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Cytotoxic effects of NSL-1406, a new thienopyrimidine derivative, on leukocytes and osteoclasts

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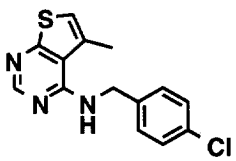
Abstract: We synthesized a series of thienopyrimidine derivatives and examined their cytotoxic effects on several cell lines. One of the derivatives, NSL-1406, was shown to exert potent cytotoxic effects on leukemia cell line including P388 cells and J774 cells. It was also inhibitory on mouse osteoclasts and suppressed the *in vitro* bone resorption by osteoclasts at nanomolar concentrations.

Hematopoietic precursor cells in bone marrow differentiate into several types of cells that collaboratively play important roles in the maintenance of homeostasis and host defense. Disturbance of the subtle balance between the activity of these cells can lead to development of various inflammatory diseases. Many compound that modulate the activity of leukocytes, have been developed as anti-inflammatory drugs. For example, steroids, which are potent suppressors of various types of leukocytes, have been widely used as immuno-suppressive drugs or anti-inflammatory drugs. Inhibitors of production of various proinflammatory mediators have also been used or are still being developed, such as inhibitors of the inducible type of cyclooxygenase,[1,2] of tumor necrosis factor (TNF) production by macrophages[3,4] or of an interluekin (IL)-1 processing enzyme.[5,6] Osteoclasts are also cells of monocyte/macrophage linkage and derived from haematopoietic precursor cells.[7,8] Since osteoclasts are primary bone-resorbing cells, they are one of the targets of chemotherapy for osteoporosis.[9,10]

During the development of anti-osteoporosis drugs, we identified a thienopyrimidine derivative **1**, which showed a potent cytotoxicity on P388 murine leukemia cells. Thienopyrimidine derivatives are known to have various biological activities, such as antimicrobial,[11] antifungal,[12] anti-inflammatory[13] and anti-hypertensive[14] effects. However, to our knowledge, no previous reports have described a compound having a thienopyrimidine structure, which exerts potent cytotoxicity on P388 cells.

In the present study, we synthesized a series of derivatives of **1** and examined their cytotoxic effects on several types of cell lines. NSL-1406 (**5**), a derivative of **1**, showed a potent and specific cytotoxicity against several cell lines of leukemia cells, but not against non-haematopoietic, adhesive cells. At submicromolar concentrations, this compound also exerted cytotoxic effects on osteoclasts and prevented bone resorption by these cells. Our results suggest that **5** and its related compounds have a potency as an anti-inflammatory or anti-osteoporosis agent.



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Results

Structure-activity relationship studies

We synthesized a series of derivatives of **1**[15] and examined the cytotoxic effect on P388 cells, as determined by WST-1 assay or by counting the number of viable cells. As shown in Table 1, the cytotoxic effect of **1** against P388 cells were noted at submicromolar concentrations (IC_{50} = 170 nM in WST-1 assay).

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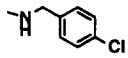
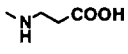
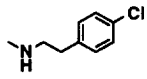
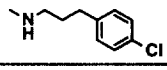
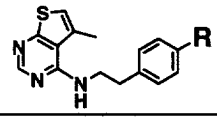
Table 1. Cytotoxicity of derivatives of 1 on P388

compd	R	IC_{50} (M)[a]	LD_{50} (M)[b]
1		1.7 (± 0.17) X 10^{-7}	7.4 (± 0.56) X 10^{-5}
2	Cl	3.3 (± 0.31) X 10^{-5}	4.9 (± 0.33) X 10^{-4}
3		5.5 (± 0.37) X 10^{-6}	8.5 (± 0.11) X 10^{-5}
4		1.2 (± 0.11) X 10^{-5}	2.5 (± 0.15) X 10^{-4}
5		5.2 (± 0.43) X 10^{-9}	3.5 (± 0.18) X 10^{-5}
6		1.3 (± 0.10) X 10^{-6}	9.8 (± 0.97) X 10^{-5}

P388 cells were incubated with each compound for 24 h and the cytotoxicity was examined. [a] IC_{50} values were defined as a dose giving a 50 % decrease in the formation of formazan dye as compared with that of a control group (see ref.16,17). [b] LD_{50} values were determined as a dose giving a 50 % decrease in the living cell number. Values are means (± sem) of three experiments

Table 2. Cytotoxicity of derivatives of 5 on P388



compd	R	IC_{50} (M)[a]	LD_{50} (M)[b]
5	Cl	5.2 (± 0.43) X 10^{-9}	3.5 (± 0.18) X 10^{-5}
7	Br	1.1 (± 0.10) X 10^{-9}	5.8 (± 0.42) X 10^{-5}
8	OCH_3	7.4 (± 0.58) X 10^{-9}	4.6 (± 0.33) X 10^{-5}
9	H	2.9 (± 0.18) X 10^{-8}	4.3 (± 0.14) X 10^{-5}
10	F	2.9 (± 0.14) X 10^{-8}	6.8 (± 0.74) X 10^{-5}
11	NH_2	2.2 (± 0.15) X 10^{-8}	3.9 (± 0.11) X 10^{-5}
12	OH	1.9 (± 0.18) X 10^{-7}	8.6 (± 0.55) X 10^{-5}

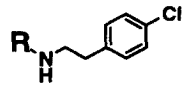
[a,b] see Table 1.

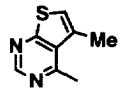
Based on the initial modification of **1**, we found that compounds **2**-**4**, which do not possess a 4-chlorobenzylamine structure, were more than 30 times less active than **1**. These findings suggest that the 4-chlorobenzylamine structure is very important for the potent cytotoxic activity. Accordingly we optimized this structure in the next series of experiments. Firstly, we examined the effect of distance between thienopyrimidine ring and 4-chlorophenyl group. The most potent activity was noted in compound **5** containing the ethylamine structure as a spacer (IC_{50} = 5.2 nM), while the insertion of an additional methylene group resulted in a 10-fold decrease in the cytotoxic activity. The LD_{50} values, as determined by viable cell counts, were to a large extent not affected by these modifications of **1**.

Next, we examined the effects of the substitutions at the 4-position of the phenyl group in **5**. As shown in Table 2, a slight increase in cytotoxicity was observed in **7** with a bromine atom at this position. The cytotoxicity of **8** with a methoxy group was similar to that of **5**. Other compounds with a hydrogen (**9**) or fluorine (**10**) atom, or an amino group (**11**) were five times less active than **5**. Furthermore, substitution with a hydroxyl group (**12**) resulted in a 10-fold decrease in cytotoxic activity.

As shown in Table 3, the presence of a sulfur atom in the thiophenopyrimidine ring seems to be essential for a potent cytotoxic effect since the introduction of a purine ring (**14**) instead of thiopheno[3,2-*d*]pyrimidine group resulted in a 1000-fold decrease in cytotoxicity. Compound **15** with a thiopheno[2,3-*d*]pyrimidine ring showed a slightly lower cytotoxicity, suggesting the importance of the sulfur atom position. Elimination of the methyl group (**13**) of **5** also resulted in a slight decrease in cytotoxicity.

Table 3. Cytotoxicity of derivatives of 5 on P388



compd	R	IC_{50} (M)[a]	LD_{50} (M)[b]
5		5.2 (± 0.43) X 10^{-9}	3.5 (± 0.18) X 10^{-5}
13		3.5 (± 0.26) X 10^{-8}	7.3 (± 0.59) X 10^{-5}
14		7.3 (± 0.14) X 10^{-6}	9.6 (± 0.77) X 10^{-5}
15		2.9 (± 0.28) X 10^{-8}	4.1 (± 0.22) X 10^{-5}

[a,b] see Table 1.

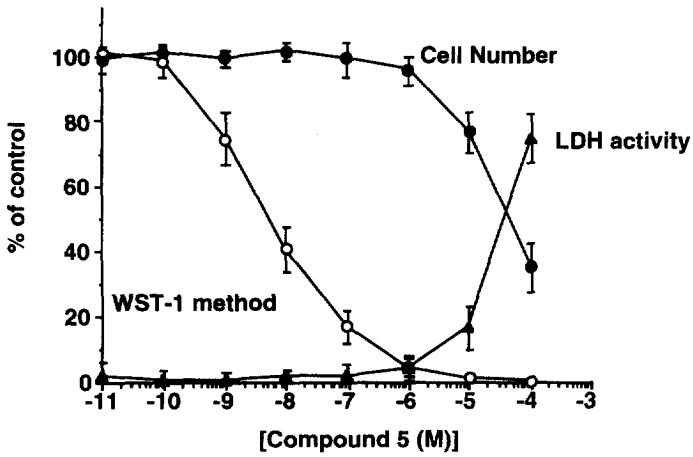


Figure 1 Cytotoxic effects of 5 (NSL-1406) on P388 .
P388 cells were incubated with **5** at various concentrations for 24 h and served for the evaluation of the cytotoxicity and cell viability. Cytotoxicity was determined by the WST-1 staining method (open circles). The number of the living cells (closed circles), as measured by trypan blue exclusion, and LDH activity in the culture medium (closed triangles) were measured to evaluate the effects on cell viability. Each point represents as a percent of the control value. Mean ± SEM, n=5.

The results of these structure-activity relationship studies suggest that the presence of thiopheno[3,2-*d*]pyrimidine ring and a 4-substituted phenethylamine structure is essential for the potent cytotoxic effect. We next examined the *in vitro* profile of one of the potent derivatives **5**.

Cytotoxic effects on several cell lines

The cytotoxic effects of **5** on P388 leukemia cells are shown in Fig.1. The formation of WST-1 formazan by P388 leukemia cells was inhibited in a dose-dependent manner with an IC_{50} value of 5.2 nM. At doses larger than 10^{-7} M, almost no formazan dye formation was observed. Microscopic examination showed that the cell number and cell morphology did not change even after treatment with **5** for 24 h, suggesting that **5** did not cause cell death. To further evaluate the cell-killing activity of **5**, the viability of the cells was measured by trypan blue exclusion. When P388 cells were treated with **5** for 24 h, the number of viable cells decreased only at the higher concentrations with an LD_{50} value of about 70 μM. Measurements of lactose dehydrogenase (LDH) activity in culture media, as a parameter of the degree of cell lysis, also suggested that cell death occurred only at concentrations > 10^{-5} M. These results suggest that at lower concentrations, **5** exerts its cytotoxic effects on P388 cells without killing the cells. These cytotoxic effects of **5** were reversible and disappeared after washing (data not shown).

Table 4 shows the effects of **5** on various types of tumor cells. In J774 cells and HL-60 cells, which are both leukemia cell lines of hematopoietic origin, the IC_{50} values for WST-1 formazan formation were at nanomolar

Table 4 Cytotoxicity of compound 5 on several cell lines

Cell line	IC_{50} (M)[a]	LC_{50} (M)[b]
J774	5.0 (± 0.60) X 10^{-9}	4.3 (± 0.41) X 10^{-5}
HL-60	8.5 (± 0.32) X 10^{-9}	6.7 (± 0.60) X 10^{-5}
ECV-304	3.4 (± 0.60) X 10^{-5}	5.3 (± 0.35) X 10^{-5}
A437	2.0 (± 0.22) X 10^{-5}	5.1 (± 0.48) X 10^{-5}

[a,b] see Table 1

concentrations, which were about 10,000 times lower than the LD_{50} values. In contrast, in two different adhesive cell lines, ECV-304 and A437 cells, **5** showed a relatively low cytotoxicity following 24 h-treatment, with IC_{50} values of 34 μM and 20 μM, respectively. LD_{50} values were almost similar to IC_{50} values.

Effects of NSL-1406 on osteoclasts

As described above, **5** seemed to exhibit specific cytotoxic effects on cells of hematopoietic origin. These results lead us to investigate the effect of this compound on osteoclasts, which are derived from the monocyte-macrophage linkage.

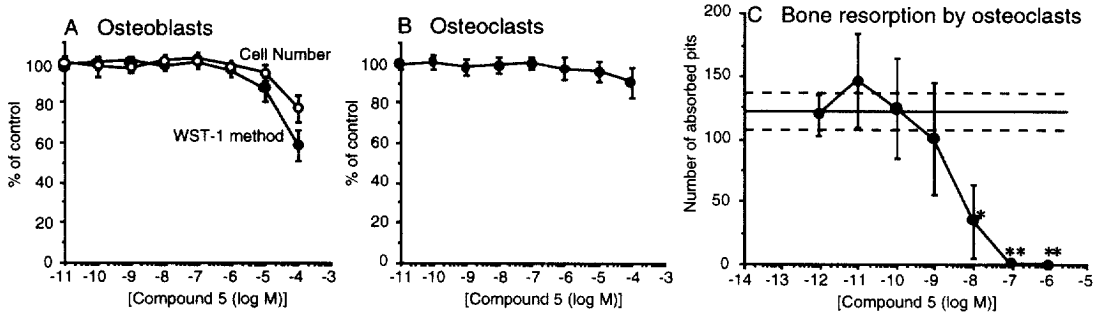


Figure 2 Cytotoxic effects of compound 5 on mouse osteoblasts and osteoclasts.
A; Effects on osteoblasts. B; Effects of on osteoclast-like cells. C; Effects on the *in vitro* pit formation by osteoclast-like cells. The solid line represents the mean number of pits of the control group and the dashed lines represent the standard error of the mean. *P< 0.05, **P < 0.001. Mean ± SEM, n=5. (see Ref. 18)

Co-culture of osteoblasts and bone marrow cells for 5 days on collagen gel results in the induction of osteoclast-like multinucleated cells, which are TRAP-positive.[19] Collagenase digestion of co-cultures recovered the crude osteoclast preparation, in which the purity of the osteoclast-like cells was about 5% while the remainder cells were osteoblast-like cells. Treatment of the osteoblast preparation or co-cultures with **5** at various doses for 24 h showed that the sensitivity of osteoclasts and osteoblast to **5** was different, as we expected. As shown in Fig.2A, osteoblast-like cells were highly resistant to **5** and no apparent cytotoxicity was observed even at 10^{-5} M, as determined by WST-1 assay and viable cell count. However, a significant cytotoxicity was noted at 10^{-4} M. As shown in Fig.2B, the number of TRAP-positive osteoclast-like cells did not change after incubation with **5** at all concentrations tested. However, the cells did not appear to be fully spread and exhibited small round configurations at higher doses of **5**. The bone resorbing activity of irregularly-shaped osteoclasts was examined by the pit assay. In this assay crude osteoclast preparations were cultured on dentine slices in the presence of **5** for 24 h and the number of resorbed pits on the slices is determined. As shown in Fig.2C, the number of resorbed pits decreased with higher doses of **5**, with an IC_{50} value of 4 nM. Osteoclasts attached to the surface of the dentine slices exhibited various irregular configurations.

Discussion

We have shown in the present study that **5**, a novel thienopyrimidine derivative containing a thiopheno[3,2-*d*] pyrimidine structure, exhibited potent cytotoxic effects on several cell types of hematopoietic origin, such as P388 leukemia cells and osteoclasts. Many thienopyrimidine derivatives have been reported to exert various biological activities. Pyrazolo[3,4-*d*]pyrimidine derivatives have an antifungal activity,[12] thienoisothiazole derivatives have an anti-inflammatory activity,[13] thieno[3,4-*d*] pyrimidine-2,4-diones derivatives have anti-hypertensive activity[14] and 2,4-dioxo-thienopyrimidine-1-acetic acid derivatives prevent sorbitol accumulation in the sciatic nerve and lens.[20] Although some of their actions could be explained by an α1-antagonistic activity or aldose reductase inhibitory activity, the mechanism of action of these thienopyrimidine derivatives have not been fully elucidated. It is unlikely that the inhibitory effects of **5** on cells of hematopoietic origin can be explained by these mechanisms. The WST-1 assay is a colorimetric assay of mammalian cell survival and proliferation, originally developed by Ishiyama *et al.*[17] In this assay, WST-1, a tetrazolium compound, is reduced in the mitochondria to form a water-soluble formazan dye, which is a good indicator of the number of viable cells. The formation of formazan dye, however, was not proportional to the number of viable cells as measured by trypan blue exclusion in the presence of **5**. The most possible explanation for this discrepancy is that **5** could act as an antimetabolite or LDH inhibitor and directly inhibits the reduction of WST-1 in mitochondria. To our knowledge, there are no studies that have described differences in mitochondrial enzymatic systems and metabolism in mitochondria between hematopoietic and non-hematopoietic cells.

We examined the cytotoxic effects of well-known metabolic inhibitors including antimycin A on several cell lines under similar assay conditions and showed that these metabolic inhibitors reduced formazan dye formation not only by P388 but also by non-hematopoietic cells such as WiDr cells (human adenocarcinoma of the rectosigmoid colon), A549 cells (human lung carcinoma) and A437 cells at submicromolar concentrations (data not shown). Therefore, it is unlikely that the cytotoxic effect of **5** could be due to metabolic inhibitory effects. Preliminary results from our laboratory have shown that **5** showed an antagonistic activity against human integrin αvβ3, the so-called vitronectin receptor, in the solid-phase binding assay using purified αvβ3 and vitronectin (unpublished data). This antagonistic activity was not very potent, with an IC_{50} value of 50 μM under our assay conditions. Osteoclasts also express a relatively large amount of αvβ3 integrin on their surface[21,22] and various forms of leukocytes also express this integrin.[23] The concentrations of **5** required to inhibit the binding of vitronectin to αvβ3 suggest that the actions of **5** described here could not be explained only by this antagonism. Thus, it is likely that other members of the integrin family could be involved in the cytotoxic activity. It has been reported that leukocytes express various types of integrins. Furthermore, interaction between integrin and ligand can evoke signal transduction and a series of intracellular events, including cytosolic alkalization, increased cytosolic calcium levels, tyrosine phosphorylation in proteins, induction of early gene expression and changes in the mechanical properties of cells.[24-26] Therefore, **5** may suppress cellular activities by binding to integrins on the surface of leukocytes, acting as an antagonistic or agonistic molecule. The precise mechanism underlying the cytotoxic effects of this compound is currently being investigated.

References and Notes

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15. Synthesis of **1** and its derivatives was carried out by one step condensation between 4-chloro-5-methylthieno[3,2-d]pyrimidine (Robba, M.; Lecomte, J.M.; Le-Guen, Y.*C. R. Hebd. Seances Acad. Sci. Ser. C* **1968**, *266*, 1706) and amines in the presence of diisopropylethylamine in DMF at 80 °C. In the modification of thienopyrimidine ring (Table 3), corresponding chloro-derivatives were used in the condensation with 4-chlorophenethylamine under above conditions. **5**; pale yellow solid; mp 112.5-113 °C; ^{1}H NMR (270 Mhz, $CDCl_3$) δ 2.36 (s, 3H), 2.97 (t, *J*=6.8 Hz, 2H), 3.86 (br dd, *J*=6.8, 12.7 Hz, 2H), 5.38 (m, 1H), 6.79 (s, 1H), 7.17 (d, *J*=8.3 Hz, 2H), 7.30 (d, *J*=8.3 Hz, 2H), 8.45 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 17.6, 34.6, 41.8, 115.9, 118.4, 128.4, 128.8, 130.2, 132.5, 137.3, 153.8, 158.0, 167.5, MS (ESI) m/z 303 $(M+H)^+$.
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18. Protocol used for investigating the cytotoxic effects of **5** on osteoclasts and osteoblasts was as follows. Primary osteoblasts were prepared from the calvaria of newborn ddy mice and pre-cultured for 5 days. After incubation with **5** at various concentrations for 24 h, the cytotoxic effects were determined by the WST-1 method or by counting the number of viable cells. Osteoblasts were differentiated from bone marrow cells isolated from the tibiae of 6-week-old mice by co-culture with osteoblasts for 5 days. After incubation with **5** at various concentrations for 24 h, the cells were stained for tartrate resistant acid phosphatase (TRAP) and the number of TRAP-positive cells were counted. The effects of **5** on *in vitro* bone resorption by osteoclasts were carried out by the pit assay. Osteoclast-like cells (1×10^4 cells), inducted from mouse bone marrow cells by co-culture with osteoblasts, were cultured on the dentine slices in the presence of **5**. After a 24-h incubation, cells were removed and resorbed pits formed on the slices were stained.
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