In vivo and *in vitro* sensitivity of blastic plasmacytoid dendritic cell neoplasm to SL-401, an interleukin-3 receptor targeted biologic agent

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ABSTRACT

Blastic plasmacytoid dendritic cell neoplasm is an aggressive malignancy derived from plasmacytoid dendritic cells. There is currently no accepted standard of care for treating this neoplasm, and therapeutic strategies have never been prospectively evaluated. Since blastic plasmacytoid dendritic cell neoplasm cells express high levels of interleukin-3 receptor α chain (IL3-Rα or CD123), antitumor effects of the interleukin-3 receptor-targeted drug SL-401 against blastic plasmacytoid dendritic cell neoplasm were evaluated *in vitro* and *in vivo*. The cytotoxicity of SL-401 was assessed in patient-derived blastic plasmacytoid dendritic cell neoplasm cell lines (CAL-1 and GEN2.2) and in primary blastic plasmacytoid dendritic cell neoplasm cells isolated from 12 patients using flow cytometry and an *in vitro* cytotoxicity assay. The cytotoxic effects of SL-401 were compared to those of several relevant cytotoxic agents. SL-401 exhibited a robust cytotoxicity against blastic plasmacytoid dendritic cell neoplasm cells in a dose-dependent manner. Additionally, the cytotoxic effects of SL-401 were observed at substantially lower concentrations than those achieved in clinical trials to date. Survival of mice inoculated with a blastic plasmacytoid dendritic cell neoplasm cell line and treated with a single cycle of SL-401 was significantly longer than that of untreated controls (median survival, 58 *versus* 17 days, $P<0.001$). These findings indicate that blastic plasmacytoid dendritic cell neoplasm cells are highly sensitive to SL-401, and support further evaluation of SL-401 in patients suffering from blastic plasmacytoid dendritic cell neoplasm.

Introduction

Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is an aggressive neoplasm derived from plasmacytoid dendritic cells.[1] In 2008, BPDCN was classified by the World Health Organization (WHO) as a distinct entity in the group of "acute myeloid leukemia (AML) and related precursor neoplasms".[2] Although elderly subjects are principally affected, BPDCN can also arise in young adults and children.[3,4] Approximately 90% of patients exhibit cutaneous lesions at diagnosis, which upon microscopic analysis appear as a dermal infiltrate of immature blastic cells with features of plasmacytoid dendritic cells.[5,6] Malignant cells isolated from skin, lymph nodes, bone marrow, spleen and/or other tissues usually express the following markers: interleukin-3 receptor alpha (IL-3Rα or CD123), BDCA2 (CD303), BDCA4 (CD304), TCL1 and ILT7.[7-9]

Currently, there is no consensus regarding the optimal treatment modality for BPDCN. Several treatments, including multi-agent chemotherapy regimens, symptomatic approaches (e.g. local radiation[10]), and intensive chemotherapy with allogeneic hematopoietic cell transplantation,[11-13] are generally used to treat patients. Although chemotherapy regimens used to treat patients with acute leukemia or lymphoma are often effective at inducing an initial response, the duration of response is typically brief and recurrent disease is generally resistant to chemotherapy. BPDCN patients generally succumb to cytopenias due to tumor infiltration of the bone marrow; the median overall survival has been reported to range from 9 to 32 months irrespectively of the initial presentation of the disease.[14-16] While longer overall survival has been reported with allogeneic hematopoietic cell transplantation, especially in younger patients,[4,11,13,16,17] many relapses have been observed after such transplants.[13]

The α-subunit of the human IL-3 receptor is a type I transmembrane glycoprotein belonging to the cytokine receptor superfamily.[18,19] Interleukin-3 (IL-3) the IL-3 receptor is a heterodimer associating an α chain (CD123) and a β chain (CD131). This chain is shared by IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor receptors. SL-401, a novel biologic targeted therapy directed against the IL-3R, is comprised of human recombinant IL-3 joined by an acid-

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labile group of amino acids to a diphtheria toxin (DT) payload that has been truncated at its receptor binding region.[20] Since IL-3, the natural ligand for IL-3R, binds with very high specificity and avidity,[21] SL-401 is able to transport DT efficiently and preferentially to cells that overexpress IL-3R, leading to internalization followed by receptor-mediated endocytosis and localization of SL-401 to early endosomes. After cleavage of the SL-401 DT constituent in the acidic medium of endosomes, DT translocates into the cytosol and binds to ADP-ribosylated elongation factor 2, leading to blockade of protein synthesis and cell death.[22]

Given the ubiquitous and high expression of IL-3R by BPDCN and the lack of therapies available to treat BPDCN, SL-401 is a potential therapeutic for BPDCN. The present study evaluated the cytotoxicity of SL-401 against patient-derived BPDCN cell lines (CAL-1 and GEN2.2) and primary BPDCN cells isolated directly from 12 patients. The investigations were performed *in vitro*, as well as *in vivo* in a murine model of BPDCN. The aim of the study was to provide further support for the use of SL-401 in patients suffering from BPDCN.

Methods

Patients' cells and cell lines

Peripheral blood or bone marrow cells were obtained for diagnostic purposes from 12 BPDCN patients (Table 1) from our national network that collects data and cells from cases diagnosed in France since 2004 (authorization number #DC-2008-713). BPDCN was diagnosed from the results of histopathology and immunostaining of cutaneous lesions, blood or bone marrow.[2,8] Two established cell lines derived from BPDCN patients were used (GEN 2.2, patent #0215927, Dr. Plumas, EFS Rhone-Alpes, Grenoble, France and CAL-1, Dr. Maeda, Nagasaki University, Japan) as well as TF/H-Ras (Prof. Frankel) and $CD123^{neg}$ (MFI<800) Daudi cell lines (ACC78, DSMZ Braunschweig, Germany) as positive and negative controls, respectively. Other lymphoid and myeloid leukemic cells used to compare sensitivity to SL-401 are described in the *Online Supplementary Appendix*.

Drug and culture

The SL-401 drug (Stemline Therapeutics, New York, NY, USA) was stored at -80°C and tested at eight concentrations ranging from 365 pM to 0.08 fM (21 ng/mL to 0.4 ng/mL) in order to cover

Table 1. Characteristics of the 12 patients suffering from blastic plasmacytoid dendritic cell neoplasm (BPDCN) who provided primary BPDCN cells.

Patient number	Age/ gender	Immunophenotype (flow cytometry)	Karyotype	Cutaneous lesions	Pathology diagnosis	Sites of disease involvement
#1	75/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ My$^-$ B$^-$ $CD7^+$ (others T$^-$)	45,XY,-5 [3], 46XY [10]	no	NA	LN, BM blood, spleen
#2	59/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^-$ $CD304^+$ $CD33^+$ (others My$^-$) B$^-$ $CD7^+$ (others T$^-$)	46,XY,del(6q)(q16q25),der(7)t(7;12)(p11;q1?3),?del(7)(p21),-8x2,-9x2,-12x2,+mar [4], 46XY [11]	yes	BPDCN	LN, BM skin
#3	81/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ $CD33^+$ (others My$^-$) $CD22^+$ (others B$^-$)$CD2^+$ (others T$^-$)	46XY	yes	BPDCN	LN, BM, skin, blood
#4	69/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ $CD33^+$ (others My$^-$)$CD22^+$ (others B$^-$) $CD7^+$ (others T$^-$)	46XY	yes	BPDCN	skin, BM
#5	63/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ $TCL1^+$ My$^-$ B$^-$ T$^-$	44,X,-Y,-13[3]/44,sl,i(7)(q10)[15]/45,XY,-15[3]	yes	BPDCN	LN, BM skin blood, spleen
#6	56/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^-$ $CD304^+$ $TCL1^+$ ILT7$^-$ $CD33^+$ $CD117^+$ (others My$^-$) $CD22^+$ (others B$^-$) $CD7^+$ (others T$^-$)	NA	yes	NA	BM, skin, blood
#7	33/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^-$ $CD304^+$ $TCL1^+$ ILT7$^-$ My$^-$ B$^-$ T$^-$	44,X,Y,13[3]/44,sl,i(7)(q10)[15]/45,XY,-15[3]	yes	BPDCN	BM, skin, blood
#8	70/F	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ $TCL1^+$ ILT7$^-$ My$^-$ B$^-$ T$^-$	46,XX i(7q)	yes	BPDCN	LN, BM, skin, blood, spleen
#9	72/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^-$ $CD304^+$ $TCL1^+$ ILT7$^-$ My$^-$ B$^-$ $CD2^+$ $CD7^+$ (others T$^-$)	NA	yes	NA	LN, BM, skin, blood
#10	63/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$ $TCL1^+$ ILT7$^-$ My$^-$ B$^-$ $CD2^+$ $CD7^+$ (others T$^-$)	46XY	yes	BPDCN	BM, skin, blood
#11	58/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^+$ $CD304^+$TCL1$^+$ ILT7$^+$ $CD33^+$ (others My$^-$) B$^-$ T-	46XY	yes	BPDCN	LN, BM, skin, spleen
#12	80/M	$CD4^+$ $CD56^+$ $CD123^{++}$ $CD303^-$ $CD304^+$ $TCL1^+$ ILT7$^-$ My$^-$ B$^-$ $CD7^+$ (others T$^-$)	43-44,XY,der(2) t(2;?)(q23;?),der(4)t(4;?)(q35;?),5,der(7)t(7;?)(q31;?),-9,-10,-13,-15,-16,del(17)(p11),+1-4mar[17];82 83,idemx2[12];46,XY	yes	BPDCN	LN, BM, skin, blood, spleen

Age (years)/gender; Results of phenotypic analysis performed on blood or bone marrow samples (flow cytometry); karyotype; presence of cutaneous lesions; histopathological diagnosis. My: myeloid markers (including myeloperoxidase, CD13, CD33, CD117, CD15, CD65, CD14, CD64); T: T lymphoid markers (including membrane CD3, intracytoplasmic CD3, CD7, CD5, CD2, CD8); B: B lymphoid markers (including intracytoplasmic CD79a, intracytoplasmic CD22, intracytoplasmic Ig μ chains, CD19, CD20, CD22, surface immunoglobulin); +: positive expression; ++: high expression; -: absence of expression; NA: not available; LN: lymph nodes; BM: bone marrow.

the concentrations obtained *in vivo* in patients enrolled in clinical trials.[23,24] The effects of chemotherapy agents used in acute leukemia were also evaluated against BPDCN cells (*Online Supplementary Appendix*). BPDCN cells were incubated at $3x10^5$ cells/mL in RPMI 1640 glutamax medium (Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (Invitrogen), 1% penicillin/streptomycin (PAA Laboratoires, Velizy Villacoublay, France) with or without SL-401 or the relevant drugs under 5% CO_2 for 18 h at 37°C.

Cytotoxicity evaluation by flow cytometry

Flow cytometry was performed using a CANTO II cytometer (BD Biosciences, San Jose, CA, USA) and DIVA 6.2 software (BD Biosciences). The cytotoxic effects of SL-401 and the various drugs were evaluated using annexin-V and 7-amino actinomycin D (AV/7AAD) and a panel of different monoclonal antibodies to gate the blastic population described in the *Online Supplementary Appendix*. In the mouse model, anti-mouse and anti-human CD45 plus anti-human CD123, CD4, CD56, CD304 were used to identify BPDCN human cells (*Online Supplementary Appendix*). A defined number of calibrated 3-μm latex beads (Flowcount beads, Beckman Coulter) was added to each sample to obtain the absolute number of circulating BPDCN cells in mice, as previously described.[25]

Cytotoxicity evaluation by the MTT assay

The percentage of viable cells obtained after incubation with or without SL-401 was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, Saint Quentin Fallavier, France). Details of the culture and following analysis are provided in the *Online Supplementary Appendix.*

Mice

NOD-SCID IL2Rγc-deficient (NSG) mice were irradiated (2 Gy) and inoculated intravenously 24 h later with $1x10^6$ GEN2.2 cells and treated intraperitoneally 8 days later with five daily injections of SL-401 (2 μg/mouse/injection, total experimental dose 100 μg/kg) or with phosphate-buffered saline (PBS) only. Mice were monitored weekly by blood cell counts and flow cytometry (*Online Supplementary Appendix*).

Statistical analysis

Statistical analyses were performed using Statel software 2.6 (Adscience, Paris, France) (*Online Supplementary Appendix*).

Results

SL-401 is cytotoxic against blastic plasmacytoid dendritic cell neoplasm cell lines and primary cells

The viability of the two $CD123^{pos}$ BPDCN cell lines, GEN2.2 and CAL-1, decreased from 62 ± 6% (range, 44-96) to 5 ± 2% (range, 0-17) after treatment with SL-401 at the experimental dose of 365 pM (21 ng/mL) for 18 h (n=9, Figure 1A). Moreover the viability of these two BPDCN cell lines decreased in a dose-dependent manner (Figure 1B). Similarly, after treatment with SL-401 at the experimental dose of 365 pM (21 ng/mL), the viability of 12 samples of freshly isolated BPDCN primary cells decreased significantly from 50 ± 4% (range, 31-71) to 10 ± 1% (range, 3-17). As expected, the $CD123^{neg}$ (Daudi) cells were not sensitive to treatment with SL-401 concentrations as high as 365 pM for 18 h [viability was unchanged from 65% (range, 88-41) to 61% (range, 83-41) in cells untreated or treated with SL-401, respectively, n=3; Figure 1A,B].

SL-401 is cytotoxic against blastic plasmacytoid dendritic cell neoplasm primary cells in a concentration-dependent manner

The viability of primary malignant cells obtained from 12 BPDCN patients treated with SL-401 for 18 or 48 h decreased in a concentration-dependent manner, as assessed by flow cytometry and MTT (Figure 1C,D). As expected, $CD123^{neg}$ Daudi cells were resistant to SL-401 treatment (Figure 1C,D). For patient #10, primary BPDCN cells were obtained at both the time of diagnosis and disease relapse following treatment with chemotherapy [including CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone), methotrexate, and L-asparaginase]. Interestingly, BPDCN cells obtained at disease recurrence were slightly more sensitive to SL-401 than cells obtained at diagnosis (IC_{50}, 6 fM *versus* 79 fM, respectively; P=0.049; Figure 1E). Thus, relapsing cells were still sensitive to SL-401 in a concentration-dependent manner, indicating that BPDCN cells retain their sensitivity to SL-401 following treatment with various cytotoxic agents, thereby suggesting a lack of cross-resistance.

SL-401 is more efficient than other tested chemotherapeutic drugs – except idarubicin – at killing blastic plasmacytoid dendritic cell neoplasm cells

In order to appreciate the cytotoxic effect of SL-401 better, we also assessed the effects of other chemotherapeutic drugs. Primary malignant cells obtained from three BPDCN patients (#7, #9 and #11) were significantly more sensitive to SL-401 than to a wide variety of cytotoxic agents commonly used for the treatment of hematologic malignancies, including cytosine arabinoside, cyclophosphamide, vincristine, dexamethasone, methotrexate, Erwinia L-asparaginase, and asparaginase (Figure 2; P<0.05 for all agents). Only idarubicin was found to be more efficient than SL-401 (viability <1%, n=5; Figure 2).

Blastic plasmacytoid dendritic cell neoplasm primary cells are more sensitive to SL-401 than acute myeloid or lymphoblastic leukemia primary cells in vitro

We next assessed the viability after SL-401 treatment of primary malignant cells isolated from three previously untreated patients suffering from acute lymphoblastic leukemia and six untreated patients suffering from AML. We compared these results to those achieved following SL-401 treatment of primary BPDCN cells. The viability of the leukemic cells decreased slightly after treatment with SL-401 (365 pM for 18 h). We observed an average decrease of 13% in viability for acute lymphoblastic leukemia cells [33±20% (range, 6-73) to 26±20% (range, 4-69), n=3] and 16% for AML cells [40±8% (range, 18-75) to 36±8% (range, 11-70), n=6]. BPDCN cells were significantly more sensitive to SL-401 and had a 75% decrease in viable cells [50±5% (range, 20-71) to 11±1%, (range, 4-17) n=11; P<0.001; Figure 3].

CD123 expression correlates with SL-401 cytotoxicity in vitro

We next compared the relative expression levels of IL-3Rα (CD123) and β (CD131) chains in primary malignant

BPDCN cells, acute leukemic cells, as well as established BPDCN cell lines to the respective sensitivities to SL-401 (Table 2). Overall, sensitivity of primary BPDCN cells was related to CD123 expression, as demonstrated by the inverse relationship between cell viability and CD123 expression (Spearman test: r = -0.58, P<0.012). The high dependence of plasmacytoid dendritic cell lineage cells (including normal or leukemic plasmacytoid dendritic cells) to IL-3 may also contribute to the high sensitivity of BPDCN cells to deprivation of IL-3 signaling during SL-401 exposure. Importantly, no such relationship was observed for CD131 (Spearman test: r =

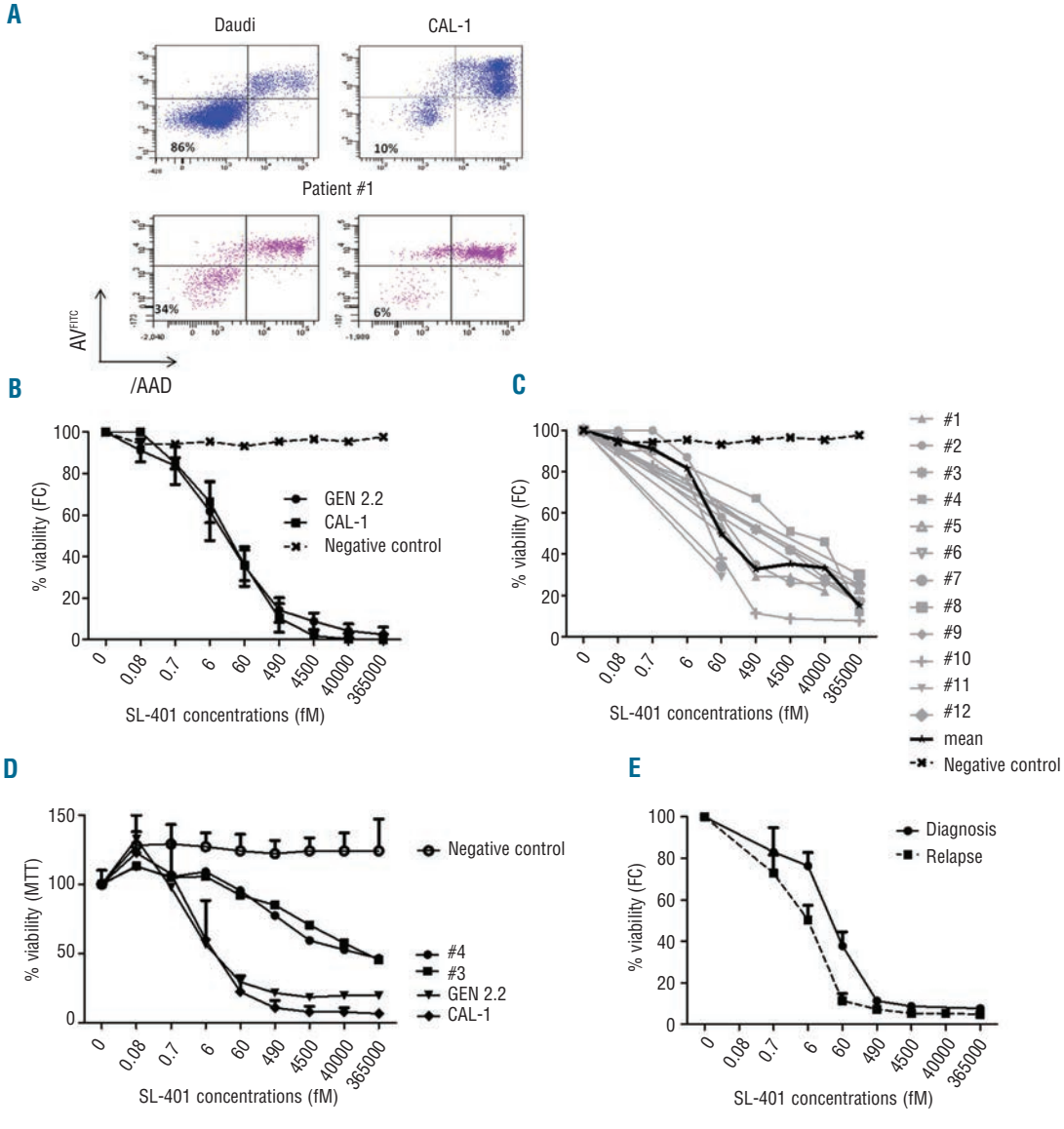

Figure 1. Sensitivity of BPDCN cells to SL-401-mediated death. (A) A representative experiment is shown. Upper panels: dot plots showing the staining of annexin V (AV ^{FITC}) and 7-AAD, on the x- and y-axes, respectively, as assessed by flow cytometry (FC), on IL-3R non-expressing Daudi cells (negative control) and CAL-1 cells (established BPDCN patient-derived cell line) after treatment with SL-401 (365 pM) for 18 h; lower panels: primary blasts from BPDCN patient #1 after treatment with SL-401 (365 pM, right hand side panel) for 18 h or no drug treatment (no SL-401, left hand side panel). The percentages indicated in each dot plot represent viable cells (AV$^-$/7-AAD$^-$ cells). (B) The percent viability (mean ± SEM) of the BPDCN cell lines CAL-1 (n=3) and GEN 2.2 (n=6) after treatment with SL-401 at different concentrations ranging from 0.08 fM to 365 pM (0-21 ng/mL) for 18 h. The Daudi cell line was used as a negative control. (C) Each gray line represents the percentage viability of primary blasts (AV$^-$/7-AAD$^-$ cells) isolated from different BPDCN patients (#1-12) according to different SL-401 concentrations (from 0.08 fM to 365 pM = 0-21 ng/mL) for 18 h. The black line represents the mean of BPDCN patients' samples as a function of SL-401 concentration; the Daudi cell line was used as a negative control. (D) Viability assessed using the MTT assay: the percentage viability of primary blasts from BPDCN patients #3 and #4 and of CAL-1 and GEN 2.2 BPDCN cell lines is dependent on SL-401 concentrations. The Daudi cell line was used as a negative control and is insensitive to SL-401-mediated death whatever the concentration of the drug used. Cells were treated with SL-401 for 48 h. The values represent the results of one experiment for patients' samples or three independent experiments for cell lines. (E) Percentage (mean ± SEM) of viable primary BPDCN cells (AV$^-$/7-AAD$^-$ cells) from patient #10 at diagnosis (n = 3) and at relapse (n=6) after incubation for 18 h with different concentrations of SL-401 or without any drug, as assessed using annexin V and 7-AAD staining and flow cytometry (FC). Untreated cells were considered as 100% viable (P=0.049).

0.01, $P<0.93$). For patient #10, the MFI of CD123 was quite similar both at diagnosis and relapse (5509 *versus* 5137, respectively).

Treatment with SL-401 significantly increases the overall survival of NSG mice inoculated with blastic plasmacytoid dendritic cell neoplasm cells

Irradiated NGS mice were inoculated with the GEN2.2 cell line ($1x10^6$ cells per mouse) through the tail vein. Eight days after inoculation, mice were treated with a daily intra-peritoneal injection of SL-401 (2 μg/day) for 5 days or with PBS as controls (n=8 mice in 3 independent experiments). In PBS-treated control mice, the mean overall survival was 17±1 days. Treatment with five daily injections of SL-401 significantly increased the overall survival of mice compared to that of control mice (58 ± 2 days; $P<0.001$; Figure 4A,B). Circulating BPDCN cells were identified as human $CD45^{pos}$, $CD123^{pos}$, $BDCA4^{pos}$, $CD4^{pos}$, $CD56^{pos}$, $CD3^{neg}$, and $CD34^{neg}$ cells (Figures 4C). Nearly all of the BPDCN cells expressed CD123 when mice developed the BPDCN (Figure 4C). This suggests that one course of SL-401 (2 μg/day for 5 days) is not sufficient to kill all of the BPDCN cells, rather that $CD123^{neg}$ BDPCN cells emerge in response to SL-401. In PBS-treated control mice, the number of BPDCN cells progressively increased until death, whereas treatment for 5 days with SL-401 successfully reduced circulating BPDCN cells to undetectable levels for 15±3 days after treatment (Figure 4D). We monitored hemoglobin and platelet counts in mice to assess leukemic cell bone marrow involvement. In PBS-treated control mice inoculated with BPDCN, hemoglobin and platelet counts progressively decreased until death. In contrast, in treated mice, these hematologic parameters reached the levels observed in irradiated control mice that were not inoculated

Table 2. Expression of IL-3Rα and β chains on primary malignant cells from patients suffering from BPDCN, acute myeloid leukemia (AML) cells, acute lymphoid leukemia (ALL) cells and cell lines –as assessed by mean fluorescence intensity – was compared with the viability after treatment with SL-401.

Cells	Mean fluorescence intensity		Viability (%)	IC_{50}
	IL-3R α (CD123)	IL-3R β (CD131)	(365 pM)	(pM)
#1	6340	2214	29▪	0.06
#2	10189	2352	38▪	0.083
#3	9095	2060	17*	0.006
#4	10746	2320	17*	NA
#7	6165	3276	27*	NA
#9	4439	2477	29*	NA
#10 diagnosis	5509	3433	6±1°	0.006
#10 relapse	5137	1068	5±1.3#	0.075
AML 1	4557	2073	83▪	NA
AML 1	2073	1870	93▪	NA
AML MLD	6941	3003	100▪	NA
AML 2	3700	1473	93▪	NA
AML 2	1068	2749	93▪	NA
ALL-T	3049	2761	94▪	NA
ALL-B	2399	2890	87▪	NA
GEN2.2	11336	5378	0,45*	0.006
CAL-1	6406	629	12,5*	0.05
TF/hras	1478	1174	75*	0.06

Expression of IL-3Rα and β chains (CD123 and CD131) –measured by flow cytometry– in BPDCN cells from patients (# 1-4, 7, 9 and 10), or in different AML, ALL and cell lines (results represent the mean of 2 determinations). Percentage of viability after culture with SL-401 (experimental dose 365 pM= 21 ng/mL) in one experiment (▪), mean±SEM of two (*), three (°) or six (#) independent evaluations and IC_{50} are indicated. AML MLD: AML with multi lineage dysplasia; NA: not available.

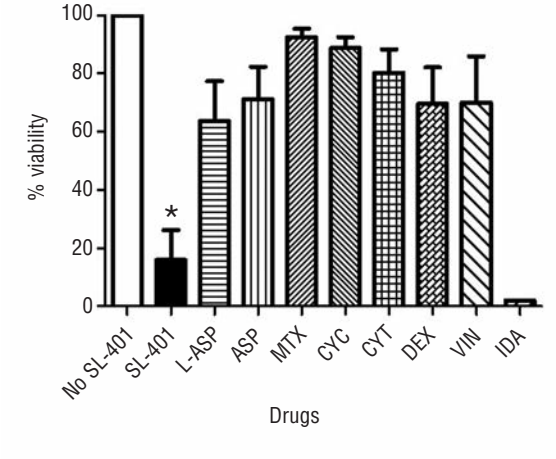

Figure 2. Sensitivity of primary BPDCN cells to SL-401 and other relevant chemotherapeutic agents. The mean percentages of viable cells ($AV^-/7\text{-}AAD^-$ cells) from three patients (# 7, 9 and 11) after treatment with with SL-401 (365 pM), Erwinia L-asparaginase (L-ASP, 10 IU/mL), asparaginase (ASP, 10 IU/mL), methotrexate (MTX, 9.9 μM), cyclophosphamide (CYC, 100 IU/mL), cytosine arabinoside (CYT, 0.329 mM), dexamethasone (DEX, 0.637 mM), vincristine (VIN, 0.0242 μM) and idarubicin (IDA, 0.158 μM). Untreated cells were considered as 100% viable. Histograms represent the mean ± SEM of three independent experiments (*$P<0.05$ between SL-401 and all other drugs pairwise except IDA).

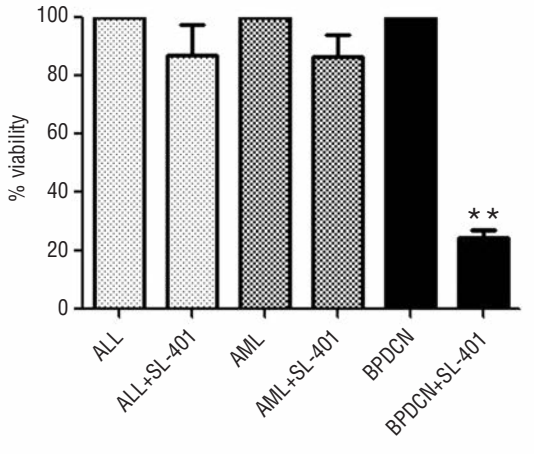

Figure 3. Comparison of sensitivity of primary blasts from patients with BPDCN, acute lymphoid leukemia (ALL), and acute myeloid leukemia (AML) to SL-401-mediated death. Leukemia blasts sampled from three patients with ALL (2 B-ALL and 1 T-ALL) and six patients with AML (1 case of AML with multilineage dysplasia, 2 cases of M1 AML, 1 case of M0 AML, and 2 cases of M2 AML) and blasts from 11 BPDCN patients (#1-10 and 12) were cultured with or without SL-401 (365 pM) for 18 h. The viability was assessed by flow cytometry ($AV^-/7\text{-}AAD^-$ cells). The bars represent the mean ± SEM of the percentage viable blastic cells, with untreated cells considered 100% viable. **$P<0.001$.

with BPDCN cells and not treated (Figure 4E). Regression of cytopenia under treatment indicates an absence, or at least, a lower level of bone marrow involvement by BPDCN cells in SL-401 treated mice. Overall, SL-401 is effective in controlling BPDCN cells *in vivo*.

Discussion

SL-401 is a biologic agent corresponding to IL-3 genetically fused to truncated DT *via* a cleavable linker. This agent induces cytotoxicity by inhibiting ribosomal function, and thereby, inhibiting protein synthesis, a mechanism that is distinct from all other anticancer therapeutics.[20] SL-401 has been demonstrated to induce a profound cytotoxicity at picomolar and subpicomolar concentrations in AML cell lines,[26] as well as in a model of human AML inoculated into immunocompromised mice.[27] Moreover, SL-401 is cytotoxic *in vivo*, in patients with advanced AML and myelosdysplastic syndrome,[23] suggesting that SL-401 targets leukemic stem cells, as well as more mature tumor cells. In contrast, SL-401 is not cytotoxic to normal hematopoietic progenitor cells,[28] which has translated into a paucity of myelosuppression in clinical trials to date.[23,28-31]

The present study was performed to evaluate and quantify the effects of SL-401 on various preclinical models of BPDCN, a malignancy that ubiquitously expresses high levels of the IL-3Rα chain, which is the target of SL-401. The results reported here demonstrate that SL-401 is highly potent against BPDCN cell lines and primary BPDCN blasts obtained from patients. Although AML cell lines and primary AML cells have demonstrated sensitivity to SL-401 with IC_{50} values in the picomolar range (10^{-12} M),[23] which are lower than plasma concentrations achieved in leukemic patients undergoing treatment with SL-401, BPDCN blasts are more sensitive, with IC_{50} values in the femtomolar range (10^{-15} M, experimental dose 0 to 21 ng/mL).[32] In addition, SL-401 produced a robust antitumor effect in an *in vivo* xenograft model using human BPDCN cells. This also indicates a potential good therapeutic index, as well as systemic activity, since mice survived more than 40 days after SL-401 treatment. The high sensitivity of BPDCN to SL-401 and the potential good therapeutic index of this agent likely reflect the high specificity of the IL-3 ligand component of SL-401 for CD123, in addition to the mechanism of action and potency for its DT payload. Since the IL-3 component of SL-401 is bound *via* an amino acid linker to a DT for which the receptor binding site is truncated, free DT is essentially inert from a toxicity standpoint. DT can only be delivered intracellularly following the binding of SL-401 to the IL-3R *via* IL-3 and internalization. Since IL-3Rα expression is limited to only a few normal tissues (plasmacytoid dendritic cells and basophils) and, in contrast, the receptor is overexpressed by BPDCN cells, SL-401 can potentially confer high therapeutic indexes for patients. Additionally, SL-401 is not a substrate for p-glycoprotein and other efflux

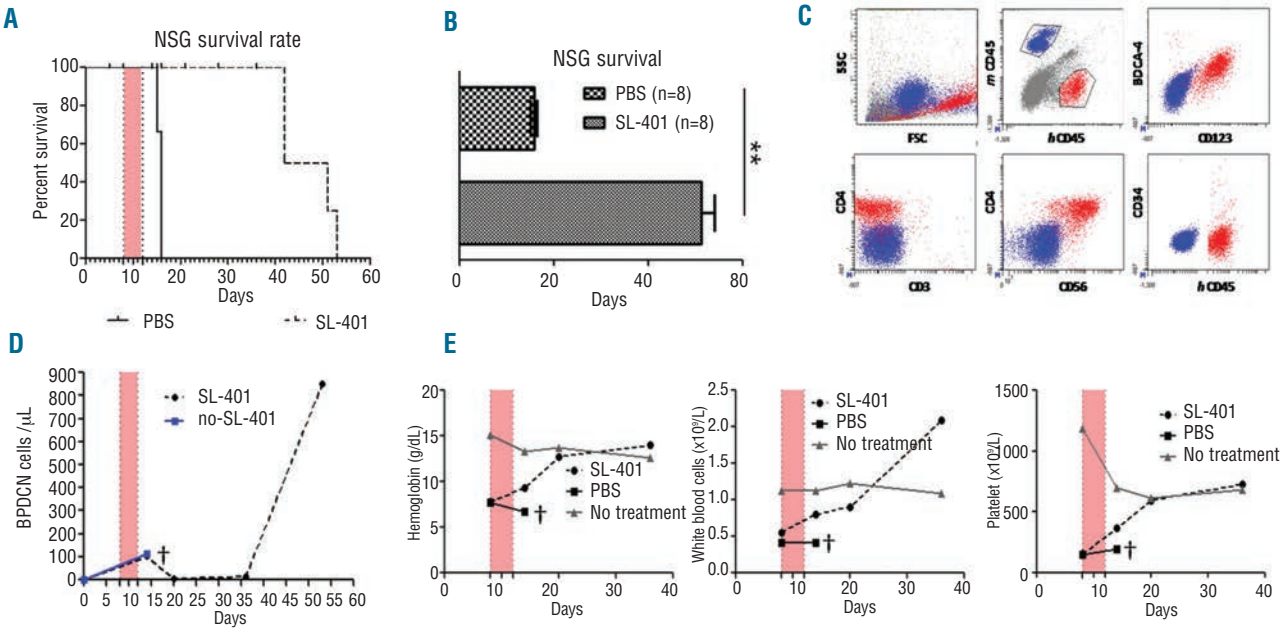

Figure 4. ***In vivo*** **efficacy of SL-401 in a NSG mouse model inoculated with BPDCN cells. NSG mice were irradiated with 2 Gy and then inoculated intravenously with 1x10⁶ GEN2.2 BPDCN cells on day 0. (A) Overall survival (OS) of BPDCN inoculated-mice treated with SL-401 (solid line; n=4) or with PBS (dotted line; n = 3). Treatment with SL-401 (2 µg/mouse intraperitoneally, experimental dose 100 µg/kg) performed daily for 5 days, was begun on day 7 (pink bar). OS from one representative experiments out of three is shown (*P*=0.3). (B) Mean OS of BPDCN inoculated-mice treated with SL-401 (n=8) or PBS (n=8) from three independent experiments (***P*<0.001). (C) one example of the immunostaining of circulating peripheral blood mononuclear cells performed at day 53 prior to sacrifice. Murine (blue) and human GEN 2.2 (red) cells are distinguishable due to specific CD45 antibody expression. Human GEN2.2 BPDCN cells express CD123, BDCA4, CD4, CD56, but not CD34 or CD3. (D) BPDCN cell count values in the blood of a mouse following treatment with SL-401 (dotted line, black circles) or with PBS (blue solid line, blue square). (E) Means of hemoglobin, as well as white blood cell and platelet counts in the blood of mice following treatment with SL-401 (n=4) or saline control (n=3). The gray line represents blood parameter values in irradiated mice that were not treated with SL-401. †: means that the mice died.**

pumps, and thus, cannot be excluded from the blastic cells. Moreover, its cytotoxic mechanism, binding to ADP-ribosylated elongation factor 2, thereby uncoupling protein synthesis blockade, does not overlap with other agents currently used.[22] IL-3 has also been shown to be a critical survival factor for plasmacytoid dendritic cells.[33] Thus, interference of this pathway by SL-401 may explain the high sensitivity of BPDCN compared with the sensitivity of other myeloid and lymphoid leukemic cells.

There is still no consensus on the best therapeutic approach for BPDCN,[3,12-15,34-38] and, overall, BPDCN remains a chemotherapy-resistant disease and may also resist the graft-*versus*-leukemia effect, since 32% of patients relapse after allogeneic hematopoietic cell transplantation according to a recent study.[13] Thus, targeted or immune-based therapies are alternative strategies to treat this aggressive leukemia.[39-41] Here, we propose that SL-401 is an efficient target-based therapy available for clinical trials and has already been shown to have favorable effects in patients with refractory or relapsed AML or myelodysplastic syndrome, although expression of CD123 is lower on myeloid blasts than on BPDCN cells.[23] We recently published data from a phase I/II clinical study involving 11 BPDCN patients. These data showed that a single cycle of SL-401 induced major responses in 78% of the patients.[24] The way to use SL-401 in BPDCN patients must, however, be discussed in the light of data from literature obtained in such patients. SL-401 can be used to consolidate the effects of first-line chemotherapy, reducing the number of relapses, which always occur after chemotherapy treatment. The combination of SL-401 with chemotherapy should make it possible to reduce chemotherapy doses, and consequently, their adverse effects, which are significant in elderly patients with comorbidities (*i.e.*, most of the patients with BPDCN). We showed here that idarubicin, at the dose we tested, induces a relevant level of cytotoxic activity *in vitro* whereas cytosine arabinoside does not. This confirms recent data showing that the BPDCN cell line CAL-1 is resistant to cytosine arabinoside.[41] Intrathecal injection of SL-401 could also be of interest since there are frequently patients with central nervous system relapse[42] and the molecular weight of SL-401 (57 KDa) predicts no diffusion across the blood-brain barrier. For patients who undergo allogeneic hematopoietic cell transplantation, SL-401 treatment can be used before allografting to minimize the level of minimal residual disease, which is the most important prognostic factor in a recent study on allografted BPDCN patients,[13] or as a consolidation treatment after allogeneic hematopoietic cell transplantation.[13] In support of this latter use, we observed that, *in vitro*, the blastic cells from a relapsing BPDCN patient were still sensitive to SL-401-mediated death. Moreover, the IL-3Rα chain (CD123) was still expressed on the surface at relapse (CD123 MFI at diagnosis: 5509 *versus* 5137 at relapse, patient #10). In the mouse model after one course of SL-401, all the BPDCN cells at relapse also expressed CD123. This supports the hypothesis that patients suffering from BPDCN can be treated with SL-401 as first-line or second-line therapy and, maybe, even with several courses of SL-401. Overall, immune-based therapy using SL-401 appears to be an appropriate way to treat BPDCN patients. New approaches based on immunomodulators[41] or demethylating agents[40] must be further evaluated and compared – or associated – with SL-401.

In conclusion, we demonstrate that clinical grade SL-401, which specifically targets IL-3R, efficiently kills primary BPDCN cells in culture and significantly improves the overall survival of mice inoculated with BPDCN receiving a single cycle of SL-401. This provides a strong rationale for the use of SL-401 in the treatment of patients suffering from BPDCN. As BPDCN is a rare subtype of leukemia, an international clinical trial using SL-401 should now be conducted to validate these results prospectively.

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