Vol. 93, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1980 Pages 675-686

STRUCTURAL REQUIREMENTS OF QUASSINOIDS FOR THE INHIBITION OF CELL TRANSFORMATION

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Received December 20,1979

SUMMARY : The effects of eleven quassinoids on Rous sarcoma virus induced cell transformation and on growth of normal cells were examined. At concentrations of 0.15-1 µg/ml they inhibited foci formation (76-99 %) without toxic effects on normal cells. The most active compounds also affected virus production by transformed cells. In intact normal and transformed cells, protein and DNA synthesis was equally affected after 3 hours of exposure to quassinoids of both cell types. RNA synthesis was not inhibited. This study has shown that the structural requirement of a C-15 ester in the quassinoids for antileukemic activity *in vitro* and *in vivo* is not essential for their antitransforming activity.

INTRODUCTION

Several quassinoids (1), the bitter principles of the plant family Simaroubaceae, have shown significant antineoplastic activity *in vivo* against the P-388 murine lymphocytic leukemia (PS) and potent inhibitory activity *in vitro* against cells derived from human carcinoma of the nasopharynx (KB) (2-4) and against cells derived from the PS leukemia (4). One of the bruceolide 1 (5) ester quassinoids, bruceantin 2 (3) has recently been placed on clinical trial by the US National Cancer Institute. Studies (6) of the structure/activity relationships for several quassinoids have established some of the structural requirements for optimal antineoplastic activity, particularly in the P-388 mouse leukemia, *viz.* : i) ester groups at C-15 and/or C-6 ; ii) a ring A oxygenated functionality as in the glaucarubolone 6 (1) or bruceolide 1 (5) esters ; iii) an epoxymethano bridge between C-8 and C-11 or between C-8 and C-13 ; and iv) the presence of a free hydroxyl group in ring A and at C-12.

Liao *et al.* (7) using HeLa cells, have shown that the antitumor activity of the bruceolide 1 esters at the molecular level is due to the irreversible inhibition of protein synthesis.

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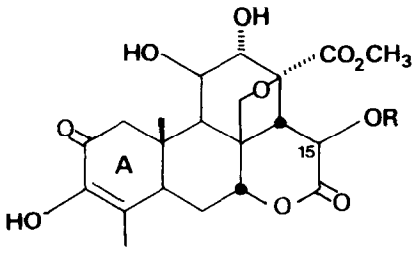
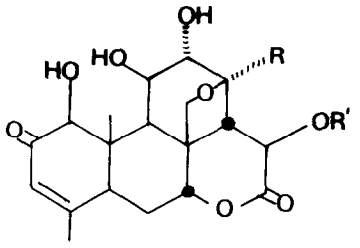


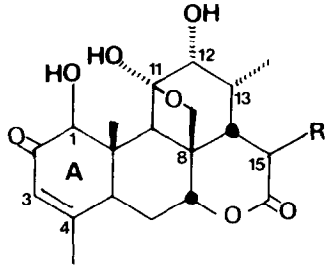
Bruceolide 1 R = H
Bruceantin 2 R = $COCH=C(CH_3)-CH(CH_3)_2$

Bruceine A 3 R = $COCH_2-CH(CH_3)_2$
Bruceine B 4 R = $COCH_3$

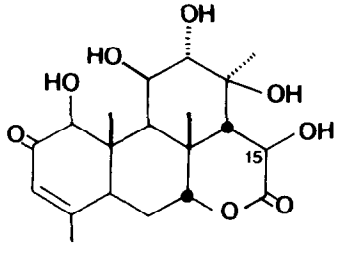


Simalikalactone D 10 R = CH_3, R' = $COCH(CH_3)-C_2H_5$

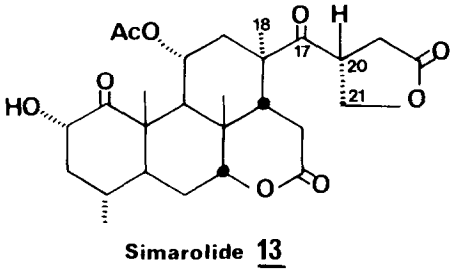
Isobruceine A 11 R = CO_2CH_3, R' = $COCH_2CH(CH_3)_2$



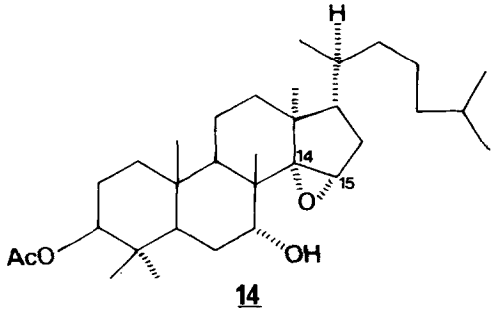
Chaparrinone 5 R = H
Glaucarubolone 6 R = OH
Glaucarubinone 7 R = $OCOC(CH_3)(OH)CH_2CH_3$
Castelanone 8 R = $OCOCH_2-CH(CH_3)_2$
Soularubinone 9 R = $OCOCH_2-C(CH_3)_2-OH$



Soulameanone 12



Simarolide 13



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Our own interest in the study of cell transformation induced by oncogenic viruses and our search for molecules having specific effects on biosynthesis of macromolecules has led us to examine the behaviour of several quassinoids (together with a triterpene epoxide) on the transformation of chick embryo fibroblasts (CEF) by Rous Sarcoma virus (RSV).

MATERIALS AND METHODS

Secondary cultures of CEF were prepared and cultivated as described previously in Eagle's MEM with 5 % calf serum and antibiotics (8). The action of the inhibitors on focus formation was tested as described earlier (8). The virus used was a clonal isolate (SR4) of Schmidt-Ruppin strain RSV, type D. Quassinoids were dissolved in dimethylsulfoxide (DMSO), diluted in the medium and sterilized by filtration. When added to the cultures the solutions of the inhibitors contained less than 0.01 % DMSO. The incorporation of radioactive precursors into proteins and nucleic acids following different exposure times of normal or transformed cells to quassinoids was followed by a standard procedure (9). The following labelled materials supplied by the Commissariat à l'Energie Atomique, Saclay, France, were used : L-leucine [^{3}H] 40 Ci/mM, 10 µCi/ml/dish, thymidine [^{3}H] 40 Ci/mM, 2 µCi/ml/dish, uridine [^{3}H] 55 Ci/mM, 4 µCi/2ml/dish.

RESULTS AND DISCUSSION

We have examined the following quassinoids : bruceine A, 3 (5) bruceine B 4 (5), chaparrinone 5 (1), glaucarubolone 6 (1), glaucarubinone 7 (1), castelanone 8 (10), soularubinone 9 (11), simalikalactone D 10 (1), isobruceine A 11 (12), soulameanone 12 (13) and simarolide 13 (14,15). All, except 13, have the same degree of oxidation in ring A. Bruceine A and B have a diosphenol group while the others have an unsaturated α ketol group. An epoxymethane bridge is located between C-8 and C-11 in 5-9, and between C-8 and C-13 in 3, 4, 10 and 11. Soulameanone lacks the epoxymethane bridge. Chaparrinone 5 is devoid of an ester side chain at C-15, whereas glaucarubolone 6 and soulameanone 12 have a hydroxy group at this position. Simarolide 13 is an acetyl derivative of a C_{25} quassinoid and has like soulameanone 12 a methyl group at C-8. We have also studied the triterpene epoxide 14 (16) which has been synthesized from oxo-3-tirucalla-7,24-diene (17), the supposed biogenetic precursor of the quassinoids.

As shown in Table I, all the quassinoids tested, except soulameanone and simarolide, inhibited RSV induced transformation of CEF in a concentration range of 0.1-0.5 µg/ml. Castelanone was somewhat less active (I_{50} = 0.78 µg/ml). The triterpene 9 had no significant effect up to 5 µg/ml. Maximal inhibition of foci for-

Table I Effect of some quassinoids on RSV-induced foci formation.

	I_{50} µg/ml	I_{50} [µM]	I max µg/ml	I max [µM]	% inhibition
3 Bruceine A	0.15	0.3	0.25	0.52	95
4 Bruceine B	0.48	1.0	0.6	1.25	95
5 Chaparrinone	0.10	0.2	0.15	0.30	76
6 Glaucarubolone	0.15	0.28	0.30	0.56	96
7 Glaucarubinone	0.35	0.90	0.50	1.30	92
8 Castelanone	0.16	0.40	0.20	0.50	83
9 Soularubinone	0.17	0.30	0.25	0.50	99
10 Simalikalactone D	0.78	1.60	1.00	2.00	88
11 Isobruceine A	0.10	0.2	0.25	0.50	94
12 Soulameanone	> 20.00	50.00			
13 Simarolide	> 20.00	40.00			
14 Triterpene	> 5.00	10.00			

Experimental conditions : Secondary CEF were plated, infected one day later with 100 focus forming units (FFU) of RSV and overlaid with 0.8 % Difco agar. The inhibitors in 1.5 ml of liquid medium were added on top of the gelled underlayer one hour after infection. Control cultures were overlaid with inhibitor-free medium. After 2 days of exposure, the inhibitor containing overlayer was replaced by the standard growth medium. Foci of transformed cells were counted 5 days later.

*Higher concentrations were toxic to normal cells.

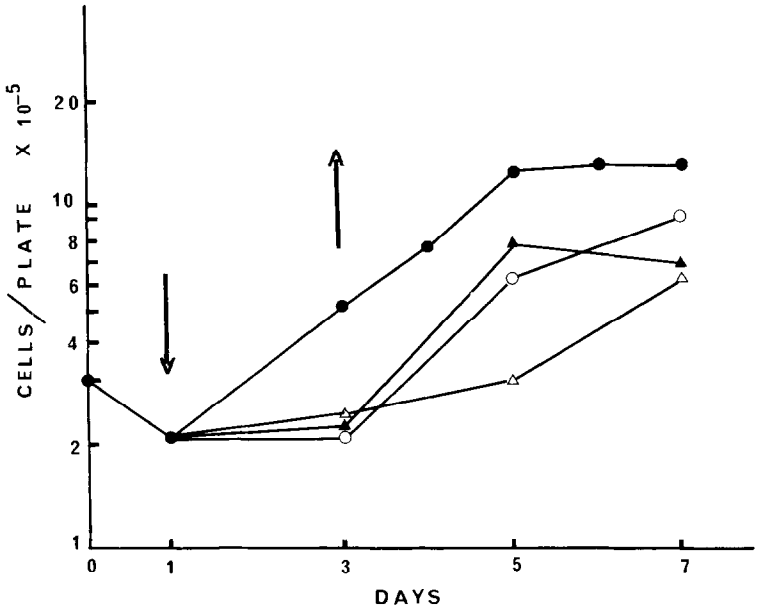

Fig. 1 : Effect of quassinoids on growth of normal CEF. 3 x 10^5 cells were seeded in plates of 35 mm in triplicate in Eagle's MEM. One day later, before addition of the inhibitors, indicated by the arrow↓ cells were counted in a haematimeter. On the third day cells were counted again and the inhibitors were removed by medium renewal, indicated by the arrow↑. In order to check the reversibility of growth arrest, cells were counted two days later and their number compared with control cultures, treated identically with inhibitor free media. Cells were incubated at 37°C. The mean values of three counts are given.

● Control, ○ chapparrinone 0.5 µg/ml (1.3 µM), ▲ glaucarubinone 0.25 µg/ml (0.5 µM), △ isobruceine A 0.3 µg/ml (0.6 µM).

mation by active quassinoids ranged between 76 and 99 %. Thus, it seems that the presence of the epoxymethane bridge is required for the anti-transforming activity. Whether the bridge is between C-8 and C-11 or between C-8 and C-13 seems to be of little importance. The presence of an ester side chain is not indispensable for the inhibitory activity, since chaparrinone 5 and glaucarubolone 6 are active.

The effects of the most active compounds, glaucarubinone 7 and isobruceine A 11, at concentration of 0.5 µM on virus production by transformed cells were studied. After 24 hours of contact the former inhibited the virus production by 40-50 % and the latter by 70 %.

The growth curves (Fig. 1) show that the concentrations necessary to inhibit cell transformation do not provoke irreversible growth arrest of normal cells.

Table II/a. Inhibition of leucine incorporation after various exposure times of CEF to quassinoids.

	Conc µg/ml	1h	3h	5h	24h
Control	0	128 095	126 250	109 993	116 320
4 Bruceine B	0.50*	32 428 (75)		9 374 (92)	3 786 (96)
5 Chaparrinone	0.50*	72 556 (43)	46 071 (44)	49 272 (55)	34 896 (70)
6 Glaucarubolone	0.20*	59 000 (54)	67 000 (47)	47 091 (57)	8 826 (93)
7 Glaucorubinone	0.25*	35 866 (72)	19 233 (84)	6 445 (94)	8 878 (92)
11 Isobruceine A	0.30*	5 600 (96)	10 109 (92)		
12 Soulameanone	0.50	107 473 (16)	107 015 (15)	76 861 (30)	81 424 (30)
13 Simarolide	0.20	109 350 (15)		72 014 (35)	78 348 (33)

Table II/b. Inhibition of thymidine incorporation after various exposure times of CEF to quassinoids

	Conc g/ml	1h	3h	5h	24h
Control	0	145 567	92 489	123 880	104 108
4 Bruceine B	0.5*	55 267 (62)		8 133 (94)	1 500 (99)
5 Chaparrinone	0.5*	140 067 (4)	31 703 (66)		19 780 (81)
6 Glaucarubolone	0.20*	80 333 (45)	51 738 (44)		2 382 (98)
7 Glaucarubinone	0.25*	33 487 (77)	5 675 (94)	5 929 (95)	6 724 (94)
11 Isobruceine A	0.3*	18 055 (88)	2 555 (97)	890 (99)	1 365 (99)
12 Soulameanone	0.50	110 872 (24)			48 655 (53)
13 Simarolide	0.20	139 670 (4)			30 812 (71)

Cells were labelled for 30 minutes at 37°, washed and radioactivity measured in the trichloroacetic acid (TCA) insoluble material. % of inhibitions are calculated with respect to untreated control cultures and are given in brackets. Results are expressed in cpm/mg of proteins, and are the mean values of two experiments.

*The highest active and non toxic concentration for CEF.

Liao et al. reported (7) that the antitumor activity of bruceantin 2 and derivatives is due to the inhibition of protein synthesis. The ester side chain at C-15 is thought to be important for the transport of the drug into intact cells and the partial unsaturation in ring A for the inhibition of protein synthesis. We have measured the effect of several structurally different quassinoids on the incorporation of radioactive leucine, thymidine and uridine into CEF. None of the compounds tested interfered significantly with uridine incorporation and with the transport of the labelled precursors into the cells (Results not shown). The effect on protein and on DNA synthesis are given in Table IIa,b and the following observations can be made :

1) Except for soulameanone 12 and simarolide 13, the quassinoids tested strongly inhibited protein synthesis (70-98 % inhibition after 24 hours of presence in the cell culture). Thus it seems that the partial unsaturation in ring A (as in 12) is not the only structural requirement for protein biosynthesis inhibition.

2) Those quassinoids which inhibited protein synthesis inhibited also DNA synthesis to about the same extent.

3) Surprisingly both simarolide and soulameanone which have no effect on transformation and only a slight effect on protein synthesis, quite strongly inhibited the incorporation of thymidine, simarolide being particularly effective.

The reversibility of the inhibition of protein and DNA synthesis by compounds 5, 6, 7, 11 and 12 is shown in Table III. The inhibition of protein synthesis by chaparrinone 5, glaucarubolone 6 and by soulameanone was reversible upon medium renewal after 1 hour. The effect on DNA synthesis by these substances was also entirely but more slowly reversible. The effects of glaucarubinone 7 and of isobruceine A 11 on both DNA and protein synthesis were irreversible after 5 hours under the same conditions.

The rate of inhibition of protein and DNA synthesis by these two quassinoids was also studied and the results are given in Fig. 2. As can be seen, 0.3 µg/ml isobruceine A 11 inhibits more strongly and more rapidly protein synthesis than DNA synthesis. The

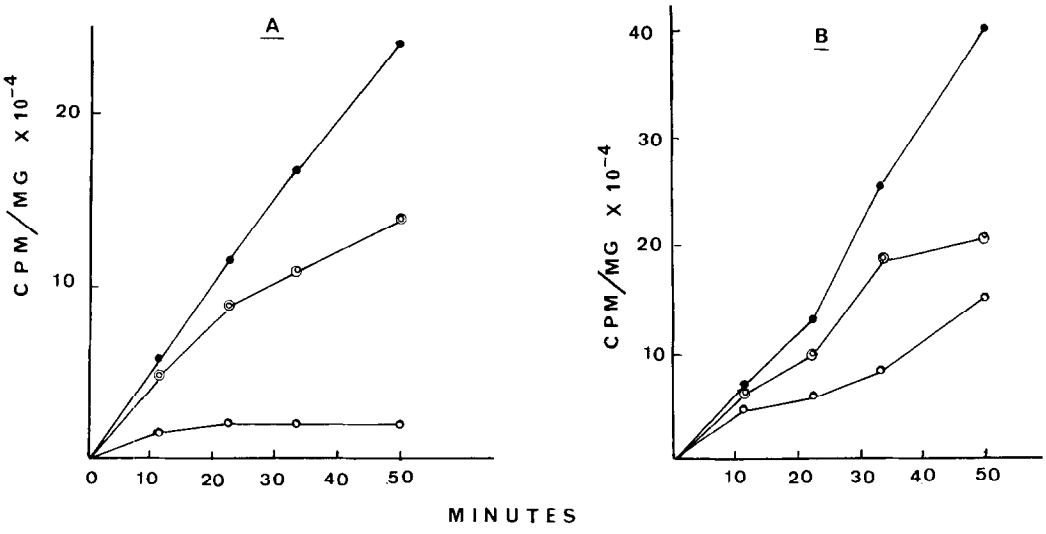

Fig. 2 : Rate of inhibition of leucine (A) and thymidine (B) incorporation into CEF in the presence of 0.3 µg/ml of isobruceine A and 0.25 µg/ml of glaucarubinone.

Secondary CEF were plated at 5 x 10^5 cells/plate of 35 mm. Culture medium was removed and radioactive precursors were added in new medium with isobruceine A (o-o), with glaucarubinone ⊙—⊙ or without the quassinoids (●-●). At different times, labelling medium was discarded and radioactivity measured in TCA precipitable material.

inhibition by glaucarubinone 7 is much slower and much weaker than the inhibition by isobruceine A, and the two biosynthesis are inhibited to about the same extent after 20 minutes of contact with the cells. However there are no differences between the effects of the two compounds on protein and DNA synthesis after an exposure time of 5 hours (Table III). Finally we studied the effect on leucine and thymidine incorporation by the two compounds at various concentrations. The I_{50} of glaucarubinone was 0.04 µg/ml for protein biosynthesis and 0.05 µg/ml for DNA biosynthesis. The I_{50} of isobruceine A 11 was 0.005 µg/ml for both. Thus compound 11 is the most potent inhibitor of cell transformation and of DNA and protein synthesis among the quassinoids tested.

In conclusion, in this model the triterpene epoxide 14 has neither a significant effect on cell transformation nor on the synthesis of macromolecules. At concentrations which inhibit cell transformation, quassinoids inhibit protein and DNA synthesis. In contrast to the results of Liao et al. (7) with bruceantin analogues in HeLa cells, our results indicate that the absence of the

Table III/a. Reversibility of the inhibition of leucine incorporation after 3 hours of exposure of CEF to some quassinoids

		Conc μg/ml	3hrs exposure time	Time following reversal (hrs) 1	2	5
	Control		126 250	139 164	115 946	128 255
5	Chaparrinone	0.50*	46 071 (44)	150 357 (0)	118 833 (0)	110 833 (14)
6	Glaucarubolone	0.20*	67 000 (47)	150 769 (0)	105 667 (8)	126 588 (1)
7	Glaucarubinone	0.25*	19 233 (85)	35 375 (75)	28 882 (75)	31 633 (75)
11	Isobruceine A	0.30*	10 109 (92)	44 107 (68)	20 833 (82)	16 833 (87)
12	Soulameanone	0.50	107 025 (15)	139 730 (0)	87 294 (25)	98 849 (23)

Table III/b. Reversibility of the inhibition of thymidine incorporation after 3 hours of exposure of CEF to some quassinoids

	Conc µg/ml	3hrs exposure time	Time following reversal (hrs) 1	2	5
Control		168 554 (0)	228 691 (0)	297 827 (0)	227 700 (0)
5 Chaparrinone	0.50	28 340 (83)	110 000 (52)	237 571 (20)	245 714 (0)
6 Glaucarubolone	0.20	84 909 (50)	162 136 (29)	273 075 (8)	308 235 (0)
7 Glaucarubinone	0.25	7 686 (96)	10 000 (96)	12 196 (96)	23 800 (90)
11 Isobruceine A	0.30	3 266 (98)	2 643 (99)	2 200 (99)	5 176 (98)
12 Soulameanone	0.50	128 667 (24)	198 780 (13)	225 857 (24)	207 792 (9)

After 3 hours of incubation of the cells were labelled to determine the extent of incorporation. From the rest of the cells inhibitors were eliminated by medium renewal and incorporation was performed as before after various times, for 30 minutes at 37°. After labelling, cells were washed and radioactivity measured in the trichloroacetate acid (TCA) insoluble material. % of inhibitions are calculated with respect to untreated controls and are given in brackets. Results are expressed in cpm/mg of proteins and are the mean values of two experiments.

* The highest active and non toxic concentration for CEF.

side chain at C-15 does not interfere with the inhibitory effect in whole cells. Furthermore, partial unsaturation in ring A is not the sole requirement to inhibit protein synthesis.

Quassinoids may prove to be a new class of potential antiviral compounds due to their inhibitory effect on infected cells at low concentration. However, the direct relationship between antitransforming activity and inhibition of protein and DNA synthesis has not yet been established. The site of action at the molecular level of these substances is currently under investigation in our laboratory.

REFERENCES

1. Polonsky, J., (1973), Fortschr. Chem. Org. Naturstoffe , 30, 101-150.
2. Wall, M.E., and Wani, M.C., (1970), Int. Symp. Chem. Nat. Prod. 7, 614 ; Wani, M.C., Taylor, H.L., Thompson, J.B. and Wall, M. E., (1978), Lloydia, 41, 578-583 ; Seida, A.A., Kinghorn, A.D. Cordell, G.A., and Farnsworth, N.R., (1978), Lloydia, 41, 584-587.
3. Kupchan, S.M., Britton, R.W., Lacadie, J.A., Ziegler, M.F., and Sigel, C.W., (1975), J. Org. Chem., 40, 648-654.
4. Polonsky, J., Varon, Z., Jacquemin, H., and Pettit, G.R., (1978), Experientia, 34, 1122-1123.
5. Polonsky, J., Baskevitch, Z., Gaudemer, A., and Das, B.C., (1967), Experientia, 23, 424-426.
6. Kupchan, S.M., Lacadie, J.A., Howie, G.A., and Sickles, B.R., (1976), J. Med. Chem., 19, 1130-1133 ; Wall, M.E., and Wani, M.C., (1978), J. Med.Chem., 21, 1186-1187.
7. Liao, L.L., Kupchan, S.M., and Horwitz, S.B., (1976), Molec. Pharmacol., 12, 167-176.
8. Robert-Géro, M., Lawrence, F., Farrugia, G., Berneman, A., Blanchard, P., Vigier, P., and Lederer, E., (1975), Biochem. Biophys. Res. Comm., 65, 1242 -1249.
9. Schneider, W.C., (1945), J. Biol. Chem., 161, 293 - 303.
10. Polonsky, J., Varon, Z., and Soler, E., (1979), C.R. Ser.C, 288, 269-271.
11. Mai Van T. , Polonsky, J., and Sévenet, T., manuscript in preparation.
12. Polonsky, J., Baskevitch-Varon, Z., and Sévenet, T., (1975), Experientia, 31, 1113-1114.
13. Polonsky, J., Mai Van, T., Varon, Z., Prangé, T., Pascard, C., Sévenet, T. and Pusset, J., Tetrahedron in press.
14. Polonsky, J., (1964), Proc.Chem.Soc., 292-293.
15. Brown, W.A.C., and Sim, G.A., ibid p. 293-294.
16. Merrien, A.M., Meunier, B., and Polonsky, J., in preparation
17. Polonsky, J., Baskevitch-Varon, Z., and Das, B.C., (1976), Phytochemistry, 15, 337 -339.