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Initial Testing of Lenalidomide by the Pediatric Preclinical Testing Program

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Abstract

Background—Lenalidomide, a novel immunomodulatory agent, is reported to modulate stem cell differentiation, and have direct antiproliferative activity as well as inhibit inflammation and hyperalgesia. On the basis of this varied pharmacological profile, lenalidomide is under investigation as a treatment for a range of oncologic indications.

Procedures—Lenalidomide was evaluated against the PPTP *in vitro* panel using 96 hour exposure at concentrations ranging from 1 nM to 10 μ M. It was tested against the PPTP *in vivo* panels at a dose of 30 mg/kg administered orally (PO) oncedaily for a planned for 6 weeks.

Results—*In vitro* activity was not observed at concentrations up to 10 μ M. Lenalidomide was well tolerated, and induced significant differences in EFS distribution compared to control in 7 of 37 (18.9%) of the evaluable solid tumor xenografts and in 0 of 8 (0%) of the evaluable ALL xenografts. The best response in the solid tumor panel was PD2 [progressive disease with growth delay (EFS T/C > 1.5)], observed in 4 of 37 (10.8%) solid tumor xenografts. A single ALL xenograft showed a PD2 response.

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Conclusions—Direct antiproliferative effects of lenalidomide were not observed *in vitro*. *In vivo* lenalidomide demonstrated low activity against tumors in immune-deficient mice. Our results suggest that lenalidomide's utility in the pediatric clinical setting may depend upon its ability to induce antitumor activity through effects on host immune and stromal cells rather than through direct effects on tumor cells.

Keywords

Preclinical Testing; Developmental Therapeutics; Lenalidomide

INTRODUCTION

Lenalidomide is the lead compound in a new class of immunomodulatory drugs characterized by a wide range of effects, including the enhancement of T cells and natural killer (NK) cell activity, inhibition of angiogenesis and tumor cell proliferation, modulation of stem cell differentiation, and inhibition of inflammation and hyperalgesia [1,2]. On the basis of this varied pharmacological profile, lenalidomide is under investigation as a treatment for a range of oncologic indications.

The direct antiproliferative activity of lenalidomide has been demonstrated *in vitro* using various hematopoietic tumor cell lines, including multiple myeloma, Burkitt lymphoma, acute myeloid leukemia (AML), and non-Hodgkin's lymphoma (NHL) [3]. Lenalidomide had no direct antiproliferative effect against a chronic lymphocytic leukemia (CLL) cell line *in vitro*, and potentiation of T and NK cell-mediated cytotoxicity against CLL *in vivo* has been postulated as a mechanism of action of lenalidomide in CLL clinical studies [4,5]. *In vivo* tumor growth models have demonstrated that lenalidomide inhibits growth of multiple myeloma cells [6].

The antiangiogenic activity of lenalidomide has been shown using human endothelial cells and arterial explant models *in vitro*, and inhibition of angiogenesis by lenalidomide is postulated as the mechanism of reduced growth of solid tumors [7]. Lenalidomide, in combination with sorafenib, inhibited angiogenesis *in vitro* and reduced tumor growth in an ocular melanoma xenograft model [8]. Lenalidomide, in combination with sunitinib and cyclophosphamide, also inhibited angiogenesis *in vitro* and effectively blocked tumor growth in ocular melanoma, colon cancer, pancreatic cancer, and cutaneous melanoma models [9].

The Food and Drug Administration (FDA) approved lenalidomide for the treatment of patients with transfusion-dependent anemia due to low- or intermediate-1-risk myelodysplastic syndromes (MDS) associated with a deletion 5q cytogenetic abnormality with or without additional cytogenetic abnormalities. Subsequently, lenalidomide in combination with dexamethasone was approved for the treatment of patients with multiple myeloma who received at least one prior therapy. Phase 2 studies have also demonstrated that lenalidomide has activity in non-Hodgkin lymphoma [10] and chronic lymphocytic leukemia [11]. Lenalidomide was evaluated against the *in vitro* and *in vivo* panels of childhood tumor xenografts of the PPTP.

MATERIALS AND METHODS

In vitro testing

In vitro testing was performed using DIMSCAN, a semiautomatic fluorescence-based digital image microscopy system that quantifies viable (using fluorescein diacetate [FDA]) cell numbers in tissue culture multiwell plates [12]. Cells were incubated in the presence of sorafenib for 96 hours at concentrations from 1.0 nM to 10.0 μ M and analyzed as previously described [13].

In vivo tumor growth inhibition studies

CB17SC-M *scid*^{-/-} female mice (Taconic Farms, Germantown NY), were used to propagate subcutaneously implanted kidney/rhabdoid tumors, sarcomas (Ewing, osteosarcoma, rhabdomyosarcoma), neuroblastoma, and non-glioblastoma brain tumors, while BALB/c nu/nu mice were used for glioma models, as previously described [13-16]. Human leukemia cells were propagated by intravenous inoculation in female non-obese diabetic (NOD)/*scid*^{-/-} mice as described previously [17]. Female mice were used irrespective of the patient gender from which the original tumor was derived. All mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee of the appropriate consortium member. Ten mice (solid tumors) or eight mice (leukemias) were used in each control or treatment group. Tumor volumes (cm³) [solid tumor xenografts] or percentages of human CD45-positive [hCD45] cells [ALL xenografts] were determined as previously described [13] and responses were determined using three activity measures as previously described [13].

Determination of response

Responses were determined using three activity measures as previously described [13]. For individual mice, progressive disease (PD) was defined as < 50% regression from initial volume during the study period and > 25% increase in initial volume at the end of study period. Stable disease (SD) was defined as < 50% regression from initial volume during the study period and $\leq$ 25% increase in initial volume at the end of the study. Partial response (PR) was defined as a tumor volume regression $\geq$ 50% for at least one time point but with measurable tumor ($\geq$ 0.10 cm³). Complete response (CR) was defined as a disappearance of measurable tumor mass ($<$ 0.10 cm³) for at least one time point. A complete response was considered maintained (MCR) if the tumor volume was $<$ 0.10 cm³ at the end of the study period. For treatment groups only, if the tumor response was PD, then PD was further classified into PD1 or PD2 based on the tumor growth delay (TGD) value. TGD values were calculated based on the numbers of days to event. For each individual mouse that had PD and had an event in the treatment groups, a TGD value was calculated by dividing the time to event for that mouse by the median time to event in the respective control group. Median times to event were estimated based on the Kaplan-Meier event-free survival distribution. If a mouse had a TGD value $\leq$ 1.5, that mouse was considered PD1. If the TGD value was $>$ 1.5, the mouse was considered PD2. Mice that had PD but did not have an event at the end of the study were coded as PD2. An in-depth description of the analysis methods is included in the **Supplemental Response Definitions** section.

Statistical Methods

The exact log-rank test, as implemented using Proc StatXact for SAS®, was used to compare event-free survival distributions between treatment and control groups. P-values were two-sided and were not adjusted for multiple comparisons given the exploratory nature of the studies.

Drug Information and Formulation

Celgene, through the Cancer Therapy Evaluation Program (NCI), provided lenalidomide to the Pediatric Preclinical Testing Program. Lenalidomide was prepared as a suspension in 1% (w/v) carboxymethylcellulose in sterile water for injection, USP (1% CMC, medium viscosity). Suspensions were stored at 4°C and used within 8 days of preparation, and stirred for 30 min at room temperature prior to dosing. The agent was administered by oral gavage at a dose of 30 mg/kg daily for a planned treatment duration of 6 weeks. Lenalidomide was provided to each consortium investigator in coded vials for blinded testing.

RESULTS

Lenalidomide *in vitro* testing

The 23 cell lines of the PPTP *in vitro* panel were evaluated for sensitivity to lenalidomide over a range of 1 nM to 10 µM. Even at the highest concentration lenalidomide exhibited little or no *in vitro* cytotoxicity or growth inhibition against any cell line (**Table I**).

Lenalidomide *in vivo* testing

Lenalidomide was tested against the PPTP solid tumor xenografts using a dose of 30 mg/kg administered by oral gavage daily for a planned treatment duration of 6 weeks. The dose was selected because it should produce systemic exposures in the range of those achieved in pediatric phase 1 trials at a dose of 70 mg/m² (a dose that was well-tolerated in children with cancer). In mice a dose of 50 mg/kg produced a lenalidomide area under the curve (AUC) systemic exposure of 30,000 ng/ml*hr [18], while in children a dose of 70 mg/m² produced a lenalidomide AUC of 11,750 ng/ml*hr [19]. Thus, the 30 mg/kg dose used by the PPTP is estimated to produce an AUC that is approximately 50% higher than that achieved in the clinic in children at the 70 mg/m² dose, making it an appropriate dose to use for *in vivo* testing.

Lenalidomide was very well tolerated at the 30 mg/kg dose with death of 5 of 418 control animals (1.2%) and 4 of 427 (0.9%) of treated animals. All 45 tested xenograft models were considered evaluable for efficacy. Complete details of testing are provided in Supplemental Table I, including total numbers of mice, number of mice that died (or were otherwise excluded), numbers of mice with events and average times to event, tumor growth delay, as well as numbers of responses and T/C values.

Lenalidomide induced significant differences in event-free survival (EFS) distribution compared to controls in 7 of 37 (18.9%) of the evaluable solid tumor xenografts and in 0 of 8 (0%) of the evaluable ALL xenografts **Table II**. For those xenografts with a significant difference in EFS distribution between treated and control groups, the EFS T/C activity

measure additionally requires an EFS T/C value of > 2.0 for intermediate activity and indicates a substantial agent effect in slowing tumor growth. High activity further requires a reduction in final tumor volume compared to the starting tumor volume. Lenalidomide did not induce tumor growth inhibition meeting criteria for intermediate or high EFS T/C activity in any of the solid tumor or leukemia xenografts studied. No xenografts achieved an objective response, with the best response in the solid tumor panel being PD2 [progressive disease with growth delay (EFS T/C > 1.5)], which was observed in 4 of 37 (10.8%) solid tumor xenografts. A single ALL xenograft showed a PD2 response.

DISCUSSION

Lenalidomide demonstrated only modest activity consisting of tumor growth delay against both solid tumor and leukemia models in this study. However, It is useful to consider the PPTP results in light of the clinical activity profile that lenalidomide has demonstrated against adult cancers. Lenalidomide has proven activity against multiple B-cell malignancies including multiple myeloma [20-22], chronic lymphocytic leukemia (CLL) [23], mantle-cell lymphoma [24,25], diffuse large B-cell lymphoma [24], grade 3 follicular lymphoma [10,24], and small lymphocytic lymphoma [10]. Lenalidomide has direct effects on these B-cell malignancies as illustrated by its *in vitro* inhibition of multiple myeloma cell proliferation and by its ability to increase expression in these myeloma cells of tumor suppressor genes such as p21waf1/cip1, SPARC, IRF8, ING4 and p57 kip2 [26]. Additionally, lenalidomide induces upregulation of B-cell activation markers such as CD40 and CD86 in CLL cells exposed *in vitro* to concentrations in the 0.5 to 1 μ M range [27]. Lenalidomide also promotes up-regulation of CD154 antigen on the surface of CLL cells at concentrations attainable in the clinic, which leads to increased IgM and IgG production by normal B cells and to upregulation of BIM, DR5, and p73 [28]. Lenalidomide has additionally shown preliminary evidence of activity in a phase 2 trial for T-cell lymphomas, with partial responses noted for anaplastic, angioimmunoblastic, and PTCL-u histologies [29].

Lenalidomide is highly active for patients with lower-risk, transfusion-dependent MDS that harbors deletion at chromosome 5q31 [30,31]. Lenalidomide is also active in patients with higher risk MDS and isolated del(5q), but shows little activity if additional cytogenetic lesions are present [32]. Complete remissions to lenalidomide have additionally been achieved in patients with AML and isolated del(5q) [33-36]. Lenalidomide shows lower levels of activity in transfusion-dependent MDS patients without del(5q), with only 26% achieving transfusion-independence and with infrequent cytogenetic improvement [37]. Lenalidomide is also active against del(5q)-associated primary myelofibrosis and post-polycythemia vera myelofibrosis [38].

The activity of lenalidomide for patients with hematologic malignancies with del(5q) may be the result of its effect on the dual specificity phosphatases Cdc25C and PP2A α (coregulators of the G(2)-M checkpoint), which are within the common deleted region (CDR) for the del(5q) phenotype [39]. Lenalidomide directly inhibits phosphatase activity of Cdc25C and indirectly inhibits the phosphatase activity of PP2A. Treatment of del(5q) AML cells, but not nondel(5q) AML cells, with lenalidomide induces G(2) arrest and apoptosis.

Small interfering RNA (shRNA) suppression of Cdc25C and PP2A α gene expression induces susceptibility to lenalidomide in both U937 and primary nondel(5q) MDS cells [39]. These data suggest a role for allelic haploinsufficiency of the lenalidomide-inhibitable Cdc25C and PP2A α phosphatases in the selective drug sensitivity of del(5q) MDS. Consistent with a role for these two phosphatases in response to lenalidomide, secondary resistance to the agent in del(5q) MDS is associated with over-expression and activation of the Cdc25C and PP2A [40]. Another gene within the CDR on which lenalidomide shows a specific effect is SPARC, a protein with antiproliferative, antiadhesive, and antiangiogenic activity. Lenalidomide induces up-regulation and increased protein expression of SPARC [41].

There are no obvious analogies in the pediatric setting to the adult cancers described above, as del(5q) is not commonly observed in pediatric malignancies. However, in light of the adult experience indicating activity of lenalidomide against a range of adult lymphoid malignancies, the limited activity of lenalidomide against the PPTP ALL xenografts is disappointing. This lack of activity may reflect the less mature stage in B-cell and T-cell development of the ALL xenografts in comparison to that of the adult malignancies in which responses have been observed. While the PPTP testing methods would identify direct anticancer effects for lenalidomide, the PPTP *in vivo* testing procedures could miss activity that was dependent upon lenalidomide's effects on host immune cells and stromal cells that are not replicated in the SCID and NOD-SCID mouse models used by the PPTP. These effects include inhibiting the production of pro-inflammatory cytokines (e.g., TNF- α , IL-6, IL-1, etc.), promoting T-cell activation and proliferation, and augmenting NK cell function [42]. As the NOD-SCID mice, which serve as hosts for the ALL xenografts, lack functional B- and T-cells and have reduced (though not absent) NK cell activity [43,44], lenalidomide responses that are dependent upon any of these cell types may be reduced or absent in NOD-SCID hosts. SCID mice, which served as hosts for most of the PPTP solid tumor models, also lack functional B- and T-cells, but have robust NK cell activity that can be enhanced by lenalidomide [45,46]. Given the substantial activity of lenalidomide against a range of adult lymphoid malignancies, testing in the clinical setting for patients with relapsed/refractory ALL the hypothesis that lenalidomide's anti-leukemia effects may be dependent upon stromal or immune cells not replicated in the PPTP models may be warranted.

Given the lack of single agent activity observed for lenalidomide against the pediatric preclinical models tested, opportunities for the use of lenalidomide in combination with other agents might be considered. One relevant effect of lenalidomide is its ability to enhance antibody-dependent cellular cytotoxicity (ADCC) [47]. The stimulation of ADCC by lenalidomide appears to be particularly relevant to natural killer (NK) cell-mediated cytotoxicity [48]. The primary effector cells for ADCC in peripheral blood are CD56^{low}CD16⁺ NK cells [49]. They express high levels of the low-affinity Fc receptor for IgG (Fc γ RIII; also known as CD16) that allows them to recognize antibody-coated target cells and mediate ADCC [26]. Lenalidomide is able to stimulate an increase in CD16 expression by CD56^{low}CD16⁺ NK cells [50,51]. Lenalidomide enhances ADCC mediated by antibodies directed against multiple antigens, including CD20 [51], CD40 [48,52], CD19 [53] and CS1 (a cell surface glycoprotein, which is highly and uniformly expressed in multiple myeloma) [54]. The pediatric phase 1 trial of lenalidomide using a once daily for

21 of 28 days schedule explored doses from 15 to 70 mg/m²/day [19], with lenalidomide being well tolerated at all dose levels. The most common histologies enrolled were non-rhabdomyosarcoma soft tissue sarcoma (n=11) and osteosarcoma (n=10), and objective responses were not observed. Significant increases in immunomodulatory function were noted across all dose levels from pretreatment to day 21 in GM-CSF, IL-2, and IL-15 levels; NK and LAK cytotoxicity; and T-reg cell and NK cell number [19]. These observations support the potential benefit of combining lenalidomide with a monoclonal antibody to enhance the antibody's ADCC effects.

In summary, lenalidomide demonstrated limited single agent activity, against the *in vitro* and *in vivo* childhood cancer models used by the PPTP. Based on these results, lenalidomide's utility in the pediatric clinical setting may depend upon its ability to induce antitumor activity through effects on host immune and stromal cells rather than through direct effects on tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table IAntitumor activity of lenalidomide against *in vitro* models of solid tumors

Cell Line	Histology	IC ₅₀	Ymin (%)
RD	Rhabdomyosarcoma	> 10 microM	90
Rh41	Rhabdomyosarcoma	> 10 microM	93
Rh18	Rhabdomyosarcoma	> 10 microM	97
Rh30	Rhabdomyosarcoma	> 10 microM	69
BT-12	Rhabdoid	> 10 microM	92
CHLA-266	Rhabdoid	> 10 microM	84
TC-71	Ewing sarcoma	> 10 microM	87
CHLA-9	Ewing sarcoma	> 10 microM	71
CHLA-10	Ewing sarcoma	> 10 microM	95
CHLA-258	Ewing sarcoma	> 10 microM	99
SJ-GBM2	Ewing sarcoma	> 10 microM	102
NB-1643	Neuroblastoma	> 10 microM	95
NB-EBc1	Neuroblastoma	> 10 microM	95
CHLA-90	Neuroblastoma	> 10 microM	95
CHLA-136	Neuroblastoma	> 10 microM	91
NALM-6	ALL	> 10 microM	88
COG-LL-317	ALL	> 10 microM	84
RS4;11	ALL	> 10 microM	99
MOLT-4	ALL	> 10 microM	65
CCRF-CEM	ALL	> 10 microM	88
CCRF-CEM (Repeat)	ALL	> 10 microM	75
Kasumi-1	AML	> 10 microM	100
Karpas-299	ALCL	> 10 microM	86
Ramos-RA1	NHL	> 10 microM	94
Median		> 10 microM	92

Table II

Antitumor activity of lenalidomide against in vivo models of solid tumors

Xenograft Line	Histology	Median Time to Event	P-value	EFS T/C	Median Final RTV	T/C	T/C Activity	EFS Activity	Response Activity	Objective Response
BT-29	Rhabdoid	12.6	0.218	0.9	>4	1.12	Low	Low	Low	PD1
KT-14	Rhabdoid	32.9	0.188	1.2	>4	0.67	Low	Low	Low	PD1
KT-12	Rhabdoid	16.2	0.469	1.4	>4	0.80	Low	Low	Low	PD1
KT-10	Wilms	11.2	0.575	1.0	>4	0.70	Low	Low	Low	PD1
KT-11	Wilms	12.6	0.477	1.1	>4	0.92	Low	Low	Low	PD1
KT-13	Wilms	9.8	0.022	0.6	>4	1.27	Low	Low	Low	PD1
SK-NEP-1	Ewing	7.8	0.174	0.9	>4	1.22	Low	Low	Low	PD1
EW5	Ewing	15.5	0.587	1.0	>4	0.97	Low	Low	Low	PD1
EW8	Ewing	6.6	0.424	1.1	>4	0.73	Low	Low	Low	PD1
TC-71	Ewing	7.3	0.619	0.9	>4	1.06	Low	Low	Low	PD1
CHL-A258	Ewing	16.3	0.556	1.0	>4	1.11	Low	Low	Low	PD1
Rh10	ALV RMS	11.5	0.266	1.0	>4	1.08	Low	Low	Low	PD1
Rh30	ALV RMS	19.9	0.126	1.4	>4	0.84	Low	Low	Low	PD1
Rh30R	ALV RMS	13.3	0.380	1.1	>4	0.71	Low	Low	Low	PD1
Rh41	ALV RMS	13.5	0.002	1.9	>4	0.65	Low	Low	Int	PD2
Rh18	EMB RMS	13.2	0.043	1.2	>4	0.71	Low	Low	Low	PD1
BT-28	Medulloblastoma	6.6	0.106	1.2	>4	0.86	Low	Low	Low	PD1
BT-45	Medulloblastoma	26.7	0.004	1.7	>4	0.57	Low	Low	Int	PD2
BT-50	Medulloblastoma	> EP	0.018	> 1.4	3.1	0.88	Low	NE	Int	PD2
BT-41	Ependymoma	> EP	0.087	.	2.0	0.51	Low	NE	Int	PD2
BT-44	Ependymoma	13.5	0.588	0.9	>4	1.02	Low	Low	Low	PD1
GBM2	Glrioblastoma	13.4	0.710	1.1	>4	0.91	Low	Low	Low	PD1
BT-39	Glrioblastoma	9.6	0.039	1.4	>4	0.79	Low	Low	Low	PD1
D645	Glrioblastoma	11.6	0.138	1.3	>4	0.78	Low	Low	Low	PD1
D456	Glrioblastoma	8.5	0.106	1.1	>4	0.87	Low	Low	Low	PD1
NB-SD	Neuroblastoma	11.9	0.331	1.0	>4	0.95	Low	Low	Low	PD1

Xenograft Line	Histology	Median Time to Event	P-value	EFS T/C	Median Final RTV	T/C	T/C Activity	EFS Activity	Response Activity	Objective Response
NB-1771	Neuroblastoma	13.4	0.267	0.8	>4	1.18	Low	Low	Low	PD1
NB-1691	Neuroblastoma	6.4	0.205	1.0	>4	1.06	Low	Low	Low	PD1
NB-EBc1	Neuroblastoma	5.1	0.747	0.9	>4	1.07	Low	Low	Low	PD1
CHLA-79	Neuroblastoma	7.1	0.559	1.1	>4	0.88	Low	Low	Low	PD1
NB-1643	Neuroblastoma	10.6	0.715	1.0	>4	1.19	Low	Low	Low	PD1
OS-1	Osteosarcoma	36.4	0.002	1.2	>4	0.69	Low	Low	Low	PD1
OS-2	Osteosarcoma	25.6	0.781	1.0	>4	1.03	Low	Low	Low	PD1
OS-17	Osteosarcoma	20.7	0.135	1.1	>4	0.90	Low	Low	Low	PD1
OS-9	Osteosarcoma	31.9	0.653	1.2	>4	0.94	Low	Low	Low	PD1
OS-33	Osteosarcoma	15.6	0.007	1.5	>4	0.70	Low	Low	Low	PD1
OS-31	Osteosarcoma	21.5	0.489	1.0	>4	0.85	Low	Low	Low	PD1
ALL-2	ALL B-precursor	15.1	0.714	1.1	>25	.		Low	Low	PD1
ALL-3	ALL B-precursor	10.3	0.855	1.3	>25	.		Low	Low	PD1
ALL-4	ALL B-precursor	11.0	0.679	1.6	>25	.		Low	Low	PD1
ALL-7	ALL B-precursor	6.0	1.000	1.0	>25	.		Low	Low	PD1
ALL-8	ALL T-cell	8.5	0.551	0.8	>25	.		Low	Low	PD1
ALL-16	ALL T-cell	29.9	0.328	1.0	>25	.		Low	Int	PD2
ALL-17	ALL B-precursor	6.9	0.356	1.2	>25	.		Low	Low	PD1
ALL-19	ALL B-precursor	4.4	0.434	0.9	>25	.		Low	Low	PD1