Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 17, pp. 2325-2328, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00422-8

ROBUSTAFLAVONE, A NATURALLY OCCURRING BIFLAVANOID, IS A POTENT NON-NUCLEOSIDE INHIBITOR OF HEPATITIS B VIRUS REPLICATION IN VITRO

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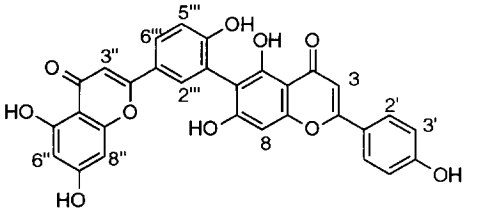
Abstract. Robustaflavone, a naturally occurring biflavanoid isolated from the seed kernel extract of *Rhus succedanea*, was found to be a potent in vitro inhibitor of hepatitis B, with an effective concentration (EC_{50}) of 0.25 μM and an in vitro selectivity index (IC_{50}/EC_{90}) of 153. Further studies suggested that inhibition of HBV DNA polymerase is the mechanism of action. © 1997 Elsevier Science Ltd.

Hepatitis B virus (HBV) represents one of the most serious health problems in the world today, and is listed as the ninth leading cause of death by the World Health Organization.[1] Approximately 300 million persons are chronically infected with HBV worldwide, with over one million of those in the United States. The only treatment approved by the Food and Drug Administration for HBV infection is interferon-α (IFN-α), which suffers from poor response rates, usually less than 40% for selected chronic HBV infections.[2]

Currently two nucleoside-based inhibitors of the HBV DNA polymerase, lamivudine[3] (3TC) and famciclovir,[4] an orally active derivative of penciclovir (PCV), are in clinical trials as potential anti-HBV agents. Unfortunately, mutant strains of HBV resistant to 3TC have already been reported in patients receiving that agent as a monotherapy,[5,6] emphasizing the need for novel molecules having potent anti-HBV activity.



Figure

As part of our program to discover novel non-nucleoside antiviral agents from natural sources, we found that robustaflavone (Figure) possesses significant anti-HBV activity. Robustaflavone was isolated from the seed-kernels of *Rhus succedanea*, using an improved modification[7] of a previously described procedure.[8] The improved method involved a dry column purification using toluene/ethanol/pyridine, which provided pure

robustaflavone in greater yields than those obtained previously, eliminated the use of benzene and decreased the volume of pyridine needed. Though flavones have been reported to possess antiviral activities,[9-13] less is known regarding antiviral activities of biflavanoids. Hinokiflavone, also isolated from *Rhus succedanea*, demonstrated inhibitory activity against genome expression of Epstein - Barr virus.[14] To our knowledge, there have been no previous reports of biflavanoids as inhibitors of hepatitis B virus replication.

Robustaflavone exhibited activity against HBV replication in a chronically infected human hepatoblastoma cell line (2.2.15),[15] inhibiting the replication of HBV (as measured by levels of extracellular viral DNA) by 50% relative to drug-free controls at a concentration of 0.25 μM (EC_{50}, mean of two trials), 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 (PCV, EC_{50} = 0.19 μM, SI = 471). Lamivudine (3TC) was clearly the most active of the agents evaluated (EC_{50} = 0.038 μM, SI = 11200). A series of 9 additional naturally occurring biflavanoids and semi-synthetic derivatives were also evaluated for inhibition of HBV replication.[16] Within this series, robustaflavone was the only compound which exhibited significant anti-HBV activity with an acceptable selectivity index, and thus the only candidate chosen for further study.

Table 1. Antiviral and cytotoxicity effects of robustaflavone and several nucleoside analogues in 2.2.15 cells.[a]

Drug	EC_{50} (μM)[b]	EC_{90} (μM)[b]	IC_{50} (μM)[c]	SI (IC_{50}/EC_{90})
Lamivudine (3TC)	0.038	0.16	1792	11200
Penciclovir (PCV)	0.19	0.92	433	471
2',3'-Dideoxycytidine (ddC)	1.4	8.4	252	30
Robustaflavone (RF)	0.25	2.2	337	153

[a]Studies were conducted as described in ref 15. [b]Effective concentration necessary to decrease extracellular HBV DNA levels by 50% (EC_{50}) or 90% (EC_{90}) relative to drug-free controls. [c]Cytotoxicity, measured by inhibition of neutral red dye uptake.

To determine a likely mechanism of action for robustaflavone, levels of extracellular and intracellular HBV DNA, mRNA and protein antigen markers were measured in 2.2.15 cells on day nine following continuous treatment.[17] As presented in Table 2, the levels of extracellular and intracellular HBV DNA were dramatically decreased relative to drug-free controls for both robustaflavone and ddC, which was used as a positive control. Neither the levels of viral mRNA (3.6 and 2.1 kb) nor the three major viral antigens (HBsAg, HBeAg, and HBcAg) were significantly affected by exposure of the cells to robustaflavone.

The results of the experiments reported in Table 2 suggest that robustaflavone acts via inhibition of HBV DNA polymerase. Because of its size, the ability of robustaflavone to penetrate the core of a mature viral particle is unlikely. Thus, inhibition of nucleic acid synthesis may occur during early stages of viral genome replication,

Table 2. Relative levels[a] of HBV nucleic acids and proteins following treatment of 2.2.15 cells with robustaflavone

	Viral DNA		Viral mRNA		Viral Antigens		
Treatment	Extracellular DNA	Intracellular DNA	3.6 kb mRNA	2.1 kb mRNA	HBsAg	HBeAg	HBcAg
Control	127 ± 8	103 ± 11	90 ± 12	101 ± 10	117 ± 11	108 ± 5	86 ± 10
ddC	1 ± 1	6 ± 1	94 ± 7	87 ± 9	90 ± 12	88 ± 10	91 ± 9
Robustaflavone	1 ± 1	5 ± 1	93 ± 10	106 ± 11	97 ± 6	86 ± 6	138 ± 8

[a]Nucleic acid and protein levels were determined following nine days of continuous exposure of confluent cultures of 2.2.15 cells to robustaflavone, ddC, and drug-free media, and are relative to the levels on day zero, before addition of drug. Experiments were conducted as described in ref 17.

such as during formation of the primer for negative-strand DNA synthesis, which occurs before encapsidation. Alternatively, it is possible that robustaflavone is itself encapsidated during assembly of the viral particle.

Robustaflavone represents a novel non-nucleoside natural product which possesses impressive activity against hepatitis B virus replication. The paucity of agents available for the treatment of HBV infection underscore the need for development of new lead compounds, especially non-nucleoside structures, which would complement the drugs currently in development, all of which are nucleoside analogues. Additionally, the recent reports concerning development of 3TC-resistance in HBV-infected patients following treatment with that drug[5,6] further emphasize the need for novel anti-HBV agents which could possibly be used as part of a combination regimen. Continued studies with robustaflavone as a lead anti-HBV agent are being pursued, including in vitro combinations with 3TC and PCV, and will be reported in the near future.[16]

Acknowledgments. We express our appreciation to Dr. Christopher Tseng (National Institute of Allergy and Infectious Diseases) for administrative support of this project. The biological assays utilized in this study were financially supported under contract N01-AI-45195 (NIAID, Georgetown University).

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7. *Improved isolation of robustaflavone.* Defatted, coarsely powered seeds of *Rhus succedanea* (16 kg, collected in Fukuoka, Japan) were exhaustively extracted with boiling 95% EtOH (150 L). Concentration of

the EtOH extracts yielded precipitates which were collected in three fractions: pigment A, 0.2%; pigment B, 0.2%; and pigment C, 2% (see ref 8). Pigment A contained a mixture of hinokiflavone and robustaflavone. Pigment A (10 g) was dissolved in 50 mL pyridine and added to 25 g dry silica gel. The pyridine was removed under reduced pressure and the dry mixture ground to a fine particle size. Into a 600 mL coarse fritted glass funnel containing a disc of filter paper over the frit was placed 250 g dry silica gel. The adsorbed pigment A was placed on top of the silica gel, and then 2.5 L of toluene:EtOH:formic acid (40:10:2) was passed through the filter. Evaporation of the eluent provided 2.01 g of a yellow solid, which contained hinokiflavone contaminated with a trace amount of robustaflavone. The silica gel in the column was dried overnight, and the top layer of silica gel containing the adsorbed pigment A was scraped from the filter and placed into a clean coarse fritted funnel containing a disc of filter paper. This material was eluted using 2.5 L toluene:EtOH:formic acid (40:10:2), followed by 4.5 L EtOH:pyridine (4:1). The former eluent provided, upon concentration, 1.1 g of a yellow solid, containing a mixture of hinokiflavone and robustaflavone. The second eluent afforded 5.65 g robustaflavone. An analytically pure sample was obtained via recrystallization from pyridine/H_2O; mp 350-352 °C (dec.); ^{1}H NMR (DMSO-d_6) δ 13.25 (s, 1 H, OH), 13.02 (s, 1 H, OH), 10.83 (s, 1 H, OH), 10.40 (s, 1 H, OH), 7.99 (d, 2 H, J = 8.9 Hz, H-2'), 7.93 (dd, 1 H, J = 8.7, 2.2 Hz, H-6'''), 7.79 (d, 1 H, J = 2.2 Hz, H-2'''), 7.05 (d, 1 H, J = 8.7 Hz, H-5'''), 6.96 (d, 2 H, J = 8.9 Hz, H-3'), 6.84 (s, 1 H, H-3''), 6.81 (s, 1 H, H-3), 6.65 (s, 1 H, H-8), 6.49 (d, 1 H, J = 2.0 Hz, H-8''), 6.20 (d, 1 H, J = 2.0 Hz, H-6''); ^{13}C NMR (DMSO-d_6) δ_{ppm} 181.8 (C-4''), 181.7 (C-4), 164.1 (C-2), 163.9 (C-2''), 163.6 (C-7''), 162.1 (C-7), 161.5 (C-9), 161.2 (C-5), 159.6 (C-5''), 159.1 (C-4'''), 157.5 (C-9''), 156.4 (C-4'), 130.9 (C-2'''), 128.6 (C-2'), 128.5 (C-6'), 127.6 (C-6'''), 121.2 (C-1'), 120.9 (C-1'''), 120.8 (C-3'''), 116.1 (C-3''), 116.0 (C-3'), 116.0 (C-5'), 108.9 (C-6), 103.7 (C-10''), 103.6 (C-10), 102.9 (C-3), 102.9 (C-5'''), 94.0 (C-8''), 93.4 (C-8), 98.8 (C-6''); HR-CIMS *m/e* 539.0970 (MH^+, requires 539.0978); FTIR (KBr) 3380, 1651, 1607, 1497, 1358, 1242, 1163, 833 cm^{-1}; UV 347 (log ε 4.38), 300 (4.42), 275 (4.44), 255 (4.71).

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(*Received in USA* 25 *June* 1997; *accepted* 11 *August* 1997)