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Further Studies on the Anticancer Activity of Citrus Limonoids

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Research in this laboratory has shown that some citrus limonoids can inhibit the development of 7,12-dimethylbenz[*a*]anthracene-induced oral tumors. The data from these studies have suggested that certain rings in the limonoid nucleus may be critical to antineoplastic activity. Using the hamster cheek pouch model, three new limonoids (ichangensin, deoxylimonin, and obacunone) have now been tested for cancer chemopreventive activity. In the first experiment, it was found that the treatments with ichangensin had no effect on tumor number or burden. In the second experiment, obacunone reduced tumor number and burden by 25 and 40%, respectively, whereas deoxylimonin reduced tumor number and burden by 30 and 50%, respectively. The results with deoxylimonin were significant, $p < 0.05$. Overall, the data indicated that changes in the A ring of the limonoid nucleus can lead to a loss of anticancer activity, whereas changes in the D ring can be tolerated without any apparent loss of biological activity.

KEYWORDS: Limonoids; oral carcinogenesis; citrus; anticancer activity

INTRODUCTION

Limonoids are a group of structurally similar triterpene derivatives found in the plant families Rutaceae and Meliaceae. Most of the early research on citrus limonoids concentrated on the fact that some of these chemicals, primarily limonin and nomilin, are extremely bitter (*1*−*6*). Taste tests showed that the bitterness threshold for limonin and nomilin in citrus juices is ∼3−6 ppm. Concentrations exceeding 6 ppm can lead to significant problems with consumer acceptance of the product. To reduce and eventually solve this bitterness problem, a considerable amount of research has been conducted to understand the movement and metabolism of these chemicals in citrus (*7*−*13*).

Recently, a number of studies have suggested that limonoids might have health-promoting properties. Most of the early work on the cancer chemopreventive activity of citrus limonoids was conducted in two laboratories. One laboratory focused primarily on limonin and nomilin. The results showed that both limonin and nomilin could inhibit the development of carcinogen-induced cancers in a variety of different animal models, including models for stomach, lung, and skin cancer (*14*−*17*). In the two-stage model for skin carcinogenesis, the data showed that nomilin was more effective as an inhibitor during the initiation stage of carcinogenesis, whereas limonin was more active during the promotional phase of carcinogenesis. These data suggested that the two limonoids, although structurally similar, may be operating by different mechanisms of action. Further support for this possibility can be found in a study on the effects of citrus limonoids on glutathione *S*-transferase activity in mice (*18*). The glutathione *S*-transferase enzymes are one of the major enzyme systems responsible for the removal of complex chemical waste including carcinogens from the cell. Cancer chemopreventive agents that induce higher levels of glutathione *S*-transferase activity are usually classified as type A blocking agents (*19*). Data for the citrus limonoids showed that at a dose of 10 mg several of the citrus chemicals could significantly increase the activity of glutathione *S*-transferases in the liver of female ICR/Ha mice. The active chemicals included nomilin, isoobacunoic acid, obacunone, and ichangin. Ineffective compounds in this study were limonin, deoxylimonin, limonol, and deacetylnomilin.

In our laboratory, we have focused on one animal model, the hamster cheek pouch model for oral carcinogenesis (*20*), and have tested nine limonoids for cancer chemopreventive activity (*21*−*25*). The results showed that three of the citrus compounds (limonin, limonin 17-β-D-glucopyranoside, and limonin carboxymethoxime) significantly reduced tumor burden with very little effect on tumor number. Because tumor burden depends on both tumor number and tumor volume, the primary effect with these chemicals was on tumor volume. Two of the limonoids (nomilin and nomilin 17-β-D-glucopyranoside) were classified as having partial activity. With these compounds, the primary effect was on tumor number with very little, if any, additional effect on tumor volume. The four remaining li-

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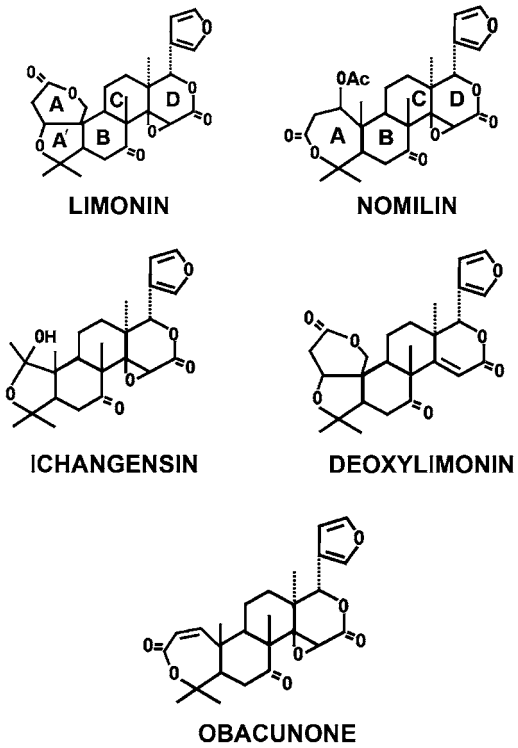

Figure 1. Structures of two reference limonoids (limonin and nomilin) and the limonoids used in this study (ichangensin, deoxylimonin, and obacunone).

monoids (limonol, nomilinic acid 17-β-D-glucopyranoside, 17, 19-didehydrolimonoic acid, and deoxylimonic acid) were inactive. This research has identified new limonoids with anticancer activity and has provided basic information on the relationship between structure and biological activity. In addition, the data showing that one set of limonoids, including limonin, classified as having significant activity, primarily reduced tumor volume, whereas a second set of limonoids, including nomilin, classified as having partial activity, reduced tumor number offer support for the idea that different limonoids maybe operating by different mechanisms of action (*16*, *18*).

As a continuation of this research three new citrus limonoids, deoxylimonin, obacunone, and ichangensin, have been tested for anticancer activity. The structures for the three test chemicals and the structure for two reference limonoids, limonin and nomilin, are given in **Figure 1**.

MATERIALS AND METHODS

Animal Care. Forty hamsters were used in the first experiment with ichangensin and 60 in the second experiment with deoxylimonin and obacunone. All of the female Syrian golden hamsters (Lak:LVG) were purchased from the Charles River Breeding Laboratories (Wilmington, MA). At the time of arrival, the hamsters were 6 weeks old, weighing 90−100 g. The animals were housed in stainless steel cages in a room maintained at 22 °C with a 12:12 h light−dark cycle. After arriving, the hamsters were given 1 week to acclimate. During this time and throughout the rest of the experiment, food (Rodent Diet 7002 by Harlan Teklad in Madison, WI) and water were provided ad libitum.

Study Design. Following the initial period of adjustment, the hamsters in the two experiments were randomly divided into groups (20 animals/group). In each experiment, the hamsters in group I served as the positive controls. In the first experiment, the left buccal pouches of the animals in the two groups were pretreated with two separate daily applications of a 50:50 mixture of DMSO and propylene glycol (group I) or a 2.5% solution of ichangensin (group II). In the second experiment, the left buccal pouches were treated with two applications of the 50:50 mixture of DMSO and propylene glycol (group I), a 2.5% solution of deoxylimonin (group II), or a 2.5% solution of obacunone (group III). The citrus limonoids were dissolved in a 50:50 mixture of DMSO and propylene glycol. The solutions of the limonoids were prepared on the day of use.

Following the initial treatments, 17 hamsters were selected from each group. The left buccal pouches of these experimental animals were painted five times per week. On an alternating week-to-week basis, the left buccal pouches of all of the hamsters were treated with a 0.5% solution of the carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA), dissolved in heavy mineral oil. On alternate days, the pouches of the animals in the first experiment were treated with either the 50:50 mixture of DMSO and propylene glycol (group I) or the 2.5% solution of ichangensin (group II). In the second experiment, the pouches were treated with either the 50:50 mixture of DMSO and propylene glycol (group I), the 2.5% solution of deoxylimonin (group II), or the 2.5% solution of obacunone (group III).

The remaining hamsters, three per group, served as negative controls. Like the experimental animals the left buccal pouches of these animals were painted five times per week. Two or three times per week the pouches of the control animals in the two experiments were treated with heavy mineral oil. On alternate days, the pouches of the hamsters in the first experiment were treated with either the 50:50 mixture of DMSO and propylene glycol (group I) or the 2.5% solution of ichangensin (group II). The pouches of the animals in the second experiment were treated with either the 50:50 mixture of DMSO and propylene glycol (group I), the 2.5% solution of deoxylimonin (group II), or the 2.5% solution of obacunone (group III).

In each experiment, all of the solutions were painted on the pouches with a no. 5 camel hair brush. Each application places ~50 μL of the solution on the surface of the pouch (*26*).

Sample and Data Collection. After a total of 70 applications, 34 with the solution of DMBA (experimental animals) or mineral oil (control animals) and 36 with the 50:50 mixture of DMSO and propylene glycol (group I) or the solution of the citrus limonoids (groups II or III), the hamsters were sacrificed. The treated pouches were excised, and the tumors were counted and measured (length, height, and width). The sum of these three measurements divided by six was used to calculate an average radius for each tumor. Using the formula for the volume of a sphere, $4/3\pi r^3$, an approximation for the volume of each tumor was calculated. The sum of the volumes of all the tumors in one pouch was defined to be the total tumor burden for that animal (*21*, *27*).

Once the gross tumor data had been collected, the tissues were mounted on heavy paper, fixed in 10% formalin, processed by routine histological techniques, and stained with hematoxylin and eosin. The data from the macroscopic and microscopic observations were used to determine tumor incidence, number, burden, and type. Student's *t* test and Chi-square analysis were used to compare the data for the experimental groups. The procedures used in these experiments are similar to the procedures used in our earlier studies with citrus limonoids (*21*−*25*).

RESULTS

Seven of the 85 animals died during the two experiments. Each of these deaths occurred relatively early. The cause of death in each case was due to respiratory problems associated with the anesthetic used to lightly anesthetize the hamsters before each treatment. At the time of death, the pouches were removed and autopsied. Signs of dysplasia were noted; however, all of the pouches were free of any apparent tumors. Each of these animals was excluded from the study.

The data for the experiment with ichangensin are given in **Table 1**. At the end of the experiment, the numbers of hamsters were 16 in group I and 17 in group II. Multiple tumors (2−14) were found in 31 of the 33 pouches. The remaining pouches, one in group I and one in group II, were free of any visible tumors. The values for tumor radii ranged from 0.7 to 4.8 mm for group I and from 0.7 to 4.7 mm for group II. From **Table**

Table 1. Ichangensin Effects on Oral Carcinogenesis

group	no. of animals	no. of tumors	av tumor no.[a]	av tumor radius (mm)	av tumor burden[a] (mm^3)
I	16	78	4.9 ± 0.8	0.81	175 ± 47
II	17	90	5.3 ± 0.7	0.83	216 ± 38

[a] Values are means ± SE.

Table 2. Deoxylimonin and Obacunone Effects on Oral Carcinogenesis

group	no. of animals	no. of tumors	av tumor no.[a]	av tumor radius (mm)	av tumor burden[a] (mm^3)
I	15	68	4.5 ± 0.6	0.86	181 ± 47
II	14	43	3.1 ± 0.5[b]	0.84	106 ± 35
III	16	54	3.4 ± 0.5	0.78	108 ± 21

[a] Values are means ± SE. [b] $p < 0.05$ when group II is compared to group I.

1, it can be seen that the treatments with ichangensin (group II) led to a slight increase in average tumor number (8%), average tumor radii (2%), and average tumor burden (23%). None of these effects proved to be significant.

Table 2 gives the results for the experiment with deoxylimonin and obacunone. The number of hamsters in the three groups ranged from 15 in group I to 14 in group II to 16 in group III. Tumors were found in all of the pouches. Most of the pouches, 15 of 15 in group I, 13 of 14 in group II, and 12 of 16 in group III, contained multiple tumors (2−9). The values for tumor radii ranged from 0.8 to 4.4 mm for group I, from 0.8 to 4.9 mm for group II, and from 0.8 to 3.8 mm for group III.

From a comparison of groups I and II, it can be seen that the treatments with deoxylimonin (group II) reduced average tumor number by >30%. Using Student's *t* test, it was found that this difference (group II versus group I) was significant, $p < 0.050$. Additional comparisons between groups II and I indicated that the paintings with deoxylimonin had very little effect on average tumor radius and reduced average tumor burden by >40%. These differences did not prove to be significant. Further analysis of the data suggested that one of the animals in group II might be an outlier. One extremely large tumor accounting for over a third (33.8%) of the total tumor burden for group II was found in the pouch from this hamster. A number of statistical tests were run (box and whiskers plots and the Komogorov−Smirnov test), each suggesting that this value should be excluded from the data set for group II. Removing this value lowers the average tumor radius for group II to 0.76 mm and the average tumor burden to 75 ± 18 mm^3. With this change, the differences in average tumor burden (group II versus group I) were significant, $p < 0.050$ (analysis of variance and Tukey's multiple-comparison test). Another way to handle extreme results is to winsorize the data (*28*). This technique forces the investigator to do the same thing to each of the experimental groups. In addition, both the high and low values for each group have to be modified. Specifically, the high and low values for each group are replaced, respectively, by the next to the highest and next to the lowest values. Winsorsizing the data one time yielded the following results for average tumor burden: 172 ± 42 mm^3 (group I), 85 ± 19 mm^3 (group II), and 106 ± 20 mm^3 (group III). The differences between groups I and II were once again significant, $p < 0.050$.

A comparison of groups I and III highlights the effects of obacunone (group III). As illustrated (**Table 2**), the treatments with obacunone led to a 25% reduction in average tumor number, a 10% reduction in average tumor radius, and a 40% reduction in average tumor burden. The differences (group I versus group III) were not significant. Even after the data for average tumor burden were winsorized, the differences between groups I and III were not significant.

Histologically, all of the tumors in the two experiments were classified as epidermoid carcinomas. Sections taken from the pouches of the control animals in the five groups were normal. During the two experiments, the weight-gain profiles for the experimental animals in the different groups were similar. No significant differences were noted. Throughout the course of the two experiments the gross appearance of the hamsters in the different groups appeared to be normal.

DISCUSSION

As illustrated in **Figure 1**, the limonoid nucleus in limonin is a collection of five rings designated the A, A′, B, C, and D rings. By comparison, the limonoid nucleus in nomilin contains only four rings. Instead of separate A and A′ rings, nomilin has one large A ring. The B, C, and D rings in nomilin and limonin are identical. As can be seen in **Figure 1**, the limonoid nuclei in ichangensin and deoxylimonin are structurally related to limonin. Obacunone is structurally similar to nomilin. The results with ichangensin, like the earlier data with 17,19-didehydrolimonoic acid (*29*), suggest that an intact A ring is required for antineoplastic activity in this tumor model. From a comparison of the structures of limonin and deoxylimonin (**Figure 1**), it can be seen that the differences in the two compounds are found in the D ring. The results with deoxylimonin, like the earlier data with limonin 17-β-D-glucopyranoside and nomilin 17-β-D-glucopyranoside, suggest (*29*) that modifications can be made to the D ring without any apparent loss of biological activity.

The minor differences between nomilin and obacunone are found in the large A ring (**Figure 1**). In addition, the results with obacunone are similar to the earlier results with nomilin and nomilin 17-β-D-glucopyranoside (*21*, *22*). In each case, there was a 20−30% reduction in tumor number. By comparison, the effects on tumor volume were minimal. Even though the effects on tumor number were not significant, the consistent pattern of the data with each of these structurally related limonoids plus the earlier data showing anticancer activity in other tumor models (*14*−*18*) suggests that each of these compounds can partially inhibit the initiation phase of oral carcinogenesis. Additional support for a classification of partial activity can be found in a recent paper (*30*) showing that obacunone can inhibit the formation of azoxymethane-induced aberrant crypt foci in rats. The results also indicated that diets containing obacunone were more effective during the initiation phase of colon carcinogenesis.

As indicated, recent studies have focused on the health-promoting properties of citrus limonoids. Most of the research has concentrated on anticancer activity; however, new studies (*31*−*33*) now indicate that limonoids have cholesterol-lowering potential and might be used to treat some forms of cancer. All of this research has increased the possibility that limonoids might eventually be used commercially for the development of new products (*29*, *34*). Most of the commercial interest has concentrated on the limonoid glucosides. There are several reasons for this emphasis. As already indicated, most of the aglycons including limonin, nomilin, and obacunone are bitter. The limonoid glucosides are tasteless (*12*, *35*). A second basic difference is solubility. Most of the aglycons are insoluble in water, whereas the limonoid glucosides are water-soluble (*12*, *35*, *36*). A third factor is human consumption. Citrus products contain high concentrations of mixed limonoid glucosides (*37*,

38). For example, the average concentrations in orange, grapefruit, and lemon juice are 320, 190, and 82 ppm. Finally, research on byproducts from juice-processing plants has shown that mixed limonoid glucosides can be isolated in large quantities from citrus molasses and seeds (*39*−*41*). The seeds are also an excellent source for the aglycons, principally a mixture of limonin and nomilin.

One potential problem with using a mixture to create a new designer food is that the health-promoting properties of all of the chemicals in the mixture must be assessed. The limonoid glucoside mixture from orange molasses contains six chemicals (*42*). The six glucosides and percent composition are limonin 17-β-D-glucopyranoside (35.9%), nomilinic acid 17-β-D-glucopyranoside (32.0%), nomilin 17-β-D-glucopyranoside (12.7%), deacetylnomilinic acid 17-β-D-glucopyranoside (8.7%), deacetylnomilin 17-β-D-glucopyranoside (6.6%), and obacunone 17-β-D-glucopyranoside (4.1%). Only three of the chemicals have been tested for cancer chemopreventive activity. As already indicated, two of them have activity in the hamster cheek pouch model, and one, nomilinic acid 17-β-D-glucopyranoside is inactive. The others, accounting for ~20% of the mixture, have not been tested. Earlier results, when coupled with the results from these experiments, suggest that obacunone 17-β-D-glucopyranoside, like obacunone, may have anticancer activity. The earlier data supporting this assumption can be found in the results for limonin and limonin 17-β-D-glucopyranoside and for nomilin and nomilin 17-β-D-glucopyranoside (*21*, *22*). In each case, the data for the aglycon and the corresponding glucoside were nearly identical. The similarity in these two sets of data suggested that the addition of one molecule of glucose to the D ring of the aglycon does not alter the antineoplastic activity of these citrus chemicals. Another possibility is that the glucose is removed and the aglycon is re-formed.

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