Biochemical Pharmacology, Vol. 40, No. 2, pp. 297–301, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

COMPARISON OF THE *IN VITRO* ACTIVITIES OF QUASSINOIDS WITH ACTIVITY AGAINST *PLASMODIUM FALCIPARUM*, ANISOMYCIN AND SOME OTHER INHIBITORS OF EUKARYOTIC PROTEIN SYNTHESIS

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(*Received* 10 *January* 1990; *accepted* 15 *February* 1990)

Abstract—Using the inhibition of incorporation of [^{3}H]hypoxanthine as an index of viability of malaria parasites, it was shown that a chloroquine-sensitive strain of *Plasmodium falciparum* (T9-96) and a chloroquine-resistant strain (K1) did not differ in their sensitivities to the quassinoids ailanthinone, bruceantin and chaparrin. Similarly, there were no differences between the strains in their sensitivities to the protein synthesis inhibitors anisomycin, deacetylanisomycin, cephalotaxine, homoharringtonine, cycloheximide, puromycin and puromycin aminonucleoside. The IC_{50} values derived for ailanthinone and bruceantin, cycloheximide, homoharringtonine and puromycin were in the nanomolar range, whereas those for the anisomycins, cephalotaxine and the aminonucleoside of puromycin were micromolar or greater. Those drugs tested which contain an ester moiety (ailanthinone, bruceantin, anisomycin, homoharringtonine) were more active than the related drugs (chaparrin, deacetylanisomycin, cephalotaxine) that do not. Cross-resistance to inhibitors of protein synthesis appeared not to accompany resistance to chloroquine.

With the ever-increasing emergence, worldwide, of strains of *Plasmodium falciparum* showing resistance to chloroquine, the need to develop new antimalarial drugs is pressing [1]. Most of the drugs currently used in the treatment of malaria have limited spectra of activity [2] and so it is important that agents with novel modes of action be made available as alternatives in the fight against this disease.

Plants of the family Simaroubaceae, particularly species of *Brucea* and *Simarouba*, have long been used in traditional medicine to treat a number of diseases, including malaria [3]. Their curative properties are attributed largely to the quassinoids or 'bitter principles' (products of the biodegradation of triterpenoids) that they contain. A large number of plant-derived quassinoids have been shown to possess significant activity against chloroquine-resistant strains of *P. falciparum in vitro*, and against experimentally-induced *P. berghei* infections in mice [4–9].

Studies of eukaryotic cells and cell-free systems have shown that bruceantin [10] and other quassinoids [11–15] disrupt normal ribosome function, causing irreversible inhibition of protein synthesis. Recently, it has been shown that seven quassinoids with differing chemical structures and differing degrees of antimalarial activity also rapidly inhibit protein synthesis in *P. falciparum in vitro* [16]. It was therefore of interest to determine and compare the possible *in vitro* antimalarial activities of some classic inhibitors of protein synthesis. The compounds chosen (Fig. 1; Table 1) are, like quassinoids, natural products, and act at discrete points in the sequence of protein synthesis (see Discussion).

Since chloroquine has no significant effect upon protein synthesis in the *in vitro* malaria model, it was also important to determine whether there was any difference in the susceptibility of chloroquine-resistant and chloroquine-sensitive strains of *P. falciparum* to quassinoids, and to protein synthesis inhibitors in general.

MATERIALS AND METHODS

Drugs. Techniques for the isolation and identification of quassinoids have previously been reported [8, 9]. Anisomycin, deacetylanisomycin, cephalotaxine, homoharringtonine, puromycin aminonucleoside, cycloheximide and chloroquine diphosphate, were obtained from the Sigma Chemical Co. (Poole, U.K.). Puromycin base was supplied by Serva Feinbiochemica (Heidelberg, F.R.G.); 1.5 mg were dissolved in 50 μL absolute ethanol with 30 μL 0.1 M HCl and further diluted with RPMI to provide a solution at the highest concentration tested. In a control test the same final concentrations of acid and alcohol did not inhibit parasite growth. All other drugs (with the exception of the anisomycins and chloroquine diphosphate, which were initially dissolved in distilled water) were first dissolved at high concentration in absolute ethanol (B.D.H., Poole, U.K.); dilutions were then carried out in serum-free, glucose-enriched RPMI 1640 culture medium (RPMI) to provide solutions at an appropriate concentration from which serial dilutions were made. The concentration of ethanol at test was always less than 0.1%.

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Table 1. Sources of the protein synthesis inhibitors used in these experiments

(A) Plant-derived	
Family Simaroubaceae	
Ailanthinone	*Ailanthus altissima* and related species; woody parts.
Bruceantin	*Brucea javanica*; fruits.
Chaparrin	*Castela nicholsoni* and *A. altissima*; woody parts.
Family Cephalotaxaceae	
Cephalotaxine	*Cephalotaxus harringtonia* (Japanese plum-yew); seeds.
Homoharringtonine	*Cephalotaxus harringtonia* (Japanese plum-yew); seeds.
(B) Non plant-derived	
Anisomycin	*Streptomyces griseolus* and *Streptomyces roseochromogenes*.
Cycloheximide	Streptomycin-producing strains of *Streptomyces griseus*, *Streptomyces noursei* and *Streptomyces naraensis*.
Puromycin	*Streptomyces alboniger*.

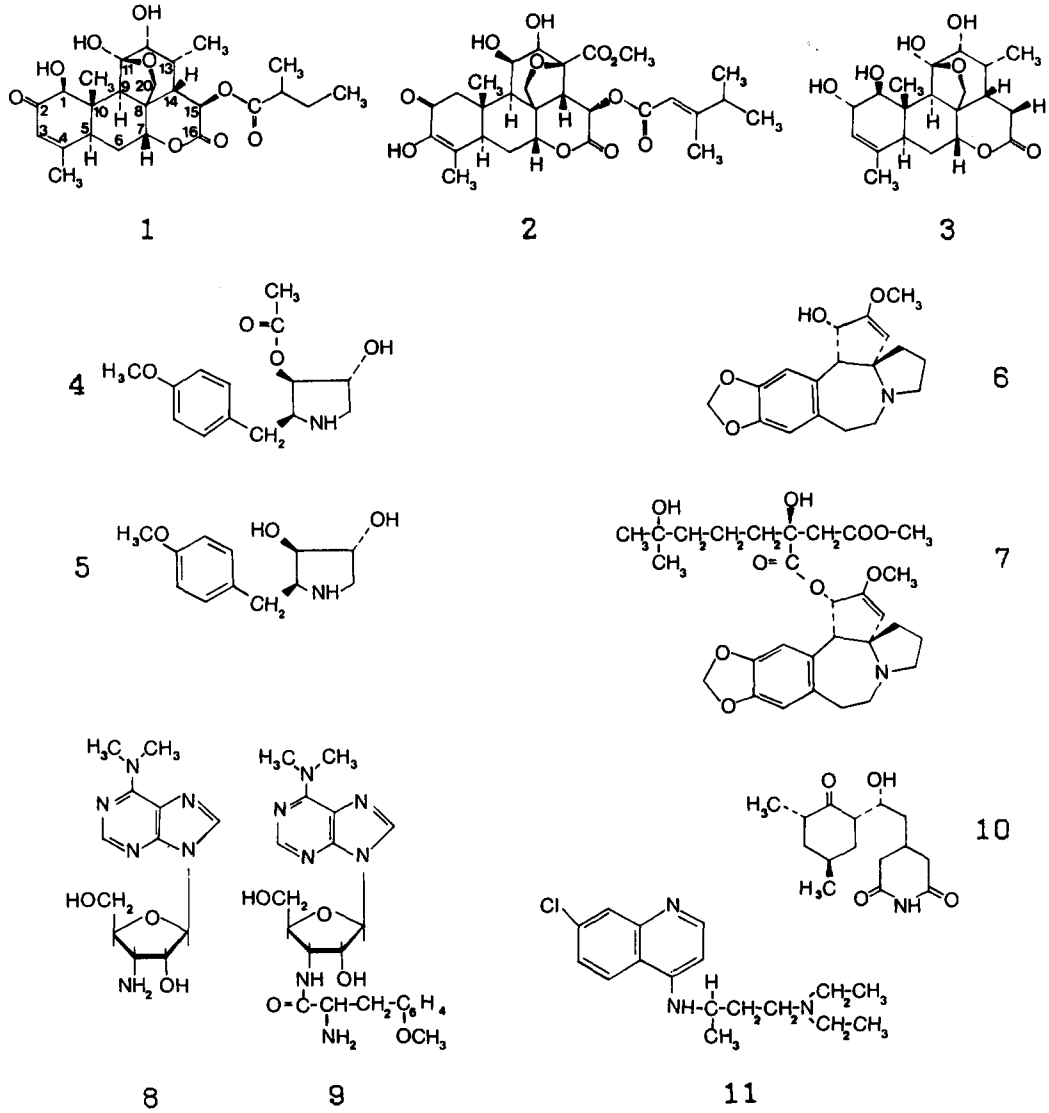

Fig. 1. Structures of the compounds and drugs tested in this study. (1) Ailanthinone; (2) bruceantin; (3) chaparrin; (4) anisomycin; (5) deacetylanisomycin; (6) cephalotaxine; (7) homoharringtonine; (8) puromycin aminonucleoside; (9) puromycin (base); (10) cycloheximide; (11) chloroquine.

Table 2. *In vitro* antimalarial activities (inhibition of incorporation of [^{3}H]hypoxanthine) of chloroquine, quassinoids and protein synthesis inhibitors against *P. falciparum*

	Mean IC_{50} K1 (SE; N) μM	Mean IC_{50} T9-96 (SE; N) μM	IC_{50} K1/T9–96
Ailanthinone	0.084 (0.033; 6)	0.090 (0.027; 6)	0.933
Bruceantin	0.013 (0.012; 6)	0.008 (0.006; 6)	1.625
Chaparrin	5.697 (4.121; 6)	6.008 (4.105; 6)	0.948
Anisomycin	0.959 (0.263; 6)	1.271 (0.621; 6)	0.754
Deacetylanisomycin	40.294 (7.971; 6)	33.565 (10.79; 8)	1.200
Cephalotaxine	28.414 (7.991; 4)	38.090 (8.882; 4)	0.746
Homoharringtonine	0.004 (0.0005; 6)	0.004 (0.0009; 6)	1.000
Cycloheximide	0.054 (0.018; 6)	0.031 (0.008; 6)	1.742
Puromycin base	0.023 (0.004; 4)	0.024 (0.006; 4)	0.958
Puromycin aminonucleoside	41.753 (11.68; 4)	36.731 (12.40; 4)	1.137
Chloroquine diphosphate	0.306 (0.102; 12)	0.005 (0.001; 12)	61.200

[G-^{3}H]Hypoxanthine ([^{3}H]hyp*; sp. act. 43.3 mCi/mg) was purchased from Amersham International (Bucks, U.K.). The lyophilized [^{3}H]hyp was dissolved in serum-free RPMI to give 40 μCi/mL. This solution was stored in 1-mL aliquots at −20° until used.

Parasite cultivation and drug sensitivity testing. T9-96 (from the WHO Culture Collection, Edinburgh, U.K.), a chloroquine-sensitive clone of *P. falciparum*, and K1, a chloroquine- and pyrimethamine-resistant strain [17] were cultured in human A^+ erythrocytes suspended in RPMI 1640 supplemented with D-glucose and 10% human A^+ serum [18, 19]. For drug sensitivity tests, vigorously growing cultures with a predominance of young ring forms were selected.

Determination of drug sensitivity was based upon the microtitre plate technique first developed by Desjardins *et al.* [20]. Each drug was tested a minimum of four times, at 12 concentrations in four-fold dilutions. The final haematocrit and parasitaemia were 2.5 and 1%, respectively; parasites were incubated for 24 hr in the presence of drugs, before adding [^{3}H]hyp (0.02 μCi per well). After a further 18–24 hr incubation parasites were harvested on to glass fibre filters using a semi-automatic cell harvester (Skatron). Filters were placed in 2.5 mL of Ecoscint scintillation fluid in 4.0 mL scintillation vials (Packard), and counting for tritium activity was carried out in a Packard Tri-Carb Scintillation Spectrometer (Model 574).

Data processing. Two series of controls were performed: one with infected red blood cells in the absence of drug (cpm equivalent to 0% inhibition), and the other with uninfected red blood cells (cpm equivalent to 100% inhibition). Using appropriate computer programmes the cpm representing [^{3}H]hyp incorporation were converted to percentage inhibition and plotted as a function of the logarithm (to the base 10) of drug concentration. Linear regression analysis was applied to the linear portion of the sigmoidal curves obtained, and the IC_{50} value (concentration of drug at which inhibition of parasite growth represents 50%) was derived for each drug. Data from different experiments were accumulated, and the mean IC_{50} value for each drug was calculated; the data obtained for the various drugs were compared.

* Abbreviations: [^{3}H]hyp, tritated hypoxanthine; IC_{50}, concentration of drug causing 50% inhibition of parasite growth in the *in vitro* screening test.

RESULTS

The mean IC_{50} values, with the standard error of each mean and the number of determinations, are presented in Table 2. In the case of chloroquine diphosphate there was a 60-fold difference in the IC_{50} values derived for the two different lines of *P. falciparum* tested. With the other drugs tested the IC_{50} values for the two strains were similar, and in no case did the difference reach even two-fold (Table 2). The IC_{50} values for homoharringtonine, cycloheximide, puromycin (base) and the quassinoids ailanthinone and bruceantin were near nanomolar; the other drugs may be considered to be considerably less potent *in vitro*, since their IC_{50} values were in the micromolar range.

DISCUSSION

Quassinoids selectively inhibit eukaryotic protein synthesis in a number of model systems [11–15], and we have recently reported an effect of quassinoids upon the chloroquine-resistant K1 strain of *P. falciparum* indicative of a primary disruptive action upon protein synthesis, followed by inhibition of nucleic acid synthesis [16]. Chloroquine was found not to alter protein synthesis significantly in those experiments, as also shown in earlier tests using *P. berghei in vitro* [21]; it may thus be concluded that

quassinoids with antimalarial properties act through a mechanism fundamentally different from that of chloroquine.

All the inhibitors of protein synthesis used in these experiments disrupt some discrete stage in the process of polypeptide chain elongation (events leading to initiation or termination of protein synthesis are not considered to be primary targets for any of the drugs used). Anisomycin and quassinoids such as bruceantin compete for binding at a site within the eukaryotic 60S ribosomal subunit [10, 22–25] and cause inhibition of ribosomal peptidyl transferase activity [10]. This enzymic action of the ribosome catalyses the release of the nascent protein from the t-RNA occupying the ribosomal P(peptidyl) site, and the redundant t-RNA is ejected from the ribosome. The polypeptide chain remains attached to the t-RNA occupying the A (aminoacyl) site; through the action of ribosomal translocase, the ribosome is relocated with respect to the m-RNA strand so that the t-RNA bearing the nascent protein now occupies the P site, and the A site is available for the next coded aminoacyl t-RNA. The enzyme-catalysed translocation of the ribosome along the m-RNA is blocked by cycloheximide.

In contrast to the blockade limited to that portion of the A site in which peptidyl transferase recognizes acceptor substrates, which is typical of inhibitors such as anisomycin [26], the *Cephalotaxus* alkaloids prevent the enzymatic binding of new aminoacyl t-RNA molecules, acceptors of the nascent peptide chain, into the ribosomal A site as a whole [26]. Indeed, the interaction of [^{3}H]anisomycin with the ribosome is itself blocked by the harringtonines [24]. Certain quassinoids, such as the grandilactones A and B (6α-tigloyloxychaparrinone and 6α-tigloyloxychaparrine), react with the ribosome in a manner more akin to that of the *Cephalotaxus* alkaloids than that of bruceantin or anisomycin [26].

Puromycin is a structural analogue of the 3′ terminus of aminoacyl-t-RNA [10, 24]. The drug occupies the A site of the larger ribosomal subunit, where it is able to form a new peptide bond by condensation with the nascent peptide chain, located in the P site. The newly-formed peptidyl-puromycin is unable to take part in any subsequent step of protein synthesis [10, 24], and is rapidly released from the ribosome.

In contrast to the other antibiotics we have tested, puromycin is a potent inhibitor of both prokaryotic and eukaryotic protein synthesis; the aminonucleoside of puromycin (which lacks the α-amino-*p*-methoxy-dihydrocinnamido group linked to the pentose sugar moiety; Fig. 1), has previously been reported to be far more effective than puromycin itself when tested against several species of *Trypanosoma*, and against some murine carcinomas and leukaemias [27–29], whilst possessing considerably less activity against bacteria [28]. In our experiments puromycin was among the most potent of the compounds tested (Table 2), and the aminonucleoside appears to be devoid of significant antimalarial activity (Table 2).

From a comparison of the *in vitro* IC_{50} values of the protein synthesis inhibitors tested, using the two lines of *P. falciparum* (Table 2), there is no reason to suggest that resistance to chloroquine has any significant influence upon sensitivity to drugs which inhibit protein synthesis. It also appears unlikely that any changes in the basic mechanisms of protein synthesis are to be found in the line of parasites which is resistant to chloroquine, since its susceptibility to ten drugs, acting at four different sites, did not differ significantly from that of the sensitive clone (Table 2).

The presence of an ester function at C-15 of quassinoids (see Fig. 1) has already been noted as an important determinant of antileukaemic [30] and antimalarial activity [7–9, 16]. Similarly, cephalotaxine is generally held to be devoid of significant antitumour activity [31], whilst its complex esters harringtonine, homoharringtonine and isoharringtonine are potent anticancer agents. In our experiments we have shown that in all cases those compounds which are esterified (ailanthinone, bruceantin, anisomycin, homoharringtonine; Fig. 1) possess a higher degree of antimalarial activity (Table 2) than do the corresponding compounds which are not (chaparrin, deacetylanisomycin, cephalotaxine; Fig. 1). In the case of the *Cephalotaxus* alkaloids this difference is nearly 10,000-fold, and the introduction of the ester moiety must be considered to be a highly significant determinant of activity. The steroid-like structure of the quassinoids (cf. Fig. 1) may facilitate their uptake into cells, and the presence of ester functions may similarly increase lipophilicity, aiding penetration to an intracellular site of action. The ester moieties of naturally-occurring quassinoids are often branched or unsaturated, and so may, in addition, be important for effective interaction or bonding with the ribosome. It is of interest to note that the affinity of deacetylanisomycin for yeast ribosomes is some 350 times less than that of anisomycin [23].

We cannot yet conclude whether the remarkable differences we have shown in the antimalarial activity of related compounds is a consequence of differences in accumulation within the parasite, or a reflection of significantly different affinities for binding at their respective sites of action. Clearly a study of both the uptake of the different drugs and of their binding affinities and other properties at their respective sites of action must be included in future studies on the use of quassinoids to treat chloroquine-resistant malaria.

Acknowledgements—The generous financial support of the Wellcome Trust, the Sir Halley Stewart Trust, the WHO/UNDP/World Bank Special Programme for Research and Training in Tropical Diseases, together with the Medical Research Council, is gratefully acknowledged. DCW is funded by the Public Health Laboratory Service. Computer programmes used to determine the antimalarial activity of drugs were devised and written by S. Grant.

REFERENCES

1. Peters W, Antimalarial drug resistance: an increasing world problem. *Br Med Bull* **38**: 187–192, 1982.
2. White NJ, Clinical pharmacokinetics of antimalarial drugs. *Clin Pharmacokinet* **10**: 187–215, 1985.
3. Key JD, *Chinese Herbs, their Botany, Chemistry and Pharmacodynamics*. Charles E. Tuttle Co., Rutland, VT, 1976.
4. Guru PY, Warhurst DC, Harris A and Phillipson JD,

Antimalarial activity of bruceantin *in vitro*. *Ann Trop Med Parasit* **77**: 433–435, 1983.
5. Trager W and Polonsky J, Antimalarial activity of quassinoids against chloroquine-resistant *Plasmodium falciparum in vitro*. *Am J Trop Med Hyg* **30**: 531–537, 1981.
6. Fandeur T, Moretti C and Polonsky J, *In vitro* and *in vivo* assessment of the antimalarial activity of sergeolide. *Planta Med* **51**: 20–23, 1985.
7. O'Neill MJ, Bray DH, Boardman P, Phillipson JD, Warhurst DC, Peters W and Suffness M, Plants as sources of antimalarial drugs: *in vitro* antimalarial activities of some quassinoids. *Antimicrob Agents Chemother* **30**: 101–104, 1986.
8. O'Neill MJ, Bray DH, Boardman P, Chan KL, Phillipson JD, Warhurst DC and Peters W, Plants as sources of antimalarial drugs, part 4: activity of *Brucea javanica* fruits against chloroquine-resistant *Plasmodium falciparum in vitro* and against *Plasmodium berghei in vivo*. *J Nat Prod* **50**: 41–48, 1987.
9. Bray DH, Boardman P, O'Neill MJ, Chan KL, Phillipson JD, Warhurst DC and Suffness M, Plants as sources of antimalarial drugs 5: activities of *Ailanthus altissima* stem constituents and of some related quassinoids. *Phytotherapy Res* **1**: 22–24, 1987.
10. Gale EF, Cundliffe E, Reynolds PE, Richmond MH and Waring MJ, Antibiotic inhibitors of ribosome function. In: *The Molecular Basis of Antibiotic Action* (Eds. Gale EF, Cundliffe E, Reynolds PE, Richmond MH, and Waring MJ), pp. 402–547. John Wiley & Sons Ltd, London, 1981.
11. Liao LL, Kupchan SM and Horowitz SB, Mode of action of the antitumor compound bruceantin, an inhibitor of protein synthesis. *Mol Pharmacol* **12**: 167–176, 1976.
12. Fresno M, Gonzáles A, Vázquez D and Jiménez A, Bruceantin, a novel inhibitor of peptide bond formation. *Biochim Biophys Acta* **518**: 104–112, 1978.
13. Beran YM, Benzie CR and Kay JE, Bruceantin, an inhibitor of the initiation of protein synthesis in eukaryotes. *Biochem Soc Trans* **8**: 357–359, 1980.
14. Willingham W, Stafford EA, Reynolds SH, Chaney SG, Lee K-Hs, Okano M and Hall IH, Mechanism of eukaryotic protein synthesis inhibition by brusatol. *Biochim Biophys Acta* **654**: 169–174, 1981.
15. Hall IH, Liou YF, Lee KH, Chaney SG and Willingham W, Antitumor agents LIX: effects of quassinoids on protein synthesis of a number of murine tumors and normal cells. *J Pharm Sci* **72**: 626–630, 1983.
16. Kirby GC, O'Neill MJ, Phillipson JD and Warhurst DC, *In vitro* studies on the mode of action of quassinoids with activity against chloroquine-resistant *Plasmodium falciparum*. *Biochem Pharmacol* **38**: 4367–4374, 1989.
17. Thaithong S, Beale GH and Chutmongkonkul M, Susceptibility of *Plasmodium falciparum* to 5 drugs: an *in vitro* study of isolates mainly from Thailand. *Trans R Soc Trop Med Hyg* **77**: 228–231, 1971.
18. Trager W and Jensen JB, Human malaria parasites in continuous culture. *Science* **193**: 673–675, 1976.
19. Fairlamb AH, Warhurst DC and Peters W, An improved technique for the cultivation of *Plasmodium falciparum in vitro* without daily medium change. *Ann Trop Med Parasit* **79**: 379–384, 1985.
20. Desjardins RE, Canfield CJ, Haynes JD and Chulay JD, Quantitative assessment of antimalarial activity *in vitro* by semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**: 710–718, 1979.
21. Homewood CA, Warhurst DC and Baggaley VC, Incorporation of radioactive precursors into *Plasmodium berghei in vitro*. *Trans R Soc Trop Med Hyg* **65**: 10, 1971.
22. Barbacid M and Vázquez D, Preparation of pure [^{3}H]anisomycin by tritium exchange labelling. *Anal Biochem* **56**: 16–25, 1973.
23. Barbacid M and Vázquez D, Ribosome changes during translation. *J Mol Biol* **93**: 449–463, 1975.
24. Vázquez D, *Inhibitors of Protein Biosynthesis*. Springer, Berlin, 1979.
25. Grollman AP, Inhibitors of protein synthesis II. Mode of action of anisomycin. *J Biol Chem* **242**: 3226–3233, 1967.
26. Jiménez A and Vázquez D, Novel inhibitors of translation in eukaryotic systems. In: *Antibiotics VI. Modes and Mechanisms of Microbial Growth Inhibitors* (Ed. FE Hahn), pp. 248–254. Springer, Berlin, 1983.
27. Hewitt RJ, Gumble AR, Wallace WS and Williams JH, Experimental chemotherapy of trypanosomiasis; reversal by purines of *in vivo* activity of puromycin, and an amino nucleoside analog, against *Trypanosoma equiperdum*. *Antibiot Chemother* **4**: 1222–1227, 1954.
28. Baker BR, Joseph JP and Williams JH, Puromycin. Synthetic studies VII. Partial synthesis of amino acid analogs. *J Am Chem Soc* **76**: 2838, 1954.
29. Tobie EJ and Highman B, Influence of the amino nucleoside of puromycin on the course and pathology of Trypanosome infections in rabbits and mice. *Am J Trop Med Hyg* **5**: 504–515, 1956.
30. Cassady JM and Suffness M, Terpenoid antitumor agents. In: *Medicinal Chemistry* (Eds. Cassady JM and Douros JD), Vol. 16, pp. 254–269. Academic Press, New York, 1980.
31. Huang M-T, Harringtonine, an inhibitor of initiation of protein synthesis. *Mol Pharmacol* **11**: 511–519, 1975.