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Thalidomide increases *in vitro* sensitivity of childhood acute lymphoblastic leukemia cells to prednisolone and cytarabine

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Abstract

Introduction: Thalidomide is a derivative of glutamic acid with anti-angiogenic, anti-inflammatory, immunomodulatory and anti-cancer properties that was found to inhibit the production of tumor necrosis factor α *in vitro*, stimulate reactive oxygen species production, and inhibit vascular endothelial growth factor receptor in acute leukemias. The purpose of this study was to determine the *in vitro* activity of thalidomide as a single agent and in combination with prednisolone or cytarabine in childhood acute lymphoblastic leukemia (ALL).
Materials and Methods: Bone marrow samples of 40 childhood ALL patients, normal lymphocytes of 9 healthy adults, and 3 lymphoid cell lines were evaluated for cytotoxicity of thalidomide (alone and in combination with prednisolone and cytarabine) using the MTT assay. Cell cycle analysis was performed by flow cytometry.
Results: Thalidomide as a single agent had weak antileukemic activity to the childhood ALL samples. However, in the presence of thalidomide the cytotoxicities of prednisolone and cytarabine were increased 3.3-fold ($p<0.001$) and 2.7-fold ($p=0.002$), respectively. Thalidomide increased apoptosis in lymphoblasts and modulated the cell-cycle arrest caused by prednisolone, but not that by cytarabine, in childhood ALL samples.
Conclusions: Thalidomide increases *in vitro* the sensitivity of childhood ALL cells to prednisolone and cytarabine.

Key words: thalidomide, prednisolone, cytarabine, drug resistance, acute lymphoblastic leukemia, children.

Abbreviations: ALL – acute lymphoblastic leukemia, IC_{50} – inhibitory concentration for 50% of cells, IL-6 – interleukin 6, ImiDs – immunomodulatory drugs, MM – multiple myeloma, MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, NF-κB – nuclear factor κB, SF – sensitization factor, TNF-α – tumor necrosis factor α, VEGF – vascular endothelial growth factor, VEGFR – VEGF receptor.

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INTRODUCTION

In the 1950's, thalidomide was recommended as a sedative, especially for pregnant women. Later, in 1960's, thalidomide was recognized as a major teratogen that caused birth defects in up to 12,000 children. It has been shown in recent studies that thalidomide has immunomodulatory, anti-inflammatory, anti-angiogenic. and anti-cancer properties [3, 5, 14, 15]. This agent has been tested in a number of *in vitro* and *in vivo* studies (reviewed by Matthews and McCoy) [5]. Due to its anti-angiogenic and anti-cancer activity, current interest in the therapeutic use of this drug is related to hematological malignancies in adults. Thalidomide presented antimyeloma activity both in a mouse model [15] and in human samples [3, 7, 14]. Thalidomide was effective in the therapy of resistant or relapsed multiple myeloma (MM), plasma cell leukemia [4], myelodysplastic syndromes [7], acute myeloid leukemia in adults [11], and in histiocytic sarcoma [1]. Lack of clinical effect of thalidomide was reported in patients with myelofibrosis with myeloid metaplasia [6].

Thalidomide and its immunomodulatory analogs (IMiDs) directly induce apoptosis or growth arrest of

MM cells, alter adhesion of MM cells to bone marrow stromal cells, inhibit the production of interleukin (IL)-6 and vascular endothelial growth factor (VEGF) in bone marrow, and the stimulate natural killer cell for anti-myeloma immunity [7]. Thalidomide and the IMiDs enhance the anti-myeloma activity of dexamethasone and, conversely, are inhibited by IL-6 [3]. There are no data concerning thalidomide activity in childhood acute lymphoblastic leukemia (ALL) so far; thus the aim of this study was to analyze the *in vitro* cytotoxicity of thalidomide as a single agent and in combination with prednisolone or cytarabine, which are two of the basic drugs used in the therapy of childhood ALL.

MATERIALS AND METHODS

Cells

Three human lymphoid cell lines were used in the study: CCRF-CEM (T-lineage ALL), Jurkat (T-lineage ALL), and Daudi (childhood B-lineage lymphoma). Bone marrow samples of 40 childhood ALL patients on initial diagnosis were obtained. Their characteristics are presented in Table 1. The median age of the patients was 7 years (range: 1–18 years). Control studies were performed on lymphocytes obtained from the peripheral blood of 9 adult healthy volunteers. The study was approved by the local Ethics Committee and written informed consent from all participants was obtained. Leukemic cells were isolated by Ficoll-Hypaque density gradient separation and resuspended in RPMI-1640 (Sigma Chemical Co, St. Louis, MO, USA) with 20% fetal calf serum (Gibco BRL Life Technologies, UK).

Table 1. Characteristics of patients

Characteristics	Number of patients (n=40)
Sex (male:female)	26:14
Percentage of leukemic cells in bone marrow (median, range)	94 (range: 90–100)
FAB subtype	L1–27, L2–13
Phenotype	precursor-B-lineage: 29, T-lineage-ALL: 11
BCR-ABL rearrangement	present: 3, absent: 37
Cytogenetics*	good risk: 6, poor risk: 5, standard: 29
In vivo prednisolone response	good: 36, poor: 4
Bone marrow early response by day 15**	M1: 33, M2: 6, M3: 1
Bone marrow response by day 33	M1: 40, M2: 0, M3: 0

* Cytogenetics: good risk was defined as hyperdiploidy over 50 chromosomes, DNA index ≥1.16, and translocation t(12:21). Poor risk included: translocation t(9:22), bcr-abl rearrangement, translocation t(4:11), hypodiploidy below 45 chromosomes, DNA index ≤0.95. Standard risk was all others.
** M1 was defined as bone marrow with less than 5% leukemic cells, M2 as bone marrow with leukemic cells in the range of 5–24%, and M3 with ≥25% leukemic cells.

Morphology was evaluated from cytospins with May-Grunwald-Giemsa staining, and viability was assessed by trypan blue dye exclusion. Only fresh leukemic samples were taken for the study and the percentage of blasts in the sample was over 90%.

Drugs

Thalidomide (Gruenenthal GmbH, Aachen, Germany) was dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) and stored at –20°C in stock. Thalidomide was tested in a concentration range of 0.0488–50 μg/ml, while in all experiments of combination with prednisolone or cytarabine it was used at a concentration of 5 μg/ml, which is relatively non-cytotoxic for leukemic cells, with DMSO as a control. Prednisolone (Jelfa, Jelenia Gora, Poland) was used in the concentration range of 0.0076–250 μg/ml and cytarabine (Cytosar, Upjohn) in the range of 0.0097–10 μg/ml.

The MTT viability assay

Cytotoxicity was measured by a viability and cell proliferation assay by measuring the ability of the cells to cleave the soluble compound 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT; Sigma) into an insoluble salt, as previously described [12]. The ability of cells to cleave MTT is indicative of the degree of mitochondrial/cellular respiration within those cells. Cells were incubated for 4 days with various concentrations of the tested drugs or in their combination on 96-well plates. Ten μl of MTT (5 μg/ml) was then added to each well. The reaction was stopped after 4 h of incubation by adding 100 μl of 0.04 N HCl in isopropanol and the optical density was measured at 550 nm (reference wavelength: 720 nm) with a Multiscan Bichromatic plate reader (Asys Hitech GmbH, Eugendorf, Austria) and DigiWin software (Asys Hitech). All experiments were performed in triplicate for each individual patient sample and were done in three runs for each cell line. The data were confirmed to be reproducible, as the median coefficient of variation within an intra-individual run was 4%. The cytotoxicity was expressed as IC_{50}, the inhibitory concentration for 50% of cells. When IC_{50} exceeded the highest tested drug concentration, the percentage of cell-kill caused by this drug was taken into account assuming that the survival of the untreated cells was 100%. The percentage of apoptotic cells in the untreated samples caused by incubation was in the range of 20–75%.

The sensitization factor (SF) was determined as the ratio of IC_{50} for prednisolon/cytarabine and IC_{50} for the respective drug in the presence of thalidomide. For the calculation of SF, all the results of the combination studies were corrected for the cytotoxicity of thalidomide as a single agent by adding the product of the percentage of cells killed by thalidomide in each tested concentration and the difference between the percentage of cells surviving at the tested drug concentration and the com-

bination of the same drug concentration + thalidomide at this concentration to the percentage of cells surviving the combination of drug + thalidomide at each tested concentration.

Cell cycle analysis

For the cell cycle analysis of apoptotic sub-G1 phase and DNA content, cells were stained with hypotonic propidium iodide solution (20 μg/ml, DNA-Prep Kit, Lot no. PN 6607055, Coulter, Miami, FL, USA) and 20,000 events were analyzed with an Epics XL flow cytometer (Coulter) after 24 h of incubation. This flow cytometer is equipped with an argon laser with an excitation wavelength of 488 nm. The cell cycle was calculated by Multicycle software (Phoenix Flow Systems, San Diego, CA). The percentage of cells in the G1, G2, and S phases were expressed as the mean ±SD.

Statistical methods

Results are given as mean values ±SD and also as median values and range. Differences in drug resistance between samples tested for cytotoxicity were analyzed by the Mann-Whitney U-test for unpaired comparisons and by the Wilcoxon signed rank test for paired comparisons. Differences in drug resistance for the cell lines were analyzed by ANOVA. All reported p-values are 2-sided; $p<0.01$ was considered statistically significant.

RESULTS

Cell lines

Thalidomide itself was not cytotoxic in the Jurkat cell line and had a weak cell-kill effect in the CCRF-CEM and Daudi cell lines. In the combination studies, the cytotoxicity of prednisolone in the presence of thalidomide increased 3-fold ($p<0.01$) in cell lines. Sensitization to cytarabine by thalidomide was observed only in the CCRF-CEM and Daudi cell lines (Table 2, Fig. 1).

Compared with untreated cells, thalidomide caused apoptosis in a median of 5% of cells and caused weak cell-cycle arrest in the G0/G1 phase in the CCRF-CEM and Daudi cell lines. In combination with prednisolone it increased the sub-G1 phase (by a median of 6% of cells, $p<0.01$) and increased the cell-cycle arrest in the G0/G1 phase (by a median of 8% of cells, $p<0.01$) caused by prednisolone in the CCRF-CEM and Daudi cell lines (Fig. 1). In combination with cytarabine, thalidomide neither increased the sub-G1 phase nor modulated the cell-cycle arrest in the S phase caused by cytarabine in all the tested cell lines.

Table 2. Sensitization to prednisolone and cytarabine by thalidomide in cell lines

Cell line	Drug resistance (IC_{50}, μg/ml)			Sensitization factor (p-value)	
	prednisolone	cytarabine	thalidomide	Pred + Thal	AraC + Thal
CCRF-CEM	9.3	0.064	30.1	3.2 ($p<0.01$)	2.4 ($p<0.01$)
Jurkat	88.4	0.133	>50 (57%)	3.0 ($p<0.01$)	1.3 ($p>0.05$)
Daudi	34.8	0.980	26.7	3.5 ($p<0.01$)	1.2 ($p<0.01$)

When the IC_{50} value exceeded the upper value of the tested drug concentration, the percentage of cells surviving in the highest concentrations was taken. Each value represents the mean of at least 3 independent experiments. The sensitization factor was calculated as IC_{50} (drug)/IC_{50}(drug + thalidomide), where drug – prednisolone or cytarabine. Pred – prednisolone, AraC – cytarabine, Thal – thalidomide.

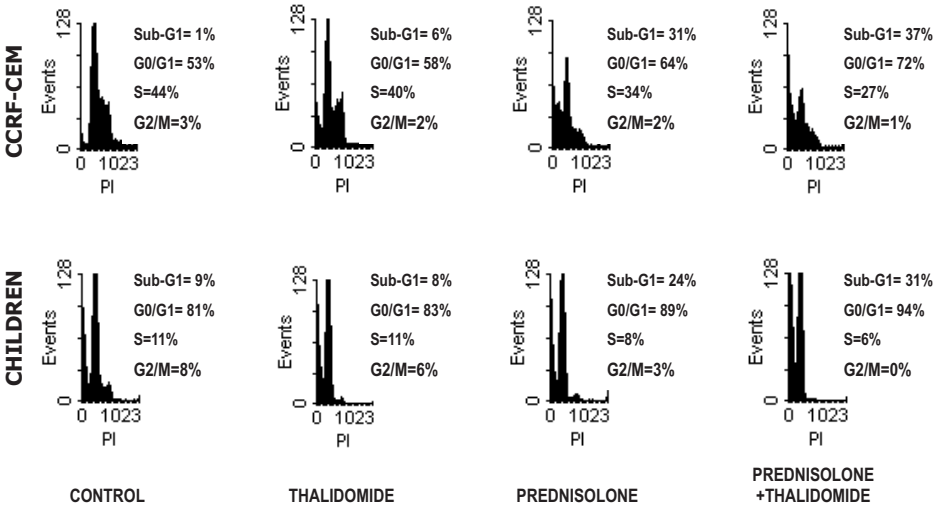

Fig. 1. Cell cycle analysis. DNA content expressed as propidium iodide (PI) staining in the presence/absence of prednisolone or thalidomide in childhood ALL samples and in CCRF-CEM cell line. Apoptotic phase expressed as sub-G1 phase shows median percentage of all analyzed cells. Cell cycle phases are presented as median values obtained from the living cells population according to the rule G0/G1+S+G2/M=100%, as calculated by Multicycle software.

Childhood leukemic samples

In ALL samples the median IC_{50} for thalidomide was 18.7 μg/ml, 44.9 μg/ml for prednisolone, and 1.47 μg/ml for cytarabine. The median value of IC_{50} for the combination prednisolone + thalidomide was 13.4 μg/ml (ie. thalidomide sensitized cells to prednisolone 3.3-fold, $p<0.001$, Fig. 2A) and IC_{50} for the combination cytarabine + thalidomide was 0.54 μg/ml (2.7-fold sensitization, $p=0.002$). No relationship between drug resistance and modulatory effect was observed (Fig. 2B).

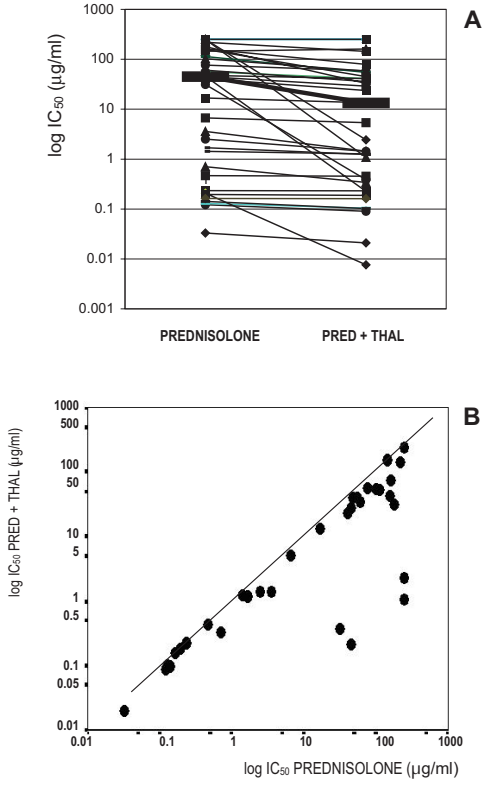

Fig. 2. Sensitization effect. **A** – IC_{50} values for individual ALL patients and their medians (in bold) for prednisolone and its combination with thalidomide. **B** – sensitivity of ALL cells in the presence of prednisolone and prednisolone + thalidomide. A line represents values y=x.

Thalidomide itself caused apoptosis in a median of 8% of cells ($p<0.001$); however it did not influence cell-cycle phases in the childhood leukemic samples. In the combination studies with prednisolone and cytarabine in ALL it increased the percentage of apoptotic cells in the sub-G1 phase (calculated as the differences in the respective phases between experiments with thalidomide + drug and drug alone) by medians of 7 and 5%, respectively ($p<0.001$). Thalidomide increased the cell-cycle arrest in the G1-phase caused by prednisolone by a median of 5% ($p<0.001$; Fig. 1), but had no impact on the cell-cycle arrest caused by cytarabine in the S-phase.

Normal lymphocytes

At the tested concentrations, thalidomide was not cytotoxic to normal lymphocytes. More than 90% of lymphocytes showed viability in the presence of the highest tested thalidomide concentration, with the percentage of apoptotic cells in the control samples being lower than 10%. No effect of thalidomide was observed in the combination studies performed on normal lymphocytes.

DISCUSSION

Leukemia is the most frequent kind of cancer in childhood, comprising about 30% of newly diagnosed patients. ALL is the most common type of leukemia, comprising about 80% of all cases. In spite of the fact that it is one of the best curable childhood neoplasms, still about 20–25% of patients relapse, and the prognosis for these children is poor. There is a need for new anti-leukemic compounds or their combination. In this study we have shown that thalidomide exerted a weak cytotoxic effect on both human lymphoid cell lines and childhood ALL samples. This activity has probably no clinical relevance. However, in combination with prednisolone and cytarabine, thalidomide significantly increased the sensitivity of leukemic cells to these agents in the childhood ALL samples.

The sensitization of lymphoblasts by thalidomide is probably dependent on various mechanisms of its action, leading to higher effectiveness of the cytotoxic activity of prednisolone and cytarabine. This effect was higher for prednisolone than for cytarabine. The modulating effect of thalidomide, detected by the MTT assay, was related to an increase in sub-G1 phase and cell-cycle arrest and was observed both in the childhood samples and the cell lines. Thus the sensitizing effect of thalidomide can be explained mainly by induction of apoptosis. Several other factors can also contribute to this effect. Firstly, thalidomide may play a role by activating nuclear factor (NF)-κB and hsp90 expression as in the myeloma multiplex model [7, 8]. This can potentiate prednisolone cytotoxicity in lymphoblasts, which are regarded as sensitive to prednisolone. The transcription factor NF-κB is one of the key factors in the glucocorticoid intracellular pathway. This can contribute to the modulation of anti-leukemic prednisolone activity in lymphoid cells. Thalidomide is known to enhance the anti-myeloma activity of dexamethasone [3], and the mechanism of the signaling pathway via NF-κB activation plays an important role in MM [9]. Secondly, an important pro-apoptotic pathway can be induced by thalidomide which acts via the caspase-8 pathway [8]. Thus thalidomide can potentiate the apoptosis process started by prednisolone or cytarabine in leukemic cells. Thirdly, reactive oxygen species induced by thalidomide [9] can increase the cytotoxic effect of prednisolone and cytarabine in sensitive cells. Fourthly, VEGF receptors are present on lym-

phoblasts [2]; thus, ALL cells might be a target for thalidomide. As a result, the anti-angiogenic properties of thalidomide can potentiate the cytotoxicity of other drugs. Prednisolone and thalidomide are drugs with anti-inflammatory properties; therefore their combination might lead to potentiation of activity, which was not the case for the combination of thalidomide with cytarabine. This phenomenon might be related to the fact that the effects of thalidomide and prednisolone on cell cycle arrest are similar in the G1 phase, but different in the combination of thalidomide and cytarabine.

While analyzing anti-leukemic effect, it should be noted that thalidomide is known to have many various effector mechanisms [5] not related to direct cytotoxicity, such as its inhibition of angiogenesis and its anti-inflammatory properties. This cannot be evaluated by the MTT assay; however, it might partially influence the results. It should be stressed, however, that the sensitization observed in our study is not necessarily a specific interaction or synergy between thalidomide and the other drugs, but could also be a non-specific effect.

In summary, thalidomide increased *in vitro* the sensitivity of childhood ALL cells to prednisolone and cytarabine as tested by means of the MTT assay. Although thalidomide is a compound which causes a number of side-effects [5], the possibility of sensitizing ALL cells by thalidomide might suggests novel therapeutic strategies, especially for its novel analogues, immunomodulatory drugs such as lenalidomide.

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REFERENCES

1. Dalle J. H., Leblond P., Decouvelaere A., Yakoub-Agha I., Preudhomme C., Nelken B. and Mazingue F. (2003): Efficacy of thalidomide in a child with histiocytic sarcoma following allogeneic bone marrow transplantation for T-ALL. Leukemia, **17**, 2056–2057.
2. El-Obeid A., Sunnuqrut N., Hussain A., Al-Hussein K., Gutierrez M. I. and Bhatia K. (2004): Immature B cell malignancies synthesize VEGF, VEGFR-1 (Flt-1) and VEGFR-2 (KDR). Leuk. Res., **28**, 133–137.
3. Hideshima T., Chauhan D., Shima Y., Raje N., Davies F. E., Tai Y. T., Treon S. P., Lin B., Schlossman R. L., Richardson P., Muller G., Stirling D. I. and Anderson K. C. (2000): Thalidomide and its analogs overcome drug resistance of human multiple myeloma cells to conventional therapy. Blood, **96**, 2943–2950.
4. Johnston R. E. and Abdalla S. H. (2002): Thalidomide in low doses is effective for the treatment of resistant or relapsed multiple myeloma and for plasma cell leukaemia. Leuk. Lymphoma, **43**, 351–354.
5. Matthews S. J. and McCoy C. (2003): Thalidomide: a review of approved and investigational uses. Clin. Ther., **25**, 342–395.
6. Merup M., Kutti J., Birgergard G., Mauritzson N., Bjorkholm M., Markevarn B., Maim C., Westin J., Palmblad J. and Swedish National Study Group on Chronic Myeloproliferative Disorders (2002): Negligible clinical effects of thalidomide in patients with myelofibrosis with myeloid metaplasia. Med. Oncol., **19**, 79–86.
7. Mitsiades N., Mitsiades C. S., Poulaki V., Chauhan D., Richardson P. G., Hideshima T., Munshi N. C., Treon S. P. and Anderson K. C. (2002): Apoptotic signaling induced by immunomodulatory thalidomide analogs in human multiple myeloma cells: therapeutic implications. Blood, **99**, 4525–4530.
8. Mitsiades N., Mitsiades C. S., Poulaki V., Chauhan D., Richardson P. G., Hideshima T., Munshi N., Treon S. P. and Anderson K. C. (2002): Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. Blood, **99**, 4079–4086.
9. Miyachi H., Azuma A., Hioki E., Iwasaki S., Kobayashi Y. and Hashimoto Y. (1996): Inducer-specific bidirectional regulation by thalidomide and phenylphthalimides of tumor necrosis factor-alpha production. Biochem. Biophys. Res. Commun., **224**, 426–430.
10. Munshi N. C., Hideshima T., Carrasco D., Shammas M., Auclair D., Davies F., Mitsiades N., Mitsiades C., Kim R. S., Li C., Rajkumar S. V., Fonseca R., Bergsagel L., Chauhan D. and Anderson K. C. (2004): Identification of genes modulated in multiple myeloma using genetically identical twin samples. Blood, **103**, 1799–1806.
11. Steins M. B., Padro T., Bieker R., Ruiz S., Kropff M., Kienast J., Kessler T., Buechner T., Berdel W. E. and Mesters R. M. (2002): Efficacy and safety of thalidomide in patients with acute myeloid leukemia. Blood, **99**, 834–839.
12. Styczynski J., Wysocki M., Balwierz W. and Kowalczyk J. R. (2002): Dexrazoxane has no impact on sensitivity of childhood leukemic blasts to daunorubicin. Leukemia, **16**, 820–825.
13. Strupp C., Germing U., Aivado M., Misgeld E., Haas R. and Gattermann N. (2002): Thalidomide for the treatment of patients with myelodysplastic syndromes. Leukemia, **16**, 1–6.
14. Richardson P. G., Schlossman R. L., Weller E., Hideshima T., Mitsiades C., Davies F., LeBlanc R., Catley L. P., Doss D., Kelly K., McKenney M., Mechlowicz J., Freeman A., Deocampo R., Rich R., Ryoo J. J., Chauhan D., Balinski K., Zeldis J. and Anderson K. C. (2002): Immunomodulatory drug CC-5013 overcomes drug resistance and is well tolerated in patients with relapsed multiple myeloma. Blood, **100**, 3063–3067.
15. Yaccoby S., Johnson C. L., Mahaffey S. C., Wezeman M. J., Barlogie B. and Epstein J. (2002): Antimyeloma efficacy of thalidomide in the SCID-hu model. Blood, **100**, 4162–4168.