index, is a practical way to estimate inhibitory activities of canventol and its analogs against carcinogenesis (although two analogs, S3 and S9, have not yet been examined). 2) indirect, low-toxicity inhibition of protein isoprenylation by canventol in cells provides new possibilities for cancer inhibition, and 3) canventol may, consequently, inhibit the function of geranylgeranylated proteins such as *rho*, which has been implicated in cell growth and metastasis,[26] as well as farnesylated $p21^{ras}$.

There are several other areas which we intend to examine. We have not yet tested single optical isomers of any of our compounds. The study of canventol and its analogs provides evidence that S3 and S9 are potential candidates for chemopreventive agents. Quite recently, we learnt from the Chemoprevention Branch of the Division of Cancer Prevention and Control of NCI that canventol tested positive in four of the 6 chemoprevention-associated biochemical assays[1] (V. E. Steele, personal communication).

Despite the fact that structure-function relationships among these 10 analogs are not yet clear, the trends of the relationships seem to be as follows: (a) the hydroxyl group of canventol and the analogs is required for the inhibitory function; for example, S11, which has carboxylic acid in place of alcohol, was not active. (b) steric bulk of the isopropyl group on the "right" side is also helpful for modulation of mevalonate incorporation into cellular lipids. These trends might be related to the grouping of the compounds into three classes in terms of the IPI index. We are now trying to design "second generation" analogs with improved activities based on this information.

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