ANTITUMOR ACTIVITY OF A FUNGAL GLUCAN TYLOPILAN AND *PROPIONIBACTERIUM ACNES* PREPARATION

JAN GRZYBEK¹, IZABELLA ZGÓRNIAK-NOWOSIELSKA², ANDRZEJ KASPROWICZ²,
BARBARA ZAWILIŃSKA², STANISŁAW KOHLMÜNZER¹

¹Department of Pharmaceutical Botany,
Collegium Medicum at the Jagiellonian University,
9 Medyczna Street, 30-688 Cracow, Poland

²Department of Virology, Institute of Microbiology,
Collegium Medicum at the Jagiellonian University,
18 Czysa Street, 21-121 Cracow, Poland

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ABSTRACT

The study evaluated the antitumor activity of tylophilan, a β - (1 $\rightarrow$ 3) (1 $\rightarrow$ 6) linked glucan isolated from fruiting bodies of *Tylophilus felleus* (Bull.: Fr.) P. Karst. (*Boletaceae*), and *Propionibacterium acnes* (*P.a.*) preparation. The antitumor effect of tylophilan and *P.a.* used alone or in combination was studied in NMRI mice inoculated i.p. with 10⁶ 180-TG Crocker tumor cells. All experiments were based on a pretreatment with tylophilan and/or *P.a.* 5 days and/or 2 h before tumor cell inoculation. Mean survival time (MST) of tumor - bearing mice was significantly prolonged in comparison to control mice by a single injection of tylophilan (25 μ g/mouse or 50 μ g/mouse) or *P.a.* (1 mg/mouse). MST was 23.6; 22.8 days in the tylophilan injected mice and 17.5 in the control animals. Tylophilan injected in conjunction with *P.a.* prolonged significantly MST in comparison to control mice as well as to tylophilan alone treated mice. We have found that *P.a.* which stimulate immune response enhanced significantly antitumor activity of tylophilan. The cytotoxicity of tylophilan at concentrations of 300, 150, 75 and 37.5 μ g/ml towards 180-TG Crocker cells in vitro studies was evaluated. All examined tylophilan concentrations showed cytotoxic activity.

KEY WORDS: antitumor, cytotoxic activities, tylophilan, polysaccharide, *Tylophilus felleus* (Bull.: Fr.) P. Karst., (*Boletaceae*), *Propionibacterium acnes* preparation.

INTRODUCTION

Tylophilan (Fig. 1) is a branched β -D-glucan, M_w 1.3 x 10⁶, isolated by Kohlmünzer and Grzybek (Kohlmünzer, Grzybek, Tanaka 1980) from fruiting bodies of *Tylophilus felleus* (Bull.: Fr.) P. Karst. (*Boletaceae*). The chemical structure of tylophilan was established by Defaye et al. (1988). A crude tylophilan extracted with hot water from fruiting bodies of *Tylophilus felleus* was examined previously. It showed the following activities: inhibition of the growth of *Neurospora crassa* and *Saccharomyces cerevisiae* strains (Kohlmünzer, Grzybek, Węgiel 1975), antimutagenic effect in seedling roots of *Triticum vulgare* (Kohlmünzer, Grzybek 1975), a marked inhibition of the growth of human-brain neoplastic glioma cells cultivated *in vitro* (Kohlmünzer, Kaluza 1975) as well as of Ehrlich carcinoma (ascites form) and Sarcoma 180 cells in mice (Chłap, Kohlmünzer 1978), an anti-inflammatory effect in the carrageenan-induced edema test (Kohlmünzer et al. 1977a, 1977b) distinct mitostatic and mitodepressive effects on meristematic plant cells (Grzybek, Kohlmünzer 1983) and an inhibitory effect with Sarcoma 180 cells implanted mice (Kohlmünzer, Grzybek, Tanaka 1980). The objective of this study was to evaluate antitumor (*in vivo*) and cytotoxic (*in vitro*) activity of tylophilan towards 180-TG Crocker tumor

cells and to evaluate the antitumor effect of *Propionibacterium acnes* preparation alone or in combination with tylophilan in the same tumor system.

MATERIALS AND METHODS*

Tylophilan was obtained from fruiting bodies of *Tylophilus felleus*, a wild growing, very common species all over Poland. The fruiting bodies were gathered at Łopuszna (Southern Poland) in August 1993. After the raw material was cut into small pieces and dried at 35°C, it was pulverized (2-3 mm), and 500 g were extracted with boiling distilled water (8 l.) for 8 h. After filtration and concentration of the filtrate to 1.5 l., an equal volume of 95% EtOH was added, thoroughly stirred, and left for 24 h at 14°C. The residue was filtered, dissolved in distilled water, deproteinized by Seville's method (Staub 1965), and reprecipitated with 95% EtOH. Then the polysaccharide was dialyzed by means of a Serva N-72 membrane against tap water for 72 h, dissolved in a 5% aqueous solution of NaOH, and precipitated with equal volume of EtOH. The

* The abbreviations in the paper concerning chemical procedure follow „Instructions to authors - 1975”, *Phytochemistry* 1975, 14, pp. 6-8.

Fig. 1. Chemical structure of the repeating unit of tylopilan acc. to Defaye et al. (1988).

procedure was repeated until a white residue was obtained. Then the polysaccharide was dissolved in distilled water and dialyzed as before. After a 24 h dialyzation against tap water a 36 h dialyzation against distilled water was done. After centrifugation, the residue was dissolved in distilled water, frozen, and lyophilized. 1.85 g of tylopilan was obtained.

Acidic hydrolysis of tylopilan

100 mg of tylopilan were dissolved in 10 ml of 2N H_2SO_4 , and 0.15 ml of $EtOH_{abs.}$ were added and heated under reflux for 5 h. After hydrolysis, the mixture was neutralized with $BaCO_3$, then filtered. The filtrate was concentrated, and the residue was dissolved in 12 ml of MeOH and analysed by means of TLC. The precoated TLC plates Kieselgel 60 F₂₅₄ Merck, impregnated with 0.02 M aqueous solution of

CH_3COONa , were used. The plates were developed with the following solvent systems (v/v):

a) $CHCl_3 - MeOH$ 60:40

b) $Me_2CO - H_2O$ 90:10

c) $Me_2CO - H_2O - CHCl_3 - MeOH$ 75:5:10:10

The following chromogenic reagents were used for detection:

1) ammonium solution of $AgNO_3$

2) anisaldehyd + H_2SO_4 and heated for 3 min. at $110^\circ C$.

The amino acids contamination of tylopilan was analysed by means of TLC (plates as above) and the following solvent systems (v/v) were used:

d) $PheOH - H_2O$ 3:1

e) $n-BuOH - HOAc - H_2O$ 4:1:1

f) $i-PrOH - H_2O$ 4:1.

Chromatograms were sprayed with a 0.2% ethanol solution of ninhydrin, then heated at $110^\circ C$ for 3 min.

Quantitative estimation of sugars of tylopilan was carried out according to Dubois's et al. method (Dubois et al. 1956). Mineral principles were estimated according to Pharmacopoea Polonica IV (1970). *Propionibacterium acnes* (P.a.) was one of several strains isolated by Kasprowicz et al. (1984). It has been studied previously (Cembrzyńska-Nowak, Jarosińska, Zgórniak-Nowosielska 1986, Roszkowski et al. 1982, Zgórniak-Nowosielska, Jarońska, Kryczko 1985a, Zgórniak-Nowosielska et al. 1985b). It was used as lyophilized whole bacterial cells inactivated by heat and suspended in PBS to a desired concentration before use. Each mouse was injected i.p. with 1 mg/mouse of P.a. at a volume of 0.1 ml.

Fig. 2. Schedules of treatment of NMRI mice implanted with 180-TG Crocker tumor cells.

Tyl. - tylopilan,

P.a. - *Propionibacterium acnes* preparation, 1mg/mouse

t - observation time (days), 25mg or 50mg/mouse

-5 - pretreatment with tylopilan 5 days before mice inoculation with 180-TG Crocker tumor cells;

-2h - pretreatment with tylopilan 2h before mice inoculation;

0 - at day 0 mice were implanted i.p. with 10^6 180-TG Crocker tumor cells;

Fig. 3. Effect of tylopihan (Tyl) (administered at 25 $\mu\text{g}/\text{mouse}$ or 50 $\mu\text{g}/\text{mouse}$) on survival of NMRI mice implanted with 10^6 180-TG Crocker tumor cells;

t – mean survival time;

-2h – Tyl was administered 2 h before tumor cells were implanted;

Fig. 4. Effect of tylopihan (Tyl) and *Propionibacterium acnes* (P.a.) on survival of NMRI mice implanted with 10^6 180-TG Crocker tumor cells;

t – mean survival time;

-5d – Tyl or P.a. were administered 5 days before tumor cells were implanted;

Fig. 5. Effect of tylophilan (Tyl) and *Propionibacterium acnes* (*P.a.*) administered alone or in combination on survival of NMRI mice implanted with 10^6 180-TG Crocker tumor cells;

t – mean survival time;

-2h -5d – Tyl and/or *P.a.* were administered 5 days and 2 hours before tumor cells were implanted.

Animals

NMRI male mice weighing 20-25 g, 6 weeks old, raised in our breeding colony, were used.

Tumor cells

180-TG Crocker cells were originally obtained from C. Chany, Institut National de la Santé et de la Recherche Médicale, Hospital Saint-Vincent-de Paul, Paris. The tumor cells have been maintained since 1989 in the Department of Virology (Cracow), by intraperitoneal passage in NMRI mice. The mice were inoculated i.p. with 10^6 cells per mouse. 10 days after inoculation ascitic tumors appeared and abdominal distention was visible.

The effect of tylophilan and/or *P.a.* on NMRI mice implanted i.p. with 180-TG Crocker tumor cells

The antitumor activity of Tylophilan and/or *P.a.* was studied according to the three different treatment schedules (Fig. 2). Ten groups of mice were used in each, twice repeated, experiments.

Tylophilan was suspended in physiological saline before use and injected i.p. at the dose of 25 µg/mouse or 50 µg/mouse 2 h before mice were implanted i.p. with 10^6 180-TG Crocker tumor cells (Fig. 3). The same doses of tylophilan or *P.a.* at the dose of 1 mg/mouse were administered i.p. into mice, 5 days before tumor cells were implanted (Fig. 4).

In the following experiments tylophilan or *P.a.* were injected alone into tumor-bearing mice or combined treatment with tylophilan or *P.a.* was applied, 5 days and 2 h before 180-TG Crocker tumor cells were implanted (Fig. 5). Control mice were implanted i.p. with the 180-TG Crocker tumor cells and observed for 40 days as the treated mice.

Statistical analysis

Wilcoxon test was used to estimate the significance of survival time as compared with the control or between the various treatment.

The effect of tylophilan on 180-TG Crocker tumor cells in vitro conditions

The 180-TG cells were obtained from the peritoneal exudate of adult NMRI mice. Successive passages of cells were carried out at two-week intervals. After collection of exudate, the cells were washed in Eagle's medium and suspended in this medium with 10% calf serum (c.s.). The following concentrations of tylophilan were used: 300, 150, 75 and 37.5 µg/ml in RPMI medium with 10% c.s.

The final concentration of cells was between 2×10^5 and 2×10^6 /ml. Cells suspended in the medium with tylophilan were incubated at 37°C for 3-4 days. The percentage of living cells was determined daily by counting in a Bürker chamber after the dead cells were made distinguishable with trypan blue. Control 180-TG cells were incubated in the same conditions in the medium without tylophilan.

RESULTS AND DISCUSSION

Chromatographic analysis (TLC) of the hydrolytic products of the isolated tylophilan excluded the presence of amino acids (and thus proteins) in it. The content of sugars in tylophilan, estimated by means of the Dubois's et al. method (Dubois et al. 1956) was 99.8%. Only glucose could be detected. Mineral substances amounted to 0.1% as determined by the Ph.P.IV method (1970).

TABLE 1. Influence of tylophilan on 180-TG Crocker cells in *in vitro* culture.

C-concentration of tylophilan solutions, in Eagle's medium, 1, 24, 48, 72, 96 – time of treatment of tumor cells with tylophilan, in h.

C (mg/ml)	% of viable cells*				
	1	24	48	72	96
300	100	36,7	1	0	0
150	100	41,0	60,5	28,8	0
75	100	78,1	69,1	41,3	5,3
37,5	100	80,2	79,2	57,3	11,5
Control	100	99,7	95,5	92,1	79,3

* mean values of two experiments

In *in vitro* studies tylophilan has shown cytotoxic activity against 180-TG Crocker tumor cells. The percentage of viable cells decreased rapidly along with all used tylophilan concentrations. (Table 1). In subsequent experiments *in vivo* NMRI mice implanted with tumor cells were pretreated with tylophilan or *P.a.* alone or tylophilan in conjunction with *P.a.* was applied. The effect of tylophilan and/or *P.a.* on NMRI mice implanted i.p. with 180-TG Crocker tumor cells is shown in Fig. 3-5. Tylophilan injected i.p. at the single dose of 25 µg/mouse or 50 µg/mouse, 2 h before tumor cell implantation, prolonged significantly the mean survival time (MST) of the treated mice in comparison to control animals. MST of tylophilan treated mice was 22.3 days and 27.3 days while of the control group of mice was 17.5 days ($p = 0.05$) (Fig. 3).

Treatment with tylophilan at the dose of 25 µg/mouse or *P.a.* at the dose of 1 mg/mouse 5 days before tumor cell implantation resulted in significant prolongation of MST of treated in comparison to control mice; MST was 23.6; 22.8 in the treated group of mice and 17.5 days in the control animals (Fig. 4).

The results of combined treatment with Tylophilan and *P.a.* or with one of these two preparations, 5 days and 2 h before tumor cells injection, are shown in Fig. 5. There was significant prolongation of MST in each treated group of mice in comparison to control. MST of mice treated with tylophilan (25 µg/mouse and 50 µg/mouse) or *P.a.* alone was 25.7; 25.3 and 29.8 days, respectively, while MST of control mice was 18.3 days. The difference of MST of mice treated simultaneously with tylophilan and *P.a.* and MST of mice injected with tylophilan alone, used at two doses, was also significant ($p = 0.01$) (Fig. 5). The MST of tylophilan alone treated mice was 25.5 days while in tylophilan combined with *P.a.* treated mice the MST increased to 32.1 da.ys.

The results indicate antitumor and cytotoxic activity of tylophilan against 180-TG Crocker cells, and expand our previous results (Chłap, Kohlmünzer 1978, Kohlmünzer, Grzybek, Tanaka 1980, Kohlmünzer, Kaluza 1975) concerning its activity against another experimental tumor systems. Generally fungal antitumor polysaccharides are considered to have no direct effect against tumor cells. Their action is believed to be exerted through the mediation of the immune mechanism of the host. But in our studies tylophilan was shown *in vitro* to have cytotoxic effect on 180-TG Crocker cells (Table 1). These observations are in accordance with our previous results, since tylophilan showed cytotoxic activity against KB and HeLa cells (Kohlmünzer, Grzybek, Tanaka 1980), and against neoplastic glioma cells (Kohlmünzer,

Kaluza 1975). One of the possible mechanisms for cytotoxic properties of tylophilan may be that it influences the permeability of the tumor cell membrane, resulting in the inhibition of DNA synthesis or disturbing mitosis. Tylophilan disturbed mitosis in meristematic plant cells (Grzybek, Kohlmünzer 1983). It induced some phenomena of nuclear structure degeneration: margination of chromatin, pycnosis, and karyorhexis. It also showed mitodepressive and mitostatic properties. These results indicate cytostatic activity of tylophilan, and shed a new light upon its biological properties. But the mechanism responsible for the antitumor and cytotoxic activities of tylophilan remains to be elucidated. On the basis of some literature (Hamuro, Chihara 1985, Wagner, Proksch 1985) concerning antitumoral polysaccharides, and our own results, one can suggest that tylophilan has both hostmediated immunostimulating effects and direct effects against experimental tumor cells. In these experiments with *Propionibacterium acnes* preparation (*P.a.*) used alone or in combination with tylophilan, both substances prolonged mean survival time of treated animals significantly. We have found that the use of *P.a.*, which stimulate immune response by several mechanism (Roszkowski et al. 1982), enhanced significantly the antitumor activity of tylophilan. *Propionibacterium acnes* (previously known as *Corynebacterium parvum*) was used by Chany, Cerutti (1982), as immunopotentiator in his studies on antitumor effect of arginine butyrate and interferon on Swiss mice grafted with the same 180-TG Crocker tumor cells, which we used in our experiments. Chany has found that antitumor resistance was greatly enhanced when treatment with interferon was preceded by immune stimulation (Chany, Cerutti 1982). The immunostimulating and antineoplastic activities of propionibacteria have been reviewed by Tuttle and Cantrell (1981), and Roszkowski et al. (1982). The authors observed antineoplastic activity in several tumor systems. As with other immunomodulators, the route of administration of propionibacteria and the dose determine their activity.

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LITERATURE CITED

- CEMBRZYŃSKA-NOWAK M., JAROŃSKA E., ZGÓRNIK-NO-
WOSIELSKA I. 1986. Interferon Production in *Propionibacterium*
acnes treated mice. Archiw. Immun. Ther. Exp. 34, 217-221.
CHANY C., CERUTTI I. 1982. Antitumor effect of arginine butyrate
in conjunction with *Corynebacterium parvum* and interferon. Int. J.
Cancer 30: 489-493.
CHŁAP Z., KOHLMÜNZER S. 1978. Effect of *Tylophilus felleus* Pre-
paration on Transplantable Tumors in Mice. Planta Med. 33: 290-291.
DEFAYE J., KOHLMÜNZER S., SODZAWICZNY K., WONG E.
1988. Structure of an antitumor water soluble D-glucan from the
carpophores of *Tylophilus felleus*. Carbohydr. Res. 173: 316-323.
DUBOIS M., GILLES K.A., HAMILTON J.K., REBERS P.A., AND
SMITH F. 1956. Colorimetric Methods for Determination of Sugars
and Related Substances. Anal. Chem. 3: 350-356.
GRZYBEK J., KOHLMÜNZER S. 1983. Biological activity of poly-
saccharides from *Tylophilus felleus* on the basis of Allium test. Herba
Hung. 22: 7-12.

- HAMURO I., CHIHARA G. 1985. Lentinan, a T-Cell-oriented Immunopotentiator. Its Experimental and Clinical Applications and Possible Mechanism of Immune Modulation. In „Immune Modulation Agents and their Mechanism”. R.L. Fenichel, and M.A. Chirigos (Eds.). M. Dekker, Inc. New York and Basel. pp. 409-435.
- KASPROWICZ A., HOEFFLER U., HECZKO P.B. 1984. Charakterystyka szczepów *Propionibacterium* wyizolowanych z przypadków trądzika młodzieńczego i od osób zdrowych. Med. Dośw. Mikrobiol. 36: 197-201.
- KOHLMÜNZER S., CHŁAP Z., KAŁUŻA J., QUINN M.L., BENOIT P., FARNSWORTH N. 1977 a. Biologische Aktivität von *Tylophilus felleus* (Bull. ex Fr.) P. Karst. Planta Med. 32: 36.
- KOHLMÜNZER S., GRZYBEK J., WĘGIEL J. 1975. Investigations on the biological activity of extracts of *Tylophilus felleus* (Bull. ex Fr.) P. Karst. by means of micological test. Pol. J. Pharmacol. Pharm. 27: 95-99.
- KOHLMÜNZER S., GRZYBEK J. 1975. Investigations on the biological activity of *Tylophilus felleus* (Bull. ex Fr.) P. Karst. by means of phytobiological test. Pol. J. Pharmacol. Pharm. 27: 649-655.
- KOHLMÜNZER S., GRZYBEK J., TANAKA M. 1980. Antitumor and cytotoxic activity of polysaccharides from *Tylophilus felleus*. Planta Med. 3: 231-232.
- KOHLMÜNZER S., KAŁUŻA J. 1975. Biologische Aktivität von *Tylophilus felleus* (Bull. ex Fr.) P. Karst. In: Gewebekultur Gehirntumorzellen. Abst. Int. Conf. Med. Plants. Marianske Lazne. p. 94.
- KOHLMÜNZER S., QUINN M.L., BENOIT P., FARNSWORTH N.R. 1977b. Antiinflammatory Activity of *Tylophilus felleus* (Bull. ex Fr.) P. Karst. Pol. J. Pharmacol. Pharm. 29: 539-541.
- PHARMACOPEA POLONICA IV. 1970. Oznaczanie ilości popiołu (substancji mineralnych). Vol. 1. PZWL. Warszawa. p. 70.
- ROSZKOWSKI W., ROSZKOWSKI K., SZMIGIELSKI S., PULVERER G., JELJASZEWICZ J. 1982. Immunostimulatory and antineoplastic activities of propionibacteria. In „Medical Microbiology”. Vol. 1. Eamson C.S.F., Jeljaszewicz J. (Eds.) Acad. Press., London, p. 433-452.
- STAUB A.M. 1965. Removal of proteins. In „Methods in Carbohydrate Chemistry”. Vol. 5. Whistler R.M. (Ed.). Acad. Press New York and London, pp. 5-6.
- TUTTLE R.L., CANTRELL J. 1981. *Corynebacterium parvum* – determinants of biologic activity. In „Augmenting agents in cancer therapy”. Hersh E.M. et al. (Eds.). Raven Press, New York, pp. 53-69.
- WAGNER H., PROKSCH A. 1985. Immunostimulatory Drugs of Fungi and Higher Plants. In „Economic and Medicinal Plant Research”. Vol. 1. Acad. Press. London, pp. 113-153.
- ZGÓRNIAC-NOWOSIELSKA I., JAROŃSKA E., KRYCZKO E. 1985a. Immunostimulation by Propionibacteria in viral infections of mice. Abstr. Int. Symp. on Prevention and Treatment of Viral Infections. Bechyne Castle, CSR, p. 52.
- ZGÓRNIAC-NOWOSIELSKA I., KRYCZKO E., JAROŃSKA E., CEMBRZYŃSKA-NOWAK M. 1985b. Immunostimulatory activity of *Propionibacterium acnes* in mice infected with *Herpes simplex* Virus. Abstr. Int. Symp. on Antiviral Substances. Smolenice. CSR, p. 31.

PRZECIWNOWOTWOROWA AKTYWNOŚĆ GRZYBOWEGO GLUKANU – TYLOPILANU ORAZ PREPARATU Z *PROPIONIBACTERIUM ACNES*

STRESZCZENIE

Badano przeciwnowotworową aktywność tylopilanu, β -(1→3) (1→6) glukanu wyizolowanego z owocników goryczaka żółciowego – *Tylophilus felleus* (Bull.: Fr.) P. Karst. (*Boletaceae*) oraz preparatu otrzymanego z *Propionibacterium acnes* (*P.a.*). Badane substancje oddzielnie lub łącznie podawano dootrzewnowo samcom myszy NMRI z wszczepionym nowotworem Crockera 180-TG. Tylopilan i *P.a.* podawano myszom 5 dni lub 2 godz. przed wszczepieniem komórek nowotworowych. Tylopilan podany w dawkach 25 μ g lub 50 μ g/mysz a *P.a.* w dawce 1 mg/mysz przedłużały w sposób statystycznie znamieny średni czas przeżycia zwierząt doświadczalnych w porównaniu z kontrolnymi. Średni czas przeżycia myszy wynosił odpowiednio 23,6 oraz 22,8 dni u myszy traktowanych tylopilanem i 17,5 dnia u myszy kontrolnych. Łączne podanie tylopilanu i *P.a.* także zwiększało średni czas przeżycia zwierząt. Stwierdzono również cytotoksyczną aktywność tylopilanu w odniesieniu do omawianego nowotworu w hodowli *in vitro*.

SŁOWA KLUCZOWE: tylopilan, polisacharyd, przeciwnowotworowa i cytotoksyczna aktywność, *Tylophilus felleus* (Bull.: Fr.) P. Karst., (*Boletaceae*), preparat z *Propionibacterium acnes*.