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Cytotoxic, antiviral (in-vitro and in-vivo), immunomodulatory activity and influence on mitotic divisions of three taxol derivatives: 10-deacetyl-baccatin III, methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) and *N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserine

Ewa Krawczyk, Mirosław Łuczak, Monika Kniotek, Maria Nowaczyk

Abstract

The aim of this study was to evaluate cytotoxic, antiviral (in-vitro and in-vivo) and immunomodulatory activity, as well as the influence on mitotic division, of three taxol derivatives representing modified parts of its molecule: 10-deacetyl-baccatin III, methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) and *N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserine. The cytotoxicity of the compounds, assessed by the formazane method, was relatively low, with a 50% cytotoxic concentration (CC50) > 500 μg mL^{-1}. Moreover, all tested compounds inhibited *Herpes simplex* type 1 virus (HSV-1) replication in non-cytotoxic concentrations in-vitro. Selectivity indices were in the range 9.5–46.7. Anti-HSV-1 activity of the compounds may be associated with their influence on mitotic division. All of the compounds decreased the number of cell divisions. Mitotic indices ranged from 40/1000 (4.0%) to 62/1000 (6.2%). One compound, 10-deacetyl-baccatin III, influenced the growth of tumours induced in mice by infection with Moloney murine sarcoma virus. The effect of the tested compounds on T lymphocyte proliferation was evaluated by measurement of the activity of tritiated thymidine incorporated into DNA of dividing cells. One compound, methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate), inhibited T lymphocyte proliferation. This paper demonstrates that modified parts of the taxol molecule possess various types of biological activity in-vitro and in-vivo. Further experiments, focused on revealing their mechanisms of action, are necessary.

Chair and Department of Medical Microbiology, Medical University of Warsaw, 5 Chalubinski St, 02-004 Warsaw, Poland

Ewa Krawczyk, Mirosław Łuczak

Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, 59 Nowogrodzka St, 02-006 Warsaw, Poland

Monika Kniotek, Maria Nowaczyk

Correspondence: E. Krawczyk, Chair and Department of Medical Microbiology, Medical University of Warsaw, 5 Chalubinski St, 02-004 Warsaw, Poland. E-mail: ewakrawczyk@gazeta.pl

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Introduction

Taxol (Figure 1A) is an effective drug used in the therapy of ovarian, breast, lung and other types of cancers (Rowinsky & Donehower 1995; Panchagnula 1998; Knox et al 2000). Its mechanisms of action include induction of microtubule polymerization and stabilization of the polymers by inhibition of depolymerization (Schiff & Horwitz 1980; Gupta et al 2003), which leads to modification of mitotic spindle structure, block of mitotic division and, in consequence, cell death (Jordan et al 1993; O'Leary et al 1998; Oldham et al 2000). Other biological actions of taxol include induction of apoptosis (Fan 1999; Wang et al 2002), immunosuppressive properties (Tange et al 2002) and immunotropic activity: taxol induces synthesis of tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) (Bogdan & Ding 1992; White et al 1998), IL-2 (Bottex-Gauthier et al 1992), IL-12 (Mullins et al 1999) cytokines, inhibits production of IL-10 (Tong et al 2000) and affects function of natural killer (NK) cells (Puente et al 1995).

An important derivative of taxol is baccatin III, used as a precursor in taxol synthesis. The mechanism of action of baccatin III is similar to that of taxol: baccatin III stimulates assembly of microtubules and blocks their depolymerization (Chatterjee et al 2001), which results in mitosis inhibition (Andreu & Barasoain 2001). However, other authors suggest that this compound influences mitotic divisions (Miller et al 1999), blocks microtubule polymerization, and has no effect on their depolymerization (Pengsuparp et al 1996). Recent experiments showed that baccatin III promotes microtubule assembly, but less effectively than taxol (Gupta et al 2003). Baccatin III is also cytotoxic (Pengsuparp et al

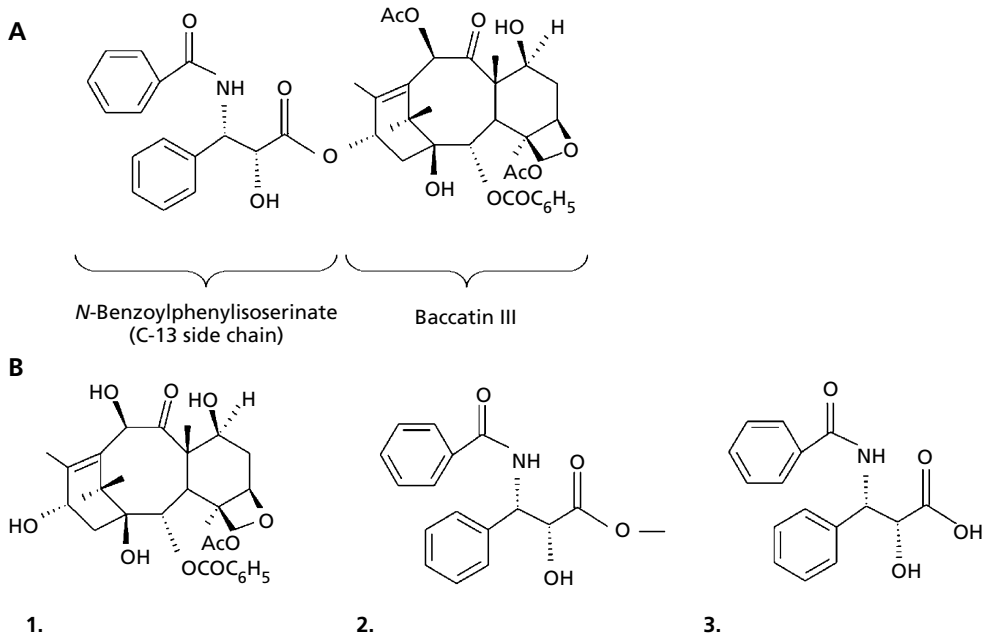

Figure 1 Chemical structure of taxol (A) and tested compounds (B): **1**, 10-deacetyl-baccatin III; **2**, methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate); **3**, *N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserine.

1996) and its derivative, 10-deacetyl-baccatin III has antifeedant activity (Daniewski et al 1998). On the other hand, the second part of the taxol molecule, the C-13 side chain -*N*-benzoylphenylisoserinate, seems to be unnecessary for biological activity of taxol (He et al 2000). Some authors suggest that baccatin III alone is functionally identical to taxol (Gupta et al 2003).

Although we have demonstrated lack of antiviral activity of taxol, derivatives of natural sesquiterpenes and taxol's C-13 side chain inhibited replication of Herpes simplex type 1 virus (HSV-1) (Krawczyk et al 2003). It was not investigated, however, whether the sesquiterpene part or the taxol part of the molecule was responsible for the antiviral action. Therefore, we deem the assessment of antiviral properties of C-13 chain derivatives (such as those used for synthesis of the *N*-benzoylphenylisoserinates of *Lactarius* sesquiterpenoid alcohols) valuable.

The aim of this study is to evaluate cytotoxic, antiviral (in-vitro and in-vivo) and immunomodulatory activity, as well as the influence on mitotic divisions, of three taxol derivatives representing modified parts of its molecule: 10-deacetyl-baccatin III, methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) and *N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserine.

Materials and Methods

The tested compounds are shown in Figure 1B and were obtained in the Institute of Organic Chemistry (Warsaw, Poland). For details on synthesis and purity, see Daniewski et al (1998) and Kopczacki et al (2001). The compounds were diluted in dimethyl sulfoxide (DMSO) (Sigma). In each experiment, the final DMSO concentration was lower than $20\,\mu L\,mL^{-1}$ (2%) and this concentration had no effect upon cells, viruses or animals.

Cytotoxicity assay

Assays of cytotoxicity and antiviral activity of the tested compounds were performed on Vero cells grown in 96-well plates (2×10^4 cells/well) for 24 h (until full-confluence monolayer was observed) in minimum essential medium (MEM; Biomed, Poland) supplemented with 10% fetal calf serum (FCS; Gibco) and Antibiotic-Antimycotic Solution (Sigma). The cells were submitted to a series of concentrations of the compounds, from $500\,\mu g\,mL^{-1}$ to $0.25\,\mu g\,mL^{-1}$, halving the concentration with each step, for 24 h at 37°C. Cytotoxicity was measured by the formazane method, using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma), according to Mosmann (1983), and was expressed as the concentration which reduced cell viability by 50% (CC50). The experiment was performed in three independent replications, with two wells for each concentration.

Antiviral assay in-vitro

Antiviral activity was assessed using series of concentrations starting from non-cytotoxic (which reduced cell viability by $\leq 10\%$) and halving the concentration with each step. Vero cells ($10^5\,mL^{-1}$) were infected with HSV-1 McIntyre strain ($HSV\text{-}1_{MC}$) (0.01 of the 50% tissue culture infective dose, TCID50, per cell). After 1 h adsorption at 37°C, the inoculum was replaced by MEM with 2%

FCS containing the tested compounds. After 24 h of cultivation (good multiplicity of infection) at 37°C, the plates were searched for viral-induced cytopathic effect (CPE). The virus titre (TCID50/mL) was calculated according to Reed & Muench (1938). The anti-HSV-1 activity was expressed as the concentration that reduced the virus titre by 50% (IC50). Aciclovir (Sigma) was used as a positive control. The experiment was performed in three independent replications; in each replication, three wells were used for each concentration.

Antiviral assay in-vivo

The TID50 of standard Moloney murine sarcoma virus (M-MSV) was $10^3\,mL^{-1}$ phosphate-buffered saline (PBS). Male NMRI mice, 4–5 weeks old, were injected with 0.2 mL of the virus/mouse into the gastrocnemius muscle of the left hind leg. On the 1st, 2nd and 3rd day after virus inoculation, the mice were injected intraperitoneally with 100 μg of the compound diluted in DMSO and 0.2 mL of PBS. The control group was infected with M-MSV and treated with PBS and DMSO. Each group consisted of 10 mice, and the experiment was performed in two independent replications. For 21 days starting from the day of infection, the growth of virus-induced tumour was assessed by measuring the calf diameter. The care and the use of the mice complied with Polish regulations and the research was conducted in accordance with the EEC Directive 86/609/EEC.

Evaluation of the influence on mitotic divisions

Vero cells (2×10^5 cells/mL; 6×10^5 cells/dish) were grown in 35 mm Petri dishes (Corning) in MEM (Biomed, Poland) supplemented with 10% FCS (Sigma) and Antibiotic-Antimycotic Solution (Sigma). A sterile microscopic cover slip was placed inside the dish. The cells were incubated at 37°C for 24 h. Then, the tested compounds were added in non-cytotoxic concentrations, which inhibited HSV-1 replication: compounds **1** and **2**, 125 $\mu g\,mL^{-1}$; **3**, 250 $\mu g\,mL^{-1}$, for 24 h at 37°C. Control cells were treated with DMSO only. Next, the cover slips covered by cells were washed in PBS (Biomed, Poland) and fixed in a 3:1 mixture of 95% ethanol (Polish Chemical Reagents, Poland) and glacial acetic acid (Sigma) for 10 min, and stored in 70% ethanol. Before staining the cells were washed in distilled water and air-dried. Staining with haematoxylin–eosin (Sigma), dehydratation and mounting were performed according to standard histological procedures. Preparations were evaluated microscopically by an observer blinded to which treatment the cells had received. The influence of the tested compounds on the mitotic divisions was assessed by calculation of the mitotic index (i.e., the number of dividing nuclei, recognized by visible condensed chromosomes, per one or two thousands counted cells).

Evaluation of the influence on T lymphocyte proliferation

Peripheral blood mononuclear cells (PBMCs), isolated from healthy blood donors, were placed in 96-well plates (10^5 cells/100 μL/well; 10^6 cells/mL; NUNC) in Parker medium (Biomed, Poland) supplemented with 10% FCS (Sigma), 0.3% gentamicin, 1% HEPES, 1% L-glutamine and 0.1% 2-mercaptoethanol. T lymphocyte mitogen–phytohemagglutinin (PHA) (Sigma) in a concentration of 10 $\mu g\,mL^{-1}$ was added. The tested compounds in non-cytotoxic concentrations of 62.5 and 31.2 $\mu g\,mL^{-1}$ or DMSO (in the same volume that was used for diluting the compounds) were added. The final DMSO concentration was 3 $\mu L\,mL^{-1}$ for higher concentrations of the compounds and 1.5 $\mu L\,mL^{-1}$ for lower concentrations. Cells submitted neither to the compounds nor to DMSO were used as a control. Incubation lasted for 72 h at 37°C. T lymphocyte proliferation was evaluated by measurement of the activity of tritiated thymidine ([^{3}H]thymidine; POLATOM, Poland) incorporated into DNA of dividing cells. For this purpose, [^{3}H]thymidine (1 μCi/well) was added to the cells for the last 17 h of incubation. Then, the cells were harvested using a Semiautomatic Cell Harvester (Skatron) and the activity of incorporated thymidine was assessed using a scintillation counter (Wallac) and expressed as the number of counts. The influence of the tested compounds on T lymphocyte proliferation was evaluated by comparing the number of counts of the cells submitted to the compounds with those submitted to DMSO only. The experiment was repeated five times, each time using PBMCs from a different donor.

Results

Cytotoxicity and antiviral assay in-vitro

The cytotoxicity of the tested compounds towards Vero cells (CC50 > 500 $\mu g\,mL^{-1}$) was considerably lower than the cytotoxicity of taxol (CC50 3.7 $\mu g\,mL^{-1}$, our previous results (Krawczyk et al 2003)). All compounds significantly inhibited HSV-1 replication. Selectivity indices were in the range 9.5–46.7 (Table 1).

Antiviral assay in-vivo

In all groups of mice, the tumours appeared on day 3–4, reached maximum size on day 8, and regressed on day 16–17

Table 1 Cytotoxicity and anti-HSV-1 activity of taxol derivatives

Compound	CC50 ($\mu g\,mL^{-1}$)	Concn range	IC50 ($\mu g\,mL^{-1}$)	Concn range	Mean SI (CC50/IC50)
1	>500	—	52.7	12.0–93.4	>9.5
2	>500	—	10.7	5.4–18.6	>46.7
3	>500	—	21.7	3.9–56.0	>23.0
Aciclovir	>250	—	1.0	0.8–1.4	>250

Results are presented as the mean of three independent experiments. Two wells (cytotoxicity assay) or three wells (in-vitro antiviral assay) were used for each concentration in each replication. CC50, 50% cytotoxic concentration; IC50. 50% inhibitory concentration; SI, selectivity index.

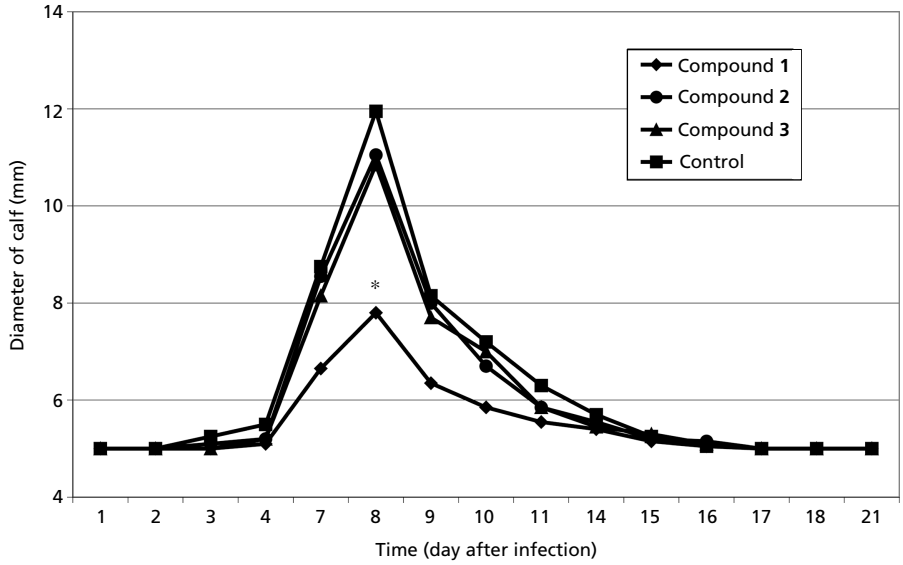

Figure 2 The influence of the tested compounds on the growth of M-MSV-induced tumour in mice. On each of the 1st, 2nd and 3rd days after intramuscular virus injection, the mice were given 100 μg of the tested compounds intraperitoneally. The diameter of the virus-injected calf was measured. Each group consisted of 20 mice; $*P < 0.05$ vs control.

after infection (Figure 2). Therefore, between-group comparison was performed only on day 8. 10-Deacetyl-baccatin III (compound **1**) significantly reduced the mean diameter of the calf (7.8 mm, s.d. 2.5) in comparison with control (12.0 mm, s.d. 3.8) by 35% (Kruskal-Wallis test: $P < 0.025$; Dunn's post-hoc test $P < 0.05$, for each replication). No effect was found with the other compounds ($P > 0.05$).

Evaluation of the influence on mitotic divisions

The mitotic index of Vero cells not submitted to the compounds was 169/2000 (8.5%). The cells grew as a monolayer and were observed in interphase and during mitotic divisions (Figure 3A). In the cells submitted to the compounds, abnormalities were seen, especially in nuclei and cell divisions, but not in the cytoplasm. Modified mitotic divisions with chromosomes lying outside of the mitotic spindle (Figure 3B) and incorrect tripolar spindles (Figure 3C) were detected. Moreover, all of the compounds significantly decreased the number of cell divisions in comparison with control ($P \leq 0.035$, χ^2 test with Yates correction). Mitotic indices ranged from 40/1000 (4.0%) to 62/1000 (6.2%) (Table 2).

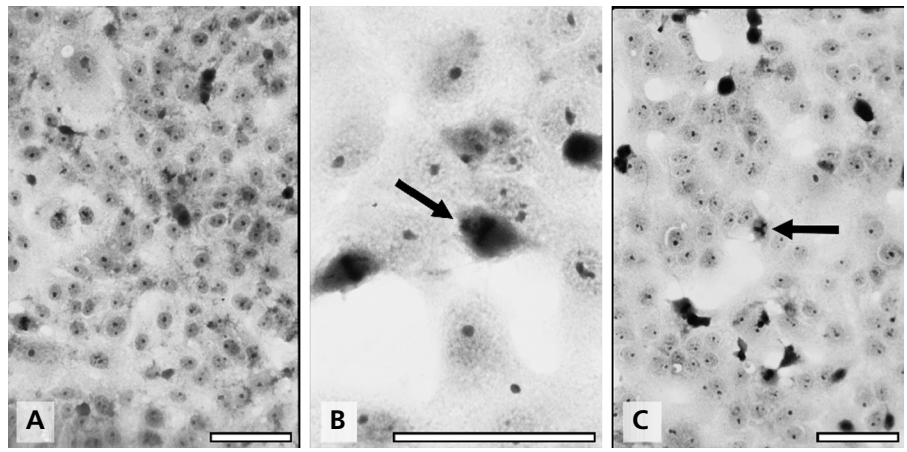

Figure 3 Control Vero cells (A), Vero cells submitted to non-cytotoxic concentration of 10-deacetyl-baccatin III (125 μg mL^{-1}) for 24 h (B; the arrow shows a cell with chromosomes lying outside of the mitotic spindle) and Vero cells submitted to non-cytotoxic concentration of 10-deacetyl-baccatin III (125 μg mL^{-1}) for 24 h (C; the arrow shows a cell with incorrect tripolar mitotic spindle). Haematoxylin–eosin staining, scale bar indicates 100 μm.

Table 2 The influence of taxol derivatives on mitotic divisions

Compound	Mitotic index	% of dividing cells	Statistical comparison with control	
			χ^2(df = 1)	P
1	106/2000	5.3	15.0	0.0001
2	62/1000	6.2	4.4	0.035
3	40/1000	4.0	19.7	< 0.0001
Control	169/2000	8.5	—	—

Evaluation of the influence on T lymphocyte proliferation

No significant difference in the number of counts was found between control cells and the cells submitted to DMSO only (Friedman test $P > 0.09$; Dunn's post-hoc test $P > 0.05$). However, significant differences between the cells submitted to the compounds and the cells submitted to DMSO only were observed (Friedman test $P < 0.001$). Methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) (compound **2**) significantly inhibited T lymphocyte proliferation, at both concentrations tested (Dunn's post-hoc test $P < 0.05$), in a dose-dependent manner (Table 3).

Discussion

We have demonstrated that cytotoxic concentrations (CC50) of the tested compounds, representing modified parts of the taxol molecule, were considerably lower than the cytotoxicity of taxol (Krawczyk et al 2003). The cytotoxic activity of taxol seems to be determined by its specific chemical structure (connection of baccatin III with *N*-benzoylphenylisoserinate side chain). This may suggest that both parts of the taxol molecule influence its biological properties, and that, contrary to some investigations (He et al 2000; Gupta et al 2003), *N*-benzoylphenylisoserinate seems to be necessary for biological activity of taxol.

Table 3 The influence of taxol derivatives on T lymphocyte proliferation

Compound	Concn of compound (μg mL^{-1})	DMSO concn (μL mL^{-1})	Mean no. of counts	Standard deviation
1	62.5	3	3232.6	550.8
	31.2	1.5	5223.4	3715.8
2	62.5	3	2322.0	2793.8
	31.2	1.5	3972.6	3559.9
3	62.5	3	11554.6	4223.7
	31.2	1.5	13465.2	6297.4
DMSO	—	3	17031.2	13917.3
	—	1.5	14137.2	6316.1
Control	—	—	23616.0	5044.8

Hence, baccatin III alone is not functionally identical to taxol. Our results are supported by other authors, who demonstrated that the taxol side chain influenced its biological properties (Andreu & Barasoain 2001), and that baccatin III was less cytotoxic than taxol (Pengsuparp et al 1996). The CC50 of baccatin III in that study was lower (4.7–29 μg mL^{-1}) than the CC50 of 10-deacetyl-baccatin III found in our study, which might result from the difference in the chemical structure of these compounds, and in the experimental models used.

In contrast to taxol (Krawczyk et al 2003), all compounds tested in this study inhibited replication of HSV-1 in-vitro. Again, it was demonstrated that not only 10-deacetyl-baccatin III, a compound with proven biological properties (Daniewski et al 1998), but also modified derivatives of the taxol side chain possessed biological (in this case, antiviral) activity. This activity was lower than that of aciclovir, but still considerable. The anti-HSV activity of the compounds may be associated with their influence on mitotic divisions. Intracellular transport and replication cycle of HSV depend on functional cytoplasmic microtubules (Topp et al 1994, 1996; Hammonds et al 1996). Compounds that affect microtubular function (including taxol) may disturb viral replication (Norrild et al 1986). Because mitotic divisions depend upon functional microtubules we suppose that the tested compounds, which interfere with cell divisions (probably through microtubules), may also modify the HSV replication cycle. So far no experiments had been performed showing that the compounds' antimitotic activity was directly associated with their influence on microtubular function. But, since these compounds are taxol derivatives, this mechanism may be considered. We demonstrated the antimitotic action of 10-deacetyl-baccatin III, a derivative of baccatin III, whose antiproliferative activity has been already shown (Andreu & Barasoain 2001). An influence on mitotic division was observed also in the case of two taxol side chain derivatives. The mechanism is not known; some data suggest that various derivatives of the taxol side chain may affect microtubular action and inhibit mitosis (Young et al 1992), although other authors have shown that there is no such activity (Fuji et al 1999). Structural differences of the compounds, and different experimental models may be the reason for this discrepancy.

M-MSV induces formation of tumours in mice (D'Andrea et al 1992). Various chemical compounds, including natural ones, influence M-MSV infection in-vivo (Panasiak et al 1990; Naesens et al 1998). It appears that 10-deacetyl-baccatin III did not affect virus replication directly. It influenced neither the timing of tumour appearance and regression, nor the number of tumour-developing mice, but affected the tumour size only. Because this compound blocked mitotic divisions, we propose that its anti-tumour activity may be another example of the antiproliferative action. However, other tested compounds, which inhibited mitosis more strongly than 10-deacetyl-baccatin III, did not influence the M-MSV-induced tumour growth. Therefore, the mechanism of action of 10-deacetyl-baccatin III still needs evaluation.

Methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) significantly inhibited PHA-induced proliferation of T lymphocytes. The mechanism of its action is not known, but, considering its antimitotic properties against Vero cells, we propose that this compound is able to inhibit proliferation of several cell types, and this action may be based on a similar mechanism. Indeed, some authors showed that taxol inhibitory activity against lymphocyte proliferation was due to its influence on microtubules (Cuthbert & Shay 1983). Why methyl (*N*-benzoyl-(2′*R*,3′*S*)-3′-phenylisoserinate) did not inhibit proliferation of tumour cells in mice needs to be evaluated.

Conclusion

In conclusion, we demonstrated that the tested chemical compounds, representing modified parts of the taxol molecule, possess biological activity in-vitro and in-vivo: they inhibit HSV replication cycle at low cytotoxicity, block mitotic divisions of Vero cells, influence M-MSV-induced tumour size and affect immune response by inhibiting PHA-induced T lymphocyte proliferation. Derivatives of baccatin III and of the taxol C-13 side chain were shown to be biologically active. Therefore, both parts of the taxol molecule may be considered as a potential source of chemotherapeutics. Their mechanisms of action remains undetermined. Hence, studies focused on revealing these mechanisms are necessary.

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