2′-CHLOROPENTOSTATIN: DISCOVERY, FERMENTATION AND BIOLOGICAL ACTIVITY

J. B. TUNAC and M. UNDERHILL

Warner-Lambert/Parke-Davis, Pharmaceutical Research
Ann Arbor, Michigan 48105, U.S.A.

(Received for Publication June 29, 1985)

2′-Chloropentostatin (2-CP) is a new nucleoside antibiotic produced by *Actinomadura* sp. ATCC 39365. A selectively sensitive assay organism, *Enterococcus faecalis* PD 05045 (MIC 0.005 μg/ml) was instrumental in the discovery of this compound. 2-CP is a tight-binding inhibitor of adenosine deaminase ($Ki = 1.1 \times 10^{-10}$ M).

The nucleoside antibiotics, vidarabine and pentostatin are co-produced in the fermentation broth of the organism *Streptomyces antibioticus* NRRL 3238[1]. Vidarabine is primarily an antiviral antibiotic with marginal antitumor activity[2], while pentostatin is an inhibitor of adenosine deaminase[3]. Adenosine deaminase catalyzes the deamination of vidarabine, consequently reducing its chemotherapeutic potential[4]. Thus, the addition of pentostatin enhances the antiviral and antitumor activity of vidarabine[5] by preventing its deamination to the nearly inactive ara-hypoxanthine.

In our antibiotics screening program, we have discovered 2′-chloropentostatin (2-CP), a new inhibitor of adenosine deaminase (Fig. 1), which is produced by a species of actinomycete belonging to the genus *Actinomadura*. The isolation and structure determination of this new antibiotic was first reported by SCHAUMBERG *et al.*[6] from our laboratory. Subsequently, ŌMURA *et al.*[21] reported the same compound.

This paper describes the assay system and biological activity of 2-CP, and the taxonomy and fermentation pattern of the organism.

Materials and Methods

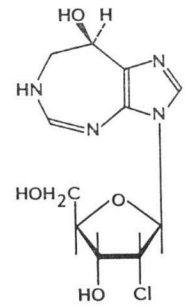
Assay Organism

The test organism, *Enterococcus faecalis* PD 05045, an antibiotic-resistant clinical isolate, was maintained in our culture collection on Bacto-Folic acid AOAC agar medium (Difco, Detroit) containing 2.5 mg/liter of folic acid. The inoculum was prepared by growing the culture at 37°C for 18 hours on Bacto-Folic acid AOAC broth medium containing 2.5 mg/ml folic acid. The culture was centrifuged and the cells resuspended in 0.85% saline to give a light transmittance of 20% at 555 nm, (Coleman 35 Spectrophotometer, Perkin-Elmer).

This suspension (1 ml) was used to inoculate 100 ml of the molten assay medium, herein designated as chloropentostatin medium (CM). The medium consisted of the following ingredients:

K_2HPO_4	3.9 g
Dextrose	25.0 g
Sodium citrate·$2H_2O$	34.4 g
Casein hydrolysate	6.2 g

Fig. 1. 2′-Chloropentostatin.
[(*R*)-3-(2-Chloro-2-deoxy-β-D-ribofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]-diazepin-8-ol].



Asparagine	375 mg	Folic acid	500 μg
L-Tryptophan	125 mg	Pyridoxine HCl	2.5 mg
Cysteine	312.5 mg	NaCl	12.5 mg
Glutathione	0.31 mg	$MgSO_4 \cdot 7H_2O$	250 mg
Thiamine HCl	250 μg	$FeSO_4 \cdot 7H_2O$	12.5 mg
Riboflavin	625 μg	$MnSO_4 \cdot H_2O$	125.0 mg
Ca pantothenate	500 μg	Tween 80	62.5 mg
Nicotinic acid	500 μg	Adenine sulfate	6.25 mg
p-Aminobenzoic acid	625 μg	Agar	15.0 g
Biotin	12.5 μg	Distilled water	1,000 ml

Antibiotic samples were applied onto the assay plates using 12.7 mm paper disks (Schleicher and Schuel). The plates were incubated at 37°C for 16~18 hours, and activity was measured as inhibitory zones.

Culture Characterization

The culture was isolated from a soil sample collected in Waynesville, N.C. The soil was subsequently plated on to an agar medium consisting of the following ingredients; glycerol 3%, L-asparagine 0.25%, potassium chloride 0.05%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, and agar 1.5%.

Culture characterization was carried out following the ISP (International Streptomyces Project) procedure[7]. In addition, Waksman starch agar (WSA)[8] and Amidex corn starch agar (ADX)[9] were used. The culture was maintained on ADX agar slants at 28°C. Morphological and color determinations of the growth of the organism were made at weekly intervals over a three-week period. Cell wall analysis was carried out following the methods of BECKER *et al.*[10].

Fermentation

Stock cultures of the organism were maintained in lyophilized vials and the working culture on ADX agar slants. The microbial growth from a two-week old slant was used to inoculate a 300-ml seed flask and incubated with shaking (New Brunswick Shaker, 5-cm throw) at 28°C. The seed medium consisted of; 0.5% yeast hydrolysate (Amberex 1003, Amber Labs.), 0.1% glucose monohydrate, 2.4% dextrin (Amidex B411, Corn Products), 0.5% hydrolyzed peptone (N-Z case, Humko-Sheffield), 0.3% spray dried meat solubles (Daylin Labs.), and 0.2% $CaCO_3$. The production of the antibiotic was carried out in 300-ml shake-flasks, 30-liter stirred-jar fermentors, and an 1,890-liter fermentor.

The production medium consisted of; 1.5% dextrin (Maltrin, Grain Processing), 1.0% lactose, 0.65% distillers solubles (Grain Processing), 0.35% peptonized milk (Humko-Sheffield), and 0.25% torula yeast (Phinelander Paper Co.). The fermentation conditions were as follows: Shake-flask, 50-ml/300-ml flask, 200 rpm shaker (Model G-53, New Brunswick Shaker); 30-liter stirred-jar fermentor, 16 liters/jar, 1.0 vol of air/vol/minute, 1,890-liter fermentor, 1,130 liters/tank, 0.375 vol of air/vol/minute (425 liters/minute), 84 rpm. The fermentation was carried out for 4~5 days at 33°C.

Biochemical and Biological Activity

The ability of 2-CP to inhibit the adenosine deaminase enzyme was evaluated following the method of CHASSIN *et al.*[11]. Briefly, the antibiotic was incubated with adenosine deaminase (EC 3.5.5.5, Type 1, Sigma Chemical Co.) for 1 hour at room temperature, and then the substrate adenosine was added. Adenosine deamination was measured by the decrease in absorbance at 265 nm as a result of the conversion of adenosine to inosine.

Results

Culture Characterization

Whole cell analyses revealed the presence of the *meso* isomer of 2,6-diaminopimelic acid and the sugar madurose (3-*O*-methyl-D-galactose). The presence of *meso* DAP would classify the organism

Table 1. Morphology and pigmentation of conidial chains and conidiophore, (14-day old culture) of *Actinomadura* sp. ATCC 39365.

Category	Culture media[a]			
	ISP-2	ISP-3	ISP-4	ISP-5
1. Mycelial color:				
Aerial	Pink and yellow	White with pink tinge	White with pink tinge	No aerial mycelia
Substratal	Brown (Coo 5m)	Light brown (Co 5b)	Brown (Coo 5b)	Brown (Coo 5m)
2. Conidia/hyphae:				
Aerial	10~50	10~50	10~50	No aerial hyphae
Substratal	None	None	None	None
3. Soluble pigment	None	None	None	None

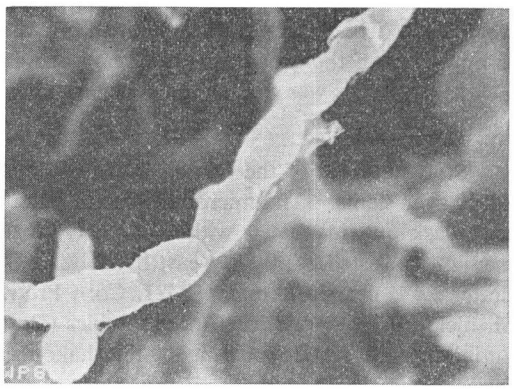
[a] ISP-2 (yeast extract - malt extract agar); ISP-3 (oatmeal agar); ISP-4 (inorganic salts - starch agar); ISP-5 (glycerol - asparagine agar).

Table 2. Carbon utilization pattern of *Actinomadura* sp. ATCC 39365.

Carbohydrate	Growth[a]
L-Arabinose	+
D-Fructose	+
D-Glucose	+
i-Inositol	±
D-Mannitol	−
Raffinose	+
L-Rhamnose	+
Sucrose	−
D-Xylose	+
Salicin	−
D-Galactose	−

[a] +; Good growth, −; no growth, ±; marginal growth.

Fig. 2. Electron micrograph of the spores of *Actinomadura* sp. ATCC 39365, 15,300 × (14-day old culture on water agar).



as cell wall chemotype III, and the presence of madurose would assign it to maduromycetes[12].

The spore chain morphology was characterized by single hooks. The organism reduced nitrate but did not liquefy gelatin nor coagulated milk; it did not produce melanin on peptone - yeast extract - iron agar (ISP-6) or on tyrosine agar. Other morphological and physiological characteristics are shown in Table 1. The carbon utilization pattern is shown in Table 2.

The spore chain morphology produced in the aerial hyphae, in the form of hooks with no substrate spores, would further assign the organism under the genus *Actinomadura*. The spores were elliptical with smooth surface (Fig. 2).

Fermentation

The production of 2-CP in the 1,890-liter fermentor was observed 24 hours after inoculation (Fig. 3). The inhibitory zone diameter at the 24-hour period was 42 mm vs. the assay organism, *E. faecalis* PD 05045. The peak production of the antibiotic was observed at the 112~120-hour periods, with inhibitory zone of 54 mm. The relationships of inhibitory zone and concentration of chloropentostatin is shown in Table 3.

The growth of the organism as measured by mycelial sedimentation (15 ml fermentation broth,

Fig. 3. Fermentation pattern of *Actinomadura* sp. ATCC 39365.

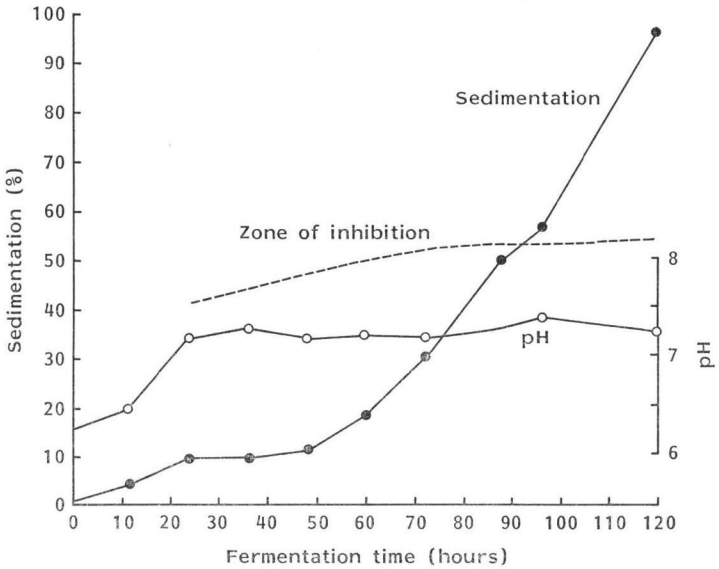


Table 3. Inhibitory zone of different concentrations of 2′-chloropentostatin vs. *Enterococcus faecalis* PD 05045.

Concentration (μg/ml)	Zone diameter (mm)[a]
0.1	29
0.2	40
2.0	47
20.0	55

[a] Paper disc diameter, 12.7 mm.

Table 4. Activity of different nucleoside and antitumor antibiotics vs. *Enterococcus faecalis* PD 05045 grown on the 2′-chloropentostatin assay medium.

Antibiotic	Inhibitory zone diameter[a] (mm)	Concentration (μg/ml)
Adriamycin	15	50
Amicetin	18	100
Angustmycin A	0	100
Azaserine	14	100
Coformycin	54	10
Cordycepin	20	50
Daunomycin	16	50
DON	26	1.0
Echinomycin	20	1.0
Formycin	0	100
Mithramycin	21	1.0
Mitomycin C	14	1.0
Puromycin	18	50
Sparsomycin	0	100
Streptozotocin	17	20
Tubercidin	32	10
Vidarabine	0	1,000
Pentostatin	29	0.1
Chloropentostatin	29	0.1

[a] Paper disc diameter, 12.7 mm.

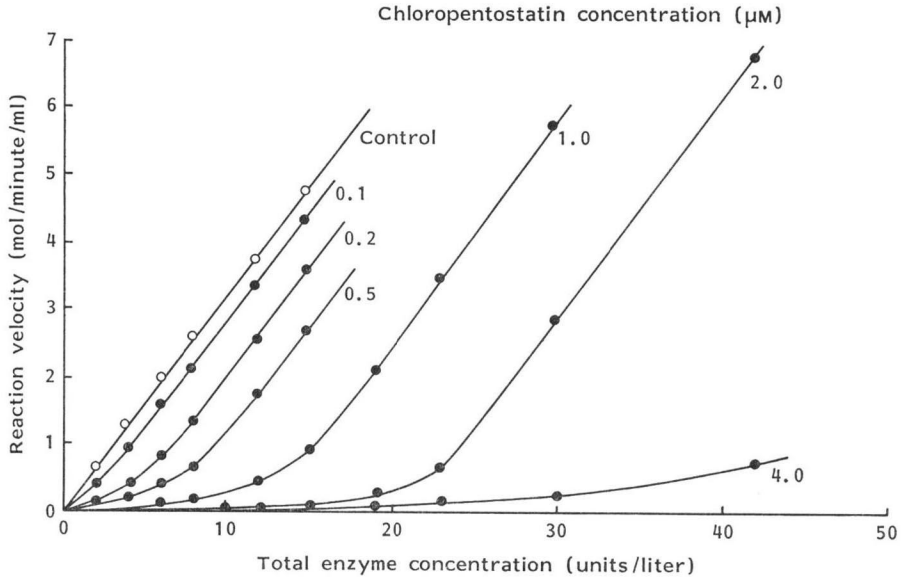
centrifuged for 12 minutes at $450\times g$, International Clinical Centrifuge) ranged from 5~15% until the 50-hour period; afterwhich there was a rapid increase in growth reaching 96% at the 120-hour period.

Selectivity of *E. faecalis* PD 05045 for 2-CP

The assay organism, *E. faecalis* PD 05045 was found resistant to a number of antibiotics, but was particularly sensitive to 2-CP (Table 4). This test organism when plated on the CM medium detected chloropentostatin level of as low as 0.005 μg/ml. However, 2-CP was inactive vs. the same organism when grown in Trypticase soy agar (Difco).

Also, 2-CP was inactive vs. other microorganisms grown in complex assay media, *e.g.*, Mycin assay agar and Penassay base agar (Difco). The organisms tested include: *Alcaligenes viscolactis*, *Micrococcus luteus*, *Torulopsis albida*, *Bacillus subtilis*, *Agrobacterium tumefaciens*.

Fig. 4. ACKERMANN-POTTER plot showing the inhibitory effect of chloropentostatin on the adenosine deaminase enzyme.



Biochemical Activity

The inhibitory effect of 2-CP on the adenosine deaminase enzyme was found to be stoichiometric or tight-binding. An ACKERMANN-POTTER[13] plot showing the stoichiometry of binding is shown in Fig. 4. The inhibitory potency (*Ki*) calculated according to the method of CHA[14], was 1.1×10^{-10} M (BORONDY, P., unpublished data).

Discussion

2′-Chloropentostatin has been demonstrated to be a tight-binding adenosine deaminase (ADA) inhibitor *in vitro*, similar to pentostatin[3]. Since the activity of ADA was found particularly high in acute lymphoblastic leukemia[15], the enzyme has served as a target for chemotherapy, particularly with pentostatin[16]. The physiology of ADA binding to 2-CP *in vivo* has not been studied, which may present a subject for future investigation.

The antitumor activity of vidarabine against murine L1210 leukemia was found to be potentiated by pentostatin[17]. Preliminary data (LEOPOLD, W., personal communication) also indicated a chemotherapeutic synergy between vidarabine-5′-monophosphate and 2-CP, although additional studies need to be carried out to determine optimum dose combinations. In carrying out studies for optimum drug dosage and determining pharmacokinetics, quantitative assay systems are necessary to evaluate the levels and distribution of the compounds. Such assay system normally involves radiolabeled compounds. MCCONNELL *et al.*[19] and SULING *et al.*[20] circumvented the use of radiolabeled compounds by the use of microbiological assay systems for both vidarabine and pentostatin. *E. faecalis* PD 05045 plated in the CM assay medium offers a selective and very quantitative assay for 2-CP. Interestingly, when adenine in the assay medium is deleted, the sensitivity of the organism to 2-CP is reversed; the organism becomes selectively sensitive to vidarabine and resistant to 2-CP (unpublished data). Moreover, in the CM assay medium devoid of adenine, the presence of 2-CP increased the sensitivity of *E. faecalis* PD 05045 toward vidarabine. Thus, this assay medium could provide an *in vitro* system for optimizing 2-CP-vidarabine combinations for *in vivo* studies.

Acknowledgment

This work was supported in part by the National Cancer Institute (USA); contract N01-CM-07379.

References

1) Dion, H. W.; P. W. K. Woo & A. Ryder: Isolation and properties of a vidarabine deaminase inhibitor, co-vidarabine. Ann. N. Y. Acad. Sci. 284: 21~29, 1977
2) Schabel, F. M.: The antiviral activity of 9-β-D-arabinofuranosyladenine. Chemotherapy 13: 321~338, 1968
3) Agarwal, R. P.; T. Spector & R. E. Parks, Jr.: Tight-binding inhibitors. IV. Inhibition of adenosine deaminase by various inhibitors. Biochem. Pharmacol. 26: 359~367, 1977
4) Brink, J. J. & G. A. LePage: Metabolism and distribution of 9-β-D-arabinofuranosyladenine in mouse tissues. Cancer Res. 24: 1042~1049, 1964
5) LePage, G. A.; L. S. Worth & A. P. Kimball: Enhancement of the antitumor activity of arabinofuranosyladenine by 2'-deoxycoformycin. Cancer Res. 36: 1481~1485, 1976
6) Schaumberg, J. P.; G. C. Hokanson, J. C. French, E. Smal & D. C. Baker: 2'-Chloropentostatin, a new inhibitor of adenosine deaminase. J. Org. Chem. 50: 1651~1656, 1985
7) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
8) Waksman, S. A.: The Actinomycetes. p. 194, Chronica Botanica Co., Waltham, Massachusetts, 1950
9) Hickey, R. J. & H. D. Tresner: A cobalt-containing medium for the sporulation of *Streptomyces* species. J. Bacteriol. 64: 891~892, 1952
10) Becker, B.; M. P. Lechevalier & H. A. Lechevalier: Chemical composition of cell wall preparations for strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
11) Chassin, M. M.; R. H. Adamson, D. W. Zaharevitz & D. G. Johns: Enzyme inhibition titration assay for 2'-deoxycoformycin and its application to the study of the relationship between drug concentration and tissue adenosine diaminase in dogs and rats. Biochem. Pharmacol. 28: 1849~1855, 1979
12) Goodfellow, M. & S. T. Williams: Ecology of actinomycetes. Ann. Rev. Microbiol. 37: 189~216, 1983
13) Ackermann, W. W. & V. R. Potter: Enzyme inhibition in relation to chemotherapy. Proc. Soc. Exp. Biol. Med. 72: 1~9, 1949
14) Cha, S.: Tight-binding inhibitors. I. Kinetic behavior. Biochem. Pharmacol. 24: 2177~2185, 1975
15) Smyth, J. F.; D. G. Poplack & B. J. Holiman: Correlation of adenosine deaminase activity with cell surface markers in acute lymphoblastic leukemia. J. Clin. Invest. 62: 710~712, 1978
16) Mitchell, B. S.; C. Koller & C. Mejias: Inhibition of adenosine deaminase by 2'-deoxycoformycin in acute lymphoblastic leukemia. J. Clin. Chem. Clin. Biochem. 17: 427~428, 1979
17) LePage, G. A.; L. S. Worth & A. P. Kimball: Enhancement of the antitumor activity of arabinosyladenine by 2'-deoxycoformycin. Cancer Res. 36: 1481~1485, 1976
18) Whiteley, R. J.; B. C. Tucker, A. W. Kinkel, N. H. Barton, R. F. Pass, J. B. Whelchel, C. G. Cobbs, A. G. Diethelm & R. A. Buchanan: Pharmacology, tolerance, and antiviral activity of vidarabine monophosphate in humans. Antimicrob. Agents Chemother. 18: 709~715, 1980
19) McConnell, W. R.; W. J. Suling, L. S. Rice, W. M. Shannon & D. L. Hill: Use of microbiological and enzymatic assays in studies on the deposition of 2'-deoxycoformycin in the mouse. Cancer Treat. Rep. 62: 1153~1159, 1978
20) Suling, W. J.; L. S. Rice & W. M. Shannon: Effects of 2'-deoxycoformycin and *erythro*-9-(2-hydroxy-3-nonyl)adenine on plasma levels and urinary excretion of 9-β-D-arabinofuranosyladenine in the mouse. Cancer Treat. Rep. 62: 369~373, 1978
21) Ōmura, S.; N. Imamura, H. Kuga, H. Ishikawa, Y. Yamazaki, K. Okano, K. Kimura, Y. Takahashi & H. Tanaka: Adechlorin, a new adenosine deaminase inhibitor containing chlorine. Production, isolation and properties. J. Antibiotics 38: 1008~1015, 1985