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Cell Transfer Therapy for Cancer: Lessons from Sequential Treatments of a Patient With Metastatic Melanoma

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Summary

The development of effective autologous cell transfer therapies for the treatment of patients with cancer has been difficult, in part because the cells used to treat each patient are different, as are the patient's tumor and immune status. Much can thus be learned by sequential treatments of the same patient with the same cells, making single modifications in the treatments to determine which factors are critical. The authors have treated a single patient with five sequential administrations of the same cells with minor modifications in the mode of administration and the immune status of the patient. The treatment of this patient strongly suggested that 1) the highly avid recognition of tumor antigens in vitro by a transferred lymphocyte population does not necessarily predict in vivo antitumor activity; 2) the administration of highly avid antitumor autologous lymphocyte populations can be far more active in mediating tumor regression in vivo when administered after nonmyeloablative chemotherapy than when administered without this prior chemotherapy; 3) intra-arterial administration of highly avid antitumor autologous lymphocytes into the blood supply of the tumor can be more effective in mediating tumor regression than the intravenous administration of these same tumor infiltrating lymphocytes; 4) one mechanism of tumor escape from immunotherapy is loss of class I MHC antigen expression by the tumor due to mutation of the beta-2 microglobulin gene.

Keywords

immunotherapy; cancer; melanoma

The adoptive immunotherapy of patients with cancer using autologous lymphocytes with antitumor reactivity provides a unique opportunity to define the principles of effective cancer treatment by correlating clinical cancer regression with the functional and phenotypic characteristics of the transferred cells and the immune state of the host. Several clinical trials have demonstrated the ability of adoptive cell transfer to mediate tumor regression in patients with metastatic cancer.^{1,2} Because the genetic and phenotypic characteristics of the tumor, the transferred immune cells, and the host can vary substantially between patients and thus lead

to confusion when data from multiple patients are collectively presented, much can be learned by analyzing the clinical impact of changes in sequential treatments administered to the same patient.

Our patient received similar populations of cells transferred five sequential times with modifications in the mode of delivery as well as the concomitant administration of a nonmyeloablative chemotherapy regimen designed to alter the host milieu into which the cells were given. By using the same cells in the same patient, several principles concerning effective adoptive immunotherapy were suggested and are the subject of this report.

MATERIALS AND METHODS

Patient Treatment

All treatments were approved by the National Cancer Institute (NCI) Investigational Review Board, the Cancer Therapy Evaluation Program of the NCI, and the Food and Drug Administration, and the patient signed an informed consent prior to each treatment. The patient received treatment 1 on NCI protocol 99-C-158 and received all subsequent treatments under a compassionate exemption.

The chemotherapy administered consisted of 2 days of cyclophosphamide at 60 mg/kg body weight followed by 5 days of fludarabine at 25 mg/m² as previously described.² On the day following the final dose of fludarabine, when circulating white cells had dropped to less than 20/mm³, the patient received an intravenous infusion of autologous lymphocytes over approximately 30 to 60 minutes. Interleukin-2 (IL-2) was administered as previously described at a dosage of 720,000 IU/kg by bolus intravenous infusion every 8 hours to tolerance.³

Cells Used for Treatment

The PBL-K3D11 cells were obtained on June 15, 2000, from peripheral lymphocytes by limiting dilution cloning using techniques previously described.^{4,5} In brief, peripheral blood mononuclear cells were stimulated in vitro with the gp100:209-217 (210M) peptide and cloned at two cells per well, using OKT-3 and IL-2 and irradiated feeders. An active microculture was again expanded with OKT-3 and IL-2 twice^{6,7} and used for treatment 1. Aliquots of these treatment 1 cells were cryopreserved and thawed and expanded an additional time in OKT-3 and IL-2 for treatments 2 through 5.

TIL-1837 was grown in complete medium with IL-2 by techniques previously described.^{1,2} A tumor deposit resected on August 2, 2000, was mechanically dispersed (Medimachine; BD Immunocytometry Systems, San Jose, CA), the tumor infiltrating lymphocytes (TILs) were grown for 26 to 36 days, and multiple aliquots were cryopreserved. For treatments 2 through 5 the TILs were thawed and expanded using OKT-3 and IL-2 for 12 to 15 days before infusion. Thus, each TIL culture was exposed to OKT-3 and IL-2 only a single time.

Assays of PBL-K3D11 and TIL-1837

Cytokine release assays of lymphocyte reactivity were performed as previously described.⁸ Aliquots of thawed T cells were cultured overnight with target cells or with T2 cells pulsed with varying concentrations of peptide, and cytokine concentrations were measured by ELISA. T-cell phenotype was determined using two-color fluorescence activated cell analysis with antibodies obtained from Beckman Coulter, Immunotech. Tetramer analysis was performed using HLA-A2/MART-1 or HLA-A2 gp100 tetramers (Beckman Coulter, Immunomics). Immunohistochemical analysis was performed on fineneedle aspirates obtained from tumors.

RESULTS

Clinical Course

Our patient was 27 years old in December 1997 when she developed a 0.8-mm-thick melanoma of the right eyelid. The tumor was excised but recurred in March 1999, and she was treated with a superficial parotidectomy, cervical lymph node dissection, 6,000 cGy to her right face and neck, and bio-chemotherapy consisting of dacarbazine, cisplatin, vinblastine, IL-2, and alpha-interferon. A subsequent recurrence was treated with lomustine chemotherapy, but the disease progressed and she was referred to the NCI in March 2000 with extensive cutaneous and subcutaneous metastases on the face and right neck. Over the next 7 months she was treated sequentially with an experimental peptide vaccine consisting of both class I and class II restricted peptides from the gp100 melanoma-melanocyte antigen and high-dose IL-2, but the disease progressed with extensive fungating metastases in the cutaneous and subcutaneous tissues of her right face and neck. At this time the patient entered the experimental adoptive cell transfer protocols that are the subject of this report.

Between October 1, 2000, and June 5, 2001, the patient was treated with five sequential adoptive cell transfers of autologous lymphocytes reactive with melanoma antigens. Each time she received the same high-dose IL-2 regimen she had previously received. The patient received two cell populations. The PBL-K3D11 cell population was derived from peripheral blood lymphocytes and the TIL-1837 population was derived from tumor infiltrating lymphocytes. The details of these five treatments and their clinical impact are shown in Table 1.

In Vitro Reactivity of Lymphocytes

The immunologic reactivity of PBL-K3D11 and TIL-1837 against gp100 and MART-1 peptides, the autologous melanoma 1910-mel (grown from a biopsy on March 13, 2001) and autologous EBV-B cells, as well as HLA-A2⁺ and HLA-A2⁻ allogeneic melanomas, for each of the five treatments is shown in Table 2. The phenotypic characteristics of the cells and their reactivity with gp100:209-217 and MART-1:26-35 (27L) HLA-A2⁺ tetramers are shown in Tables 3 and 4, respectively.

In the first treatment the patient received the PBL-K3D11 that had highly avid reactivity against the gp100:209-217 peptide, the autologous melanoma 1910-mel, and matched HLA-A2⁺ melanomas and no reactivity against autologous EBV-B cells or HLA-A2⁻ melanomas. In treatments 2 through 5, the PBL-K3D11 cells were also administered but were given in conjunction with TIL-1837, which reacted with the MART-1:26-35 peptide and similarly had reactivity against HLA-A2⁺ melanomas and the autologous 1910-mel but not autologous EBV-B cells.

The PBL-K3D11 used for treatment 1 was almost exclusively CD8⁺, although with the additional expansion using OKT3 and IL-2 required to generate enough cells for treatments 2 through 5, a CD4⁺ population appeared. PBL-K3D11 reacted exclusively with the gp100:209-217 tetramer and TIL-1837 reacted only with the MART-1:26-35 (27L) tetramer. Although minor variations in the phenotypic and functional characteristics existed in treatments 2 through 5, each of the populations contained sufficient numbers of cells of each phenotype and function to make it unlikely that clinical impact was due to these minor variations.

Following tumor stimulation in vitro, PBL-K3D11 and TIL-1837 each produced interferon-gamma, GM-CSF, and low levels of tumor necrosis factor-alpha but produced no IL-2, IL-4, or IL-10. TIL-1837 also produced low levels of RANTES cytokine (data not shown).

Clinical Impact of Treatments

Treatment 1—The first treatment, administered October 31, 2000, consisted of cyclophosphamide and fludarabine chemotherapy followed by the adoptive transfer of 1.6×10^{10} PBL-K3D11 lymphocytes, which recognized the patient's melanoma in vitro as well as the native gp100:209-217 peptide at 1 picomolar, the highest avidity we have seen in a human T cell. This treatment resulted in little to no cancer regression (Fig. 1A). Thus, the avidity of lymphocyte reactivity in vitro against a melanoma antigen is not sufficient to predict clinical effectiveness upon adoptive transfer. The reason for lack of in vivo effectiveness of these cells remains unclear. The PBL-K3D11 was derived from peripheral blood lymphocytes and thus may not have had the appropriate homing receptors necessary to reach and infiltrate tumor deposits. PBL-K3D11 was generated by limiting dilution cloning and thus had been subjected to three rounds of in vitro expansion with OKT3 and high-dose IL-2; its proliferative potential may have been compromised by this extensive culturing.

Treatments 2 and 3—Because of extensive tumor progression, the patient received a second treatment with the PBL-K3D11 cells on November 29, 2001, but in addition received 3.1×10^{10} TIL-1837, which recognized one nanomolar of the native MART-1:27-35 melanoma peptide. This treatment caused a regression of several nodules on the face, anterior and posterior to the ear, but had no impact on the bulk of the patient's disease in the neck, which continued to progress (see Fig. 1B). Because of this progression and the hypothesis that these same cells might be more active when given in conjunction with nonmyeloablative chemotherapy, on January 22, 2001, the patient received treatment 3, consisting of both the PBL-K3D11 and TIL-1837 cells 1 day after completing 7 days of chemotherapy with cyclophosphamide and fludarabine (the same chemotherapy given with treatment 1). Following this treatment the patient experienced a rapid regression of disease in the upper neck as well as an ongoing regression of the lesions on the face (see Fig. 1C). Thus, the administered cells, in conjunction with the nonmyeloablative chemotherapy, had a substantially greater impact than the cells administered in the absence of the chemotherapy. Because the identical chemotherapy administered in treatment 1 had no impact and the same cells administered in treatment 2 had minimal impact, this improved treatment appeared to be due to the combination of the cells plus the alteration of the host immunologic environment by the nonmyeloablative chemotherapy.

Treatment 4—Following the dramatic regression of melanomas on the face and upper neck after the third treatment, a metastasis on the lower neck continued to grow and formed a large ulcerating lesion above the clavicle (see Fig. 1C). Since this was an irradiated area, we hypothesized that the transferred cells that mediated the regression of the remaining melanoma may not have reached this area in sufficient amounts. Thus, on March 22, 2001, the patient received a fourth treatment with the same PBL-K3D11 and TIL-1837 that she had received on January 22, 2001, but this time the cells were infused into the thyrocervical artery and the superior thyroid artery to concentrate the cells at the location of the lower neck lesions. The blood supply of the tumor and the concentration of the cells to this area were confirmed by angiography (Fig. 2A) and computed axial tomography (see Fig. 2B). Following this intra-arterial infusion, a dramatic regression of the lower neck metastasis occurred and all of the patient's tumors in the face and neck also continued to regress (see Fig. 1D). This fourth treatment indicated that the introduction of the reactive lymphocytes into the blood supply of the tumor was more effective than the intravenous administration of the TILs and implied that traffic of lymphocytes to the tumor was a limiting factor in mediating regression of the lesion.

Treatment 5—The patient's tumor continued to regress, but at the beginning of June 2001 new tumors appeared in the lower neck. The patient received a fifth and final treatment with intra-arterial administration of the same combination of PBL-K3D11 and TIL-1837 previously

given, but no anti-tumor regression was seen. Progressing tumors were biopsied on August 2 and September 6, 2001, and immunohistochemical studies revealed that although the tumors retained expression of MART-1 and gp100 antigens, they had lost expression of HLA-A2 antigens (Table 5). In contrast, all four prior fine-needle aspirates of the patient's tumor between August 2000 and March 2001 had shown positive staining for the HLA-A2 antigen. In contrast to the autologous 1910-mel cell line obtained on March 13, 2001, an autologous tumor line, 1956-mel grown from the biopsy obtained on August 2, 2001, expressed no MHC class I antigens by FACS analysis and was not susceptible to recognition by either the PBL-K3D11 or TIL-1837 cells (see Table 1). PCR studies of this 1956-mel cell line revealed a T-to-G point mutation in codon 7 of the beta-2 microglobulin gene, resulting in a stop codon. No normal beta-2 microglobulin was produced, and thus MHC class I antigen expression was lost. Semiquantitative RT-PCR analysis revealed that both 1910-mel and 1956-mel expressed both the gp100 and MART-1 antigens.

DISCUSSION

Destruction of syngeneic tumor in experimental systems is initiated by the recognition of cell surface antigens by activated T lymphocytes. Adoptive cell transfer is an approach to cancer immunotherapy that enables the administration of large numbers of selected, highly avid, functionally characterized, and previously activated antitumor lymphocytes into a host preconditioned to optimally sustain cell survival and proliferation. Because autologous cells are administered, each cancer patient receives a different cell population, often with reactivity to antigens that can differ with respect to specificity, effector function, and avidity.^{1,2} Thus, it is often difficult to draw conclusions from summaries of data from multiple patients. Some of these problems of interpretation can be overcome by the administration of multiple sequential treatments to the same patient, each with minor modifications, to determine which modification results in cancer regression *in vivo*. The multiple treatments administered to our patient during her difficult clinical course have helped to suggest critical factors involved in developing cell transfer therapies.

The first treatment administered to our patient involved the administration of an oligoclonal, highly CD8⁺-enriched population originally derived from limiting dilution of PBLs from this immunized patient. These PBL-K3D11 cells exhibited high recognition of the patient's melanoma and recognized the gp100:209-217 peptide at 1 picomolar concentration. This treatment did not mediate *in vivo* tumor regression, in accord with published pilot trials in which patients with metastatic melanoma received cloned CD8⁺ gp100 reactive T cells without clinical impact.^{5,9,10} The cyclophosphamide and fludarabine chemotherapy that preceded this adoptive cell transfer is commonly used in patients receiving hematopoietic allografts¹¹ and was administered based on murine studies that demonstrated that cell transfer therapy was most effective when administered to an immunosuppressed host.¹² This chemotherapy causes a severe lymphopenia, neutropenia, and thrombocytopenia, which begins to recover after approximately 7 days.^{2,9} Of interest, our patient tolerated 12 doses of high-dose IL-2 with minimal toxicity, a number of doses rarely tolerated by patients with intact immune systems containing CD4 cells,¹³ thus strongly suggesting that the toxicity seen with high-dose IL-2 is largely due to secondary cytokines produced by host cells rather than a direct impact of IL-2 alone. Cyclophosphamide and fludarabine¹⁴ have minimal to no impact on the growth of melanomas, and the lack of clinical response after this treatment strongly suggested that the response seen in subsequent treatments was not due to these chemotherapeutic agents.

In the second and third treatments, the patient received PBL-K3D11 but also received a highly avid TIL population with recognition of the MART-1 antigen, albeit with a lower avidity (recognition of 1 nanomolar peptide) than PBL-KD311. This TIL population contained roughly equal numbers of CD4⁺ and CD8⁺ cells. The majority of the patient's cancer did not respond

to the cell transfer alone (treatment 2) (see Figs. 1A and B) but did regress when these same cell populations were administered in conjunction with cyclophosphamide and fludarabine chemotherapy (treatment 3) (see Fig. 1C). Because the cells alone in treatment 2 and the chemotherapy in treatment 1 each had no impact, we concluded that the effectiveness of the cells administered in treatment 3 was dependent on the prior immunosuppression of the host. Since in treatment 1 and in our previous studies⁹ we had seen no cancer regression in patients who received cloned CD8⁺ cells in conjunction with the same chemotherapy, it also strongly suggested that the heterogeneity of the CD8 cells, the presence of CD4 cells, and the derivation of cells from the tumor versus the blood were important factors in the therapeutic impact of treatment 3.

To mediate effective cancer regression, cells need appropriate reactivities but also must traffic to tumor deposits.¹⁵ Cells administered in treatment 3 had substantial antitumor impact in the upper neck, but a metastasis in the lower neck continued to grow (see Fig. 1C). The patient's previous irradiation to her neck may have altered tumor vascularity, accounting for the different distribution of cells to these tumor areas. Angiographic studies demonstrated that the tumor in the lower neck was supplied predominantly by the thyrocervical and the superior thyroid arteries (see Fig. 2) and thus, in an attempt to increase the cell traffic to these areas, the patient received treatment with the same cells used for treatment 3 injected intra-arterially into these vessels. This treatment resulted in major regression of all remaining tumor (see Fig. 1D). The intra-arterial treatment given in treatment 4 was not associated with additional chemotherapy, although following the two prior administrations of cyclophosphamide and fludarabine, the patient remained lymphopenic and immunosuppressed prior to treatment 4.

Consistently through the patient's course, tumor biopsies exhibited the expression of both MART-1 and gp100 antigens as well as the HLA-A2 class I MHC antigen. When tumors progressed in this patient about 2 months after treatment 4, we again administered cells intraarterially, although no therapeutic impact was seen. Biopsies of these progressing tumors revealed that the tumor had lost expression of HLA-A2 and a tumor cell line grown from these biopsies, 1956-mel, had lost expression of all class I MHC antigens. The regulation of tumor antigens or MHC class I antigens has previously been described as a mechanism of tumor escape from immune attack.^{16,17} The patient refused surgical resection of the growing nodules in the lower neck, and she died of progressive melanoma December 16, 2001.

Any conclusions drawn from studies of a single patient must be made with considerable caution. Minor variations in the characteristics of cells as they grow can introduce unknown variables, as can changes in the tumor during the patient's course. Despite these caveats, the treatment of this patient suggested several hypotheses worthy of further evaluation in additional patients. Strong in vitro reactivity did not necessarily translate to in vivo reactivity of these cells. Immunosuppressive chemotherapy could enhance the antitumor effects of transferred cells. Increased delivery of lymphocytes to the tumor site could be beneficial. The escape of tumor by downregulation of MHC class I antigens is stimulating efforts to develop natural killer cell populations that may be capable of mediating antitumor effects independent of the expression of MHC class I antigens.

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FIGURE 1.

Photographs of the lower face and neck of our patient. **(A)** Photo taken on November 29, 2000, after the administration of chemotherapy plus the PBL-K3D11 and immediately prior to the administration of TIL-1837. **(B)** Photo taken on January 16, 2001, demonstrating dramatic progression of tumor in the neck following the intravenous administration of PBL-K3D11 and TIL-1837. **(C)** Photo taken on March 22, 2001, 2 months after the intravenous administration of cells in conjunction with nonmyeloablative chemotherapy, demonstrating dramatic regression of tumor in the upper but not lower neck. **(D)** Ongoing regression in the face and upper neck and dramatic regression of tumor in the lower neck following the intra-arterial administration of PBL-K3D11 and TIL-1837 into the thyrocervical artery and superior thyroid arteries.

FIGURE 2.

(A) Angiographic appearance of the blood supply to the tumor of our patient following injection into the thyrocervical artery and the superior thyroid artery. A tumor blush was seen at the site of the tumor. (B) Post angiogram computerized axial tomographic scan demonstrating distribution of dye to the site of the ulcerating tumor in the lower neck.

Table 1

Oligonucleotides used in PCR for site-directed mutagenesis of P450 2B4dH/H226Y.

Mutants	Oligonucleotides
V292A	5'-ATCCTCACCGCGCTCTCGTC-3' 5'-GAGCGAGAGC <u>CGGT</u> GAGGAT-3'
F296A	5'-TGCTCTCGCTCGCCTTCGCCGGCACC-3' 5'-GGTGCCGGCGAAGGCGAGCGAGAGCA-3'
T302A	5'-GCACCGAGGCCACCAGCAC-3' 5'-GTGCTGGTGGCCTCGGTGC-3'
I363A	5'-GGACCTCGCCCCCTTCGG-3' 5'-CCGAAGGGGGCGAGGTCC-3'
V367A	5'-TTCGGGGCGCCCCACACG-3' 5'-CGTGTGGGGCGCCCCGAA-3'
V367L	5'-TTCGGGTGCCCCACACGGTC-3' 5'-GACCGTGTGGGGCAACCCGAA-3'
V477A	5'-GAGAGTGGCGGGCAACGT-3' 5'-ACGTTGCCCCGCGCACTCTC-3'
V477F	5'-GAGAGTGGCTTCGGCAACGTGC-3' 5'-GCACGTTGCCAAGCCACTCTC-3'

The nucleotides changed to make the desired mutation are underlined.

Table 2

Inhibition of P450 2B4dH/H226Y and mutants by 4-CPI and BIF: determination of IC₅₀ using a 7-EFC O-deethylation assay.

P450	BIF (μM)	IC ₅₀ 4-CPI (μM)	BIF/4-CPI
2B4dH/H226Y	0.55 ± 0.02	0.010 ± 0.003	55
V292A	0.52 ± 0.02	0.011 ± 0.001	47
F296A	0.88 ± 0.09	0.022 ± 0.008	40
T302A	0.41 ± 0.09	0.008 ± 0.000	51
I363A	0.36 ± 0.01	0.027 ± 0.005	13
V367A	0.47 ± 0.03	0.007 ± 0.001	67
V367L	0.56 ± 0.01	0.012 ± 0.002	47
V477A	0.61 ± 0.10	0.011 ± 0.003	55
V477F	0.85 ± 0.16	0.015 ± 0.002	56

Results are the mean ± standard deviation of at least three independent experiments.

Table 3

Steady state kinetic analysis of 7-EFC O-deethylation by P450 2B4dH/H226Y and mutants.

P450	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m
2B4dH/H226Y	9.9 (0.7) ^a	108 (14)	0.09
V292A	11 (0.7)	102 (12)	0.11
F296A	5.0 (0.6)	73.3 (18)	0.07
T302A	1.3 (0.1)	67.8 (10)	0.02
I363A	5.3 (0.6)	81.8 (18)	0.07
V367A	1.8 (0.1)	284 (90)	0.01
V367L	8.3 (0.3)	67.0 (5.6)	0.12
V477A	4.7 (0.3)	85.7 (9.2)	0.05
V477F	9.5 (0.3)	42.6 (3.2)	0.22

Results are the representative of at least two independent determinations. The variation between the experiments is $\leq 20\%$.

^aStandard errors for fit to Michaelis-Menten are shown in parenthesis.

Table 4
Testosterone hydroxylation by 2B4dH/H226Y and mutants at 200 μM substrate.

	Turn over (nmol/min/nmol P450)			Stereoselectivity		Regioselectivity	
	16α-OH	16β-OH	2α-OH	16α-OH:16β-OH	16-OH:2-OH		
P450							
2B4dH/H226Y	0.43	0.06	0.17	7.2	2.9	16-OH:2-OH	
V292A	0.26	ND	0.13	ND	2.0		
F296A	0.44	ND	0.34	ND	1.3		
T302A	0.01	0.03	ND	0.3	ND		
I363A ^a	3.7	1.4	0.51	2.7	10		
V367A	0.17	0.31	0.07	0.6	6.9		
V367L	0.07	ND	0.09	ND	0.80		
V477A	ND	ND	ND	ND	ND		
V477F	0.08	0.01	0.46	8.0	0.19		

Results are the mean of at least three determinations. The standard error of mean (SEM) was approximately ± 25% of the mean values. SEM was not indicated in the Table to enhance the clarity.
^aI363A also catalyzes P450 testosterone 15α-hydroxylation (12 nmol/min/nmol P450), whereas P450 2B4dH/H226Y showed non-determinable (ND) testosterone 15α-hydroxylase activity.

Table 5
Steady state kinetic analysis for testosterone hydroxylation by P450 2B4dH/H226Y and mutants.

P450	16 α -OH		16 β -OH		15 α -OH	
	k _{cat} (min ⁻¹)	K _m (μM)	k _{cat} (min ⁻¹)	K _m (μM)	k _{cat} (min ⁻¹)	K _m (μM)
2B4dH/H226Y	0.7 (0.1) ^a	175 (38)	--	--	--	--
F296A	1.0 (0.2)	96.0 (35)	--	--	--	--
I363A	6.6 (0.3)	30.4 (4.3)	2.0 (0.1)	32.6 (3.2)	12.0 (0.7)	28.4 (5.5)
V367A	--	--	0.9 (0.2)	180 (67)	--	--

Results are the representative of at least two independent determinations. The variation between the experiments is ≤ 20%.

^aStandard errors for fit to Michaelis-Menten are shown in parenthesis