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Robustaflavone, a potential non-nucleoside anti-hepatitis B agent

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Abstract

Robustaflavone, a naturally occurring biflavanoid isolated from *Rhus succedanea*, was found to be a potent inhibitor of hepatitis B virus (HBV) replication in 2.2.15 cells, with an effective concentration (EC_{50}) of 0.25 μM, and a selectivity index (SI, IC_{50}/EC_{90}) of 153. Robustaflavone hexaacetate inhibited HBV replication with an EC_{50} of 0.73 μM, but exhibited no cytotoxicity at concentrations up to 1000 μM. Combinations of robustaflavone with penciclovir and lamivudine displayed synergistic anti-HBV activity, having the most pronounced effects when the combination ratios were similar to the ratio of EC_{50} potencies. Thus, a 1:1 combination of robustaflavone and penciclovir exhibited an EC_{50} of 0.11 μM and an SI of 684, while a 10:1 combination of robustaflavone and lamivudine exhibited an EC_{50} of 0.054 μM and an SI of 894. Statistical analyses of the combination data using the Combostat® program confirmed that robustaflavone exhibited synergism with both penciclovir and lamivudine.

Keywords: Biflavanoid; Combination therapy; Hepatitis B virus; Natural product; Non-nucleoside antiviral agent

1. Introduction

Hepatitis B virus (HBV) represents one of the most serious health problems in the world today (Hoofnagle, 1990). Approximately 300 million persons are chronically infected with HBV worldwide, with over 1 million of those in the USA. The Centers for Disease Control estimates that over 300000 new cases of acute HBV infection occur in the USA each year, resulting in 4000 deaths due to cirrhosis and 1000 due to hepatocel-

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lular carcinoma (Aach, 1988). The highest incidence of HBV infection occurs in the Far East and sub-Saharan Africa, where approximately 20% of the population are chronically infected Martin and Friedman, 1992). Infection with HBV can be prevented through the use of several extremely effective recombinant vaccines (Maynard et al., 1989). These vaccines are highly immunogenic, inducing antibodies in approximately 90% of recipients (Dusheiko, 1994). Despite the availability of these vaccines, HBV infection remains the most significant viral pathogen infecting man, particularly in underdeveloped countries.

The only treatment currently approved by the Food and Drug Administration for treatment of HBV infection is interferon-α (IFN-α) (Hoofnagle and Lau, 1997). Unfortunately, IFN-α treatment suffers from poor response rates, usually less than 50% for selected chronic HBV infections. In recent years there has been considerable effort to identify 'small-molecule' antiviral agents for the treatment of HBV infection, primarily synthetic nucleoside analogs (Martin and Friedman, 1992, Dusheiko, 1994, Regenstein, 1994, Hoofnagle and Lau, 1997).

Lamivudine (3TC), approved for treatment of HIV infection, is currently being evaluated as a treatment for HBV. A recent study of 40 HIV–HBV-coinfected patients showed impressive decreases in the level of HBV replication following treatment with 3TC over a 12-month period, with no observable adverse effects (Benhamou et al., 1996). Another agent under investigation is famciclovir, an orally active prodrug of the acyclic guanine derivative penciclovir (Korba and Boyd, 1996). Famciclovir is approved for treatment of herpes zoster and acute recurrent genital herpes, and has also demonstrated promising results against HBV infection in clinical trials with little observable toxicity (Main et al., 1994). It is hoped that these agents will eventually provide promising treatment options for HBV infection.

Unfortunately, monotherapy of viral infections often results in selection for viral strains resistant to the antiviral drug being used. Indeed, clinical isolates of mutant HBV strains resistant to 3TC (Ling et al., 1996, Tipples et al., 1996) and famciclovir (Aye et al., 1996, 1997) were identified following monotherapy with those agents. It is plausible that combination therapy of HBV would provide an enhanced antiviral response, while reducing the danger of resistance selection. Combinations of agents have been demonstrated to be superior to monotherapy in the treatment of HIV (Hammer et al., 1996). Thus, the identification of compounds that inhibit HBV, particularly compounds having molecular structures differing from those of the nucleoside analogs, is of critical importance in the search for effective anti-HBV regimens.

As part of our program to discover novel non-nucleoside antiviral agents from natural sources, we assayed a series of naturally occurring biflavanoids, biflavanones and related semi-synthetic derivatives for in vitro antiviral activity against HBV. Of the analogs evaluated, only robustaflavone (Fig. 1) and two of its semi-synthetic analogs exhibited significant anti-HBV activity. Robustaflavone was isolated from the seed kernels of *Rhus succedanea*, using an improved modification (Lin et al., 1997b) of a previously described procedure (Lin and Chen, 1974a). The structure of robustaflavone, illustrated in Fig. 1, is composed of two units of apigenin (5,7,4′-trihydroxyflavone), joined at the 6-position of one flavone unit and the 3′-position of the other. The hexa-*O*-acetate and hexa-*O*-methyl ether derivatives of robustaflavone were prepared, and also found to be potent inhibitors of HBV replication, but with greatly reduced cytotoxicity. Additionally, robustaflavone was evaluated for in vitro anti-HBV activity, in combination with two nucleoside antiviral agents (lamivudine and penciclovir) which are currently undergoing clinical trials for treatment of chronic hepatitis. Robustaflavone acted synergistically with both compounds, demonstrating the potential for its use as part of a combination regimen.

2. Materials and methods

Lamivudine (3TC) and penciclovir (PCV) were purchased from Moravek Biochemical (La Brea, CA). Robustaflavone, its hexamethyl ether and hexaacetate (Lin and Chen, 1974a), amen-

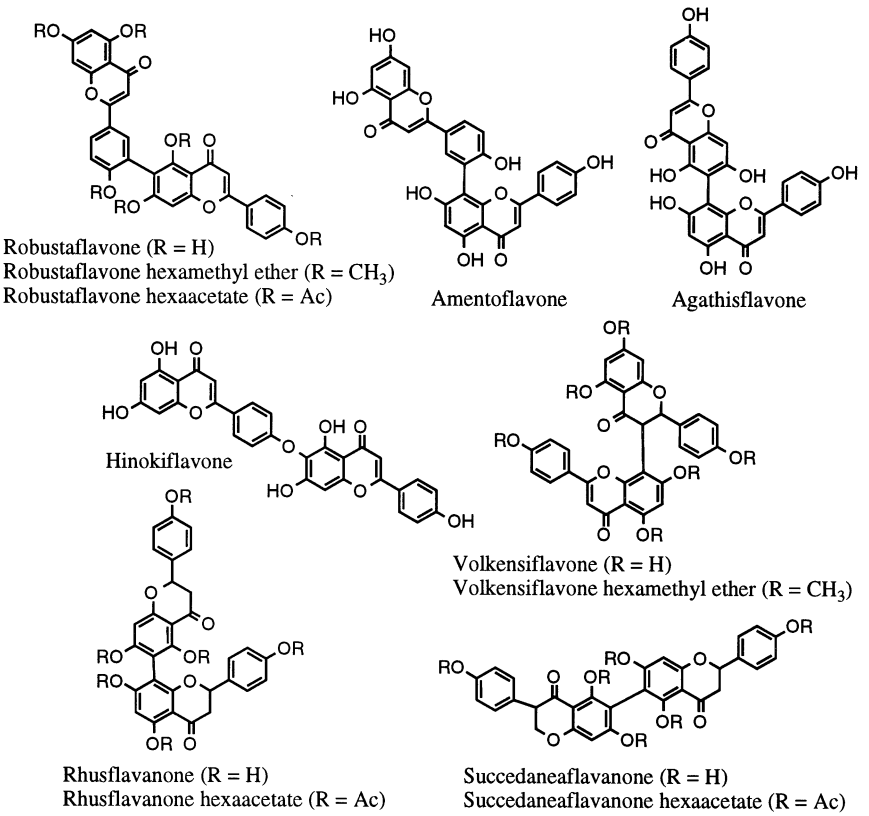

Fig. 1. Structures of naturally occurring biflavanoids and biflavanones, and related semi-synthetic derivatives.

toflavone (Chen et al., 1974), agathisflavone (Lin and Chen, 1974b), hinokiflavone (Chen et al., 1974), volkensiflavone and its hexamethyl ether (Chen et al., 1975), rhusflavanone and its hexaacetate (Chen and Lin, 1976), and succedaneaflavanone and its hexaacetate (Chen and Lin, 1975) were all purified from natural sources or prepared as previously described in the corresponding literature references. All materials tested were pure.

2.1. Assay of anti-HBV activity and toxicity in 2.2.15 cells

Determination of therapeutic concentrations (EC_{50} and EC_{90}) and toxicity concentrations (IC_{50}) were conducted as previously described (Korba and Gerin, 1992). Stock solutions of test drugs were prepared in dimethylsulfoxide. Culture medium was changed daily and analyzed for extracellular and/or intracellular HBV DNA after 9 days of continuous drug exposure. Extracellular HBV DNA was extracted from the culture medium and analyzed by a slot blot hybridization technique, using a ^{32}P-labeled *Eco*RI HBV DNA fragment as previously described (Korba and Milman, 1991), and quantitated via comparison to HBV standards on a nitrocellulose filter using an AMBIS beta scanner. Toxicity was determined by measuring inhibition of neutral red dye uptake following 9 days of continuous exposure. Effective concentration (EC_{50} and EC_{90}) and toxicity (IC_{50}) values were calculated via comparison with drug-free controls. Effective concentration values for individual agents are the mean of six cultures per concentration point. Toxicity values are the mean of three cultures per concentration point.

Table 1
Anti-HBV activity of naturally occurring biflavanoids, biflavanones and related semi-synthetic derivatives

Compound	EC_{50}	EC_{90}	SI (IC_{50}/EC_{90})
2′,3′-ddC	1.4	8.4	28
Lamivudine (3TC)	0.038	0.16	11200
Penciclovir (PCV)	0.19	0.92	471
Robustaflavone	0.25 (0.60)	2.2 (5.6)	153 (60)
Robustaflavone hexamethyl ether	2.9	28	>36
Robustaflavone hexaacetate	0.73 (3.9)	2.8 (11)	>360 (163)
Amentoflavone	>100	>100	ND
Agathisflavone	>100	>100	ND
Hinokiflavone	>100	>100	ND
Volkensiflavone	>100	>100	ND
Volkensiflavone hexamethyl ether	11	108	1.3
Rhusflavanone	>100	>100	ND
Rhusflavanone hexaacetate	7.1	62	2.8
Succedaneaflavanone	>100	>100	ND
Succedaneaflavanone hexaacetate	3.5	128	1.9

Anti-HBV activity was evaluated by measurement of extracellular HBV DNA (virion DNA). Numbers in parentheses correspond to values obtained via measurement of intracellular HBV DNA (replicative intermediates).
ND indicates that a selectivity index could not be determined because the compound did not exhibit anti-HBV activity.

2.2. Combination studies of robustaflavone, 3TC and PCV in 2.2.15 cells

Cultures were treated with combinations of agents as previously described (Korba, 1995). Briefly, antiviral agents were mixed at approximately equipotent molar concentrations based on the EC_{50} values. Serial dilutions of these mixtures were then used to treat cultures as described above along with the appropriate monotherapies. For these studies, eight cultures were used for each of six 3-fold dilutions. Statistical analyses were conducted with the Combostat® software program (Belen'kii and Schinazi, 1994).

3. Results

Of the natural products tested (Fig. 1), robustaflavone exhibited the most potent anti-HBV activity, inhibiting the replication of HBV by 50% relative to drug-free controls (EC_{50}) at a concentration of 0.25 μM, with an in vitro selectivity index (SI, IC_{50}/EC_{90}) of 153 (Table 1). In a comparison with several nucleoside antiviral agents, the anti-HBV activity of robustaflavone was superior to ddC ($EC_{50} = 1.4$ μM, SI = 30) and similar to penciclovir ($EC_{50} = 0.19$ μM, SI = 471). Lamivudine was clearly the most active of the agents evaluated ($EC_{50} = 0.038$ μM, SI = 11200).

The semi-synthetic derivatives robustaflavone hexa-*O*-acetate and robustaflavone hexa-*O*-methyl ether were approximately 3-fold and 10-fold less potent with regard to anti-HBV activity, respectively, but neither of these derivatives exhibited cytotoxicity up to a concentration of 1000 μM. Volkensiflavone hexa-*O*-methyl ether, rhusflavanone hexa-*O*-acetate and succedaneaflavanone hexa-*O*-acetate were the only other non-robustaflavone analogs to inhibit HBV replication, but all possessed unacceptable SIs (1.3, 2.8 and 1.9, respectively). Interestingly, the parent compounds of the latter three semi-synthetic derivatives (volkensiflavone, rhusflavanone and succedaneaflavanone) were all inactive against HBV replication, in contrast to the relationship of robustaflavone with its hexaacetate and hexamethyl ether derivatives. In addition to the effect of robustaflavone and robustaflavone hexaacetate on extracellular HBV DNA levels, the effect on intracellular HBV DNA (replicative intermediate) levels was measured, again in 2.2.15 cells. As shown in parentheses in Table 1, both robustaflavone and its hexaacetate inhibited pro-

Table 2
Antiviral and cytotoxicity effects of robustaflavone (RF), lamivudine (3TC), penciclovir (PCV) and drug combinations in 2.2.15 cells

Drug (or combination)	EC_{50} (μM) [a]	EC_{90} (μM) [a]	IC_{50} (μM)	SI (IC_{50}/EC_{50})	3TC or PCV (μM) [b]
RF	0.25	2.2	337	153	–
3TC	0.038	0.16	1790	11200	–
PCV	0.19	0.92	433	471	–
RF+3TC (30:1)	0.33	1.7	325	191	0.057
RF+3TC (10:1)	0.054	0.38	340	894	0.038
RF+3TC (3:1)	0.16	0.73	363	497	0.24
RF+PCV (10:1)	0.99	2.9	334	115	0.29
RF+PCV (3:1)	0.22	0.63	360	570	0.21
RF+PCV (1:1)	0.11	0.51	349	684	0.51

Single agent studies were conducted as described in Korba and Gerin (1992). Combination studies were conducted as described in Korba (1995).

[a] EC, effective concentration necessary to decrease extracellular HBV DNA levels by 50% (EC_{50}) or 90% (EC_{90}) relative to drug-free controls.

[b] Concentration of 3TC or PCV in combination with RF at varying ratios required to achieve inhibition of HBV replication by 90% (EC_{90}).

duction of replicative intermediates to a similar degree as for virion DNA.

Robustaflavone was evaluated in combination with two leading anti-HBV candidates, lamivudine (3TC) and penciclovir (PCV, the bioactive form of famciclovir), to determine if combinations of robustaflavone with either of these drugs could potentially offer synergistic benefits if used as part of an anti-HBV regimen. Combinations of antiviral agents are now accepted to be superior to monotherapy in certain instances, especially in treatment of HIV infection, because of the ability of drug combinations to suppress the selection of resistant mutants which accumulate following exposure to a single agent ((Hammer et al., 1996). Indeed, clinical isolates of HBV mutants resistant to 3TC (Ling et al., 1996, Tipples et al., 1996) and famciclovir (Aye et al., 1996, 1997) have been observed following monotherapy with these agents.

As shown in Table 2, combinations of robustaflavone (RF) with 3TC at differing ratios showed varying degrees of synergism and antagonism. It is interesting to note that the most effective ratio of RF to 3TC was 10:1, which exhibited an EC_{50} of 0.054 μM with respect to RF (0.0054 μM with respect to 3TC). The ratio of 10:1 correlates well with the EC_{50} ratio for RF and 3TC (RF/3TC = 6.6). The concentration of 3TC in the 10:1 combination (0.038 μM) necessary to achieve 90% reduction of HBV replication was lower than that required by 3TC alone (0.16 μM), illustrating the potential benefits of the combination. Ratios of RF/3TC of 30:1 and 3:1 were less effective than the 10:1 combination. Combostat® analysis (Belen'kii and Schinazi, 1994) of the RF/3TC combination data indicated that, at a molar ratio of 10:1, the combination exhibited synergism at all dilutions (Fig. 2). The RF/3TC ratio of 30:1 exhibited antagonism at lower drug concentrations, and additive or mildly synergistic effects at higher concentrations. At an RF/3TC ratio of 3:1, the combination exhibited antagonism at all drug concentrations.

The combinations of RF with penciclovir (PCV) also showed a clear synergistic effect. Because RF and PCV have similar effective concentrations (EC_{50} values of 0.25 and 0.19 μM, respectively) and cellular toxicity concentrations (IC_{50} values of 337 and 433 μM, respectively), the synergistic effects of the agents together was much more obvious. At an RF/PCV ratio of 10:1, the SI was inferior to either drug alone. At an RF/PCV ratio of 3:1, the SI improved to 570, higher than either drug alone, while an RF/PCV ratio of 1:1 exhibited an even higher SI of 684. At the 1:1 combination ratio, the concen-

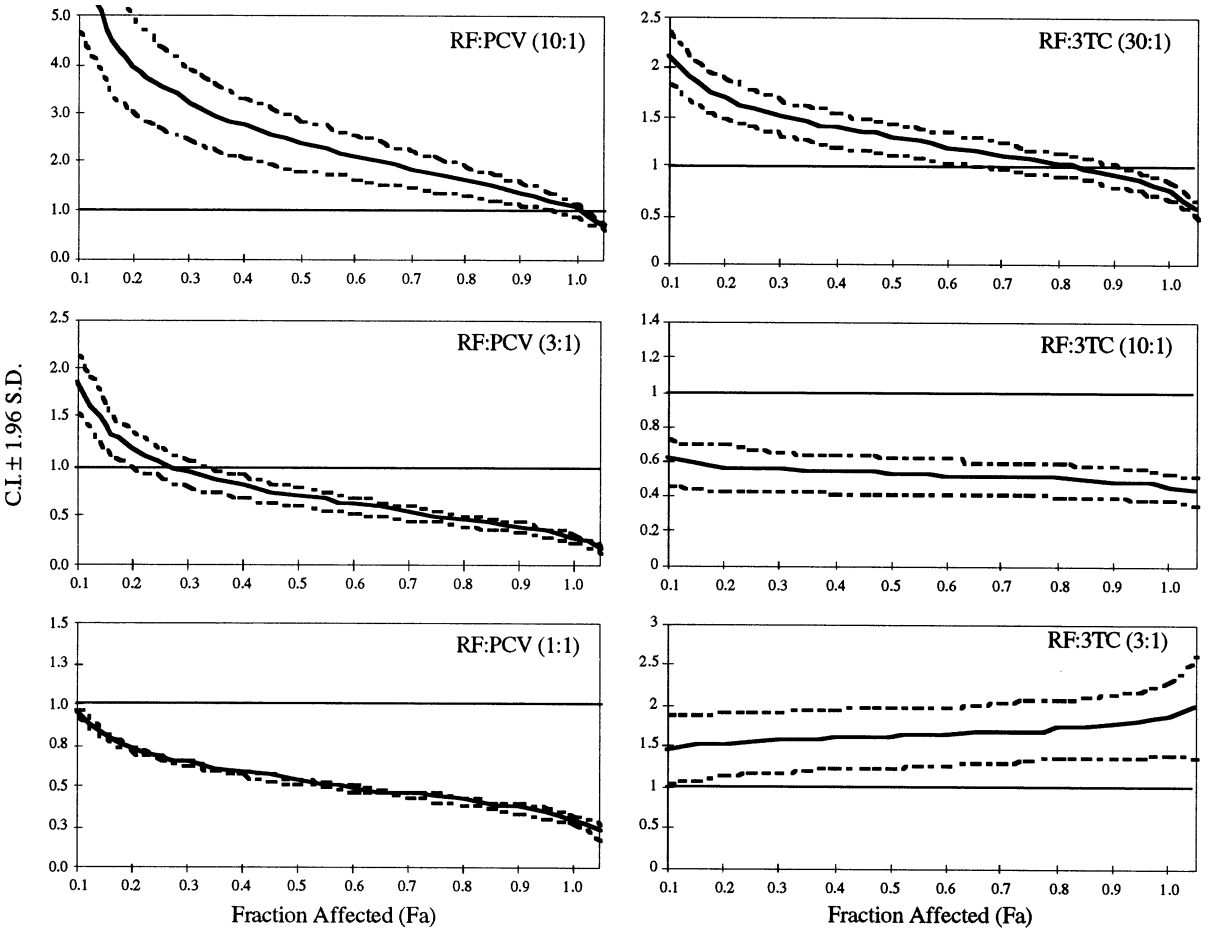

Fig. 2. Fraction affected (Fa) versus confidence interval (CI) curves for combinations of robustaflavone (RF) with lamivudine (3TC) and penciclovir (PVR) in vitro. The solid lines of the curves represent the mean ± 1.96 SD CI values. A CI of 1.0 (horizontal line) indicates additivity. CI values greater than 1.0 indicate antagonism; values below 1.0 indicate synergism. For a rigorous discussion of the Combostat® program, see Belen'kii and Schinazi (1994).

tration of PCV necessary to achieve 90% reduction of HBV replication was 0.51 μM, nearly 2-fold less than the concentration required of PCV alone (0.92 μM). Combostat® analysis of the combination data indicated that an RF/PCV ratio of 1:1 exhibited synergism at all concentrations (Fig. 2). At an RF/PCV ratio of 3:1, the combination still showed a mild degree of synergism at all concentrations except for the very lowest, while a 10:1 ratio exhibited antagonism at most concentrations. Again, the ratio exhibiting the greatest synergistic effect was that which mirrored the EC_{50} ratio for the two agents (RF/PCV = 1.3).

4. Discussion

Robustaflavone represents a novel non-nucleoside natural product possessing activity against HBV replication. Comparison of robustaflavone with a series of other naturally occurring biflavanoids and biflavanones, as well as several semi-synthetic derivatives, indicated that robustaflavone possesses unique structural features that impart the observed antiviral activity. Preliminary mechanism of action studies suggested that robustaflavone inhibited the activity of the HBV DNA polymerase enzyme; following treatment of 2.2.15 cells, levels of extracellular

and intracellular HBV DNA were dramatically reduced, while levels of viral mRNA and viral proteins were virtually unaffected (Lin et al., 1997b). It was also shown previously that robustaflavone inhibited the activity of HIV-1 reverse transcriptase, though weakly (Lin et al., 1997a). However, because both the hexa-*O*-acetate and hexa-*O*-methyl derivatives were also significantly active against HBV, it is possible that inhibition of other events in the viral replication cycle could be responsible for the observed in vitro anti-HBV activity of robustaflavone and its analogues.

Studies of robustaflavone in combination with two other potential anti-HBV agents, lamivudine (3TC) and penciclovir (PCV), indicated that robustaflavone acts synergistically with these two nucleoside analogs. The most beneficial combinations were those in which the molar ratios of robustaflavone to nucleoside drug were similar to the respective EC_{50} ratios. It is plausible that robustaflavone could potentially be utilized as part of a combination therapy to combat the selection of drug-resistant viral strains which can accumulate as a result of monotherapy, or as 'rescue' therapy for patients infected with such mutants. Further studies of robustaflavone and its hexaacetate derivative as potential anti-HBV agents are continuing.

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References

Aach, R.D., 1988. The treatment of chronic type B viral hepatitis. Ann. Intern. Med. 109, 89–91.

Aye, T.T., Bartholomeusz, A., Shaw, T., Breschkin, A., Groenen, L., Bowden, S., McMillan, J., Angus, P., McCaughan, G.W., Locarnini, S., 1996. Hepatitis B virus polymerase mutations during famciclovir therapy in patients following liver transplantation. Hepatology 24, 285A.

Aye, T.T., Bartholomeusz, A., Shaw, T., Bowden, S., Breschkin, A., McMillan, J., Angus, P., Locarnini, S., 1997. Hepatitis B virus polymerase mutations during antiviral therapy in a patient following liver transplantation. J. Hepatol. 26, 1148–1153.

Belen'kii, M.S., Schinazi, R.F., 1994. Multiple drug effect analysis with confidence interval. Antiviral Res. 25, 1–11.

Benhamou, Y., Katlama, C., Lunel, F., Coutellier, A., Dohin, E., Hamm, N., Tubiana, R., Herson, S., Poynard, T., Opolon, P., 1996. Effects of lamivudine on replication of hepatitis B virus in HIV-infected men. Ann. Intern. Med. 125, 705–712.

Chen, F.-C., Lin, Y.-M., 1975. Succedaneaflavanone—a new 6,6″-binaringenin from *Rhus succedanea*. Phytochemistry 14, 1644–1647.

Chen, F.-C., Lin, Y.-M., 1976. Rhusflavanone, a new biflavanone from the seeds of wax-tree. J. Chem. Soc. Perkin Trans. 1 1976, 98–101.

Chen, F.-C., Lin, Y.-M., Liang, C.-M., 1974. Biflavonyls from drupes of *Rhus succedanea*. Phytochemistry 13, 276–278.

Chen, F.-C., Lin, Y.-M., Hung, J.-C., 1975. Phenolic compounds from the heartwood of *Garcinia multiflora*. Phytochemistry 14, 300–303.

Dusheiko, G.M., 1994. The pathogenesis, diagnosis and management of viral hepatitis. Aliment. Pharmacol. Ther. 8, 229–253.

Hammer, S.M., Katzenstein, D.A., Hughes, M.D., Gundacker, H., Schooley, R.T., Haubrich, R.H., Henry, W.K., Lederman, M.M., Phair, J.P., Niu, M., Hirsch, M.S., Merigan, T.C., 1996. A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. New Engl. J. Med. 335, 1081–1090.

Hoofnagle, J.H., 1990. Chronic hepatitis B. New Engl. J. Med. 323, 337–339.

Hoofnagle, J.H., Lau, D., 1997. New therapies for chronic hepatitis B. J. Viral Hepatitis 4 (Suppl. 1), 41–50.

Korba, B.E., 1995. In vitro evaluation of combination therapies against hepatitis B virus replication. Antiviral Res. 29, 49–51.

Korba, B.E., Boyd, M.R., 1996. Penciclovir is a selective inhibitor of hepatitis B virus replication in cultured human hepatoblastoma cells. Antimicrob. Agents Chemother. 40, 1282–1284.

Korba, B.E., Gerin, J.L., 1992. Use of a standardized cell culture assay to assess activities of nucleoside analogs against hepatitis B virus replication. Antiviral Res. 19, 55–70.

Korba, B.E., Milman, G., 1991. A cell culture assay for compounds which inhibit hepatitis B virus replication. Antiviral Res. 15, 217–228.

Lin, Y.-M., Chen, F.-C., 1974a. Robustaflavone from the seed-kernels of *Rhus succedanea*. Phytochemistry 13, 1617–1619.

Lin, Y.-M., Chen, F.C., 1974b. Agathisflavone from the drupes of *Rhus succedanea*. Phytochemistry 13, 657–658.

Lin, Y.-M., Anderson, H., Flavin, M.T., Pai, Y.-H.S., Mata-Greenwood, E., Pengsuparp, T., Pezzuto, J.M., Schinazi, R.F., Hughes, S.H., Chen, F.-C., 1997a. In vitro anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*. J. Nat. Prod. 60, 884–888.

Lin, Y.-M., Zembower, D.E., Flavin, M.T., Schure, R.M., Anderson, H.M., Korba, B.E., Chen, F.-C., 1997b. Robustaflavone, a naturally occurring biflavanoid, is a potent inhibitor of hepatitis B virus replication in vitro. Bioorg. Med. Chem. Lett. 7, 2325–2328.

Ling, R., Mutimer, D., Ahmed, M., Boxall, E.H., Elias, E., Dusheiko, G.M., Harrison, T.J., 1996. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 24, 711–713.

Main, J., Brown, J.L., Karayiannis, P., Georgiou, P., Boyd, M., Prince, W., Thomas, H., 1994. A double-blind, placebo-controlled study to assess the effect of famciclovir on virus replication in patients with chronic hepatitis B virus infection. J. Hepatol. 21 (Suppl. 1), S32.

Martin, P., Friedman, L.S., 1992. Therapies for Hepatitis B virus: current status and future possibilities. In: Block, T.M., Junkind, D., Crowell, R.L., Denison, M., Walsh, L.R. (Eds), Innovations in Antiviral Development and the Detection of Virus Infections. Plenum Press, New York, pp. 111–120.

Maynard, J.E., Kane, M.A., Hadler, S.C., 1989. Global control of hepatitis B through vaccination: role of hepatitis B vaccine in the expanded programme on immunization. Rev. Infect. Dis. 11, S574–S578.

Regenstein, F., 1994. New approaches to the treatment of chronic viral hepatitis B and C. Am. J. Med. 96 (Suppl. 1A), 47S–51S.

Tipples, G.A., Ma, M.M., Fischer, K.P., Bain, V.G., Kneteman, N.M., Tyrrell, D.L.J., 1996. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 24, 714–717.