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The Quinoxaline Antitumor Agent (R+)XK469 Inhibits Neuroblastoma Tumor Growth

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Abstract

The quinoxaline antitumor agent (R+)XK469 mediates its effects by topoisomerase IIB inhibition. This report describes a 14 year-old with relapsed neuroblastoma who experienced disease stabilization for 14 months while receiving (R+)XK469 monotherapy. Due to this favorable response, laboratory studies were undertaken to determine efficacy in the preclinical setting. (R+)XK469 inhibited proliferation, caused G₂ cell cycle arrest of neuroblastoma cells *in vitro*, and inhibited growth of neuroblastoma xenograft tumors. These preclinical results, coupled with the favorable clinical response, demonstrate that (R+)XK469 and similar anti-tumor agents may be effective in the treatment of high-risk neuroblastoma and warrant further testing.

Keywords

neuroblastoma; topoisomerase inhibitor; relapse; phase I

Introduction

Less than 40% of children with high-risk neuroblastoma are cured, emphasizing the need for more effective treatments [1]. We describe a child with relapsed neuroblastoma, treated with the Quinoxaline Antitumor agent (R+)XK469, who experienced 14-month disease stabilization. *In vitro* and *in vivo* studies were subsequently performed to further evaluate the anti-neuroblastoma activity of (R+)XK469.

Case Report

The patient was diagnosed with a stage 4, *MYCN* non-amplified neuroblastoma at age 7½ years. She was treated with induction chemotherapy per a Pediatric Oncology Group high-risk study [2], followed by surgical resection of the primary adrenal mass. Re-evaluation with meta-iodobenzylguanidine (MIBG) scintigraphy revealed no evidence of cortical bone disease and morphologic examination of bone marrow showed no disease. However, cells

expressing GD-2 were identified by flow cytometry using anti-GD2 antibody, and tyrosine hydroxylase (TH) was detected in the bone marrow by RT-PCR (29 ng/ml on left side and 160 ng/ml on right), using a nested RT-PCR assay. For this assay, a 10 μ l calculated concentration unit value was extrapolated from a 10 fold serial dilution standard curve that was generated by seeding the NGP neuroblastoma cell line (1×10^3 to 1×10^{-2} ng/ μ l) into normal bone marrow samples. Values greater than 1×10^0 ng/ μ l were classified as positive.

The patient subsequently underwent matched unrelated donor stem cell transplant using reduced intensity conditioning with fludarabine, antithymocyte globulin and busulfan, according to an institutional protocol testing the feasibility and efficacy of allogeneic stem cell transplant in high-risk patients.

Four years following transplant, the patient relapsed in cortical bone and marrow, and was referred to the University of Chicago for further treatment. Following four cycles of intravenous (IV) cyclophosphamide and topotecan [1] persistent disease was detected in the bone marrow by morphology and in multiple cortical bone sites by MIBG and bone scan.

The patient was subsequently enrolled on a University of Chicago Phase I study of R(+)-XK469 for patients with advanced solid tumors and lymphoma (NCI protocol number 4570, local protocol number 11108B). Treatment was initiated at 2000 mg IV over one hour, on day 1 of a 21 day cycle, at 80% of the adult maximum tolerated dose (MTD), which is the classical starting dose for pediatric phase 1 studies recommended by the NCI. Due to grade 3 neutropenia and thrombocytopenia, her dose was decreased to 1600 mg, and treatment cycles were prolonged to 28 days. Grade one fatigue and nausea were experienced throughout treatment. The patient also required hospitalization for fever and neutropenia after four chemotherapy cycles. A peptostreptococcus central line infection was diagnosed and treated with linezolid.

Evaluations were performed every two cycles, and her disease remained stable for 14 months, with negative MIBG and bone scans and minimal disease identified morphologically on bone marrow sampling. After 13 treatment cycles, the patient experienced cortical bone relapse and more extensive disease was detected in bone marrow. She was taken off study and treated on a Children's Oncology Group Phase 2 trial of ABT751, but progressed after 1 cycle. Progressive disease was again detected after three cycles of cyclophosphamide and topotecan. The patient then underwent treatment with low-dose palliative MIBG therapy, but disease progressed within a month and she died from metastatic neuroblastoma.

Materials and Methods

Cell lines

Neuroblastoma cell lines NBL-W-N, SMS-KCNR and LA1-55n were grown at 5% CO₂ in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen) and L-glutamine.

Proliferation Assay

CellTiter 96 Aqueous Nonradioactive Proliferation Assay kit (Promega, Madison, WI) was used as previously described [3]. Briefly, cells were seeded and after 24 hours (R+)XK469 was added at various concentrations. Following a 72 hour incubation, MTS reagent was added for 3 hours and absorbance measured using a Synergy 2 microplate reader (Bio-Tek Instruments, Winooski, VT).

Measurement of Cell Cycle Phase Distribution by Flow Cytometry

Cells were cultured with or without 40 μ M (R+)XK469 for 48 hours, washed with phosphate buffered saline (PBS), fixed in 70% ethanol, and hypotonically lysed in 1 ml DNA staining solution [0.05mg/ml propidium iodide (Sigma, St. Louis, MO), 0.1% Triton X-100] and data analyzed on a FACSCanto flow cytometer (BD Biosciences, San Jose, CA) using Flowjo software (Tree Star, Ashland, OR). A student's t-test was used to compare responses in control and treatment groups.

Xenograft Treatment

Nude mice (Harlan, Madison, WI) underwent subcutaneous injection of 1×10^7 SMS-KCNR cells as previously described [3]. Once tumors were palpable, mice (n=8) were treated with 40 mg/kg (R+)XK469 via tail vein injection for 5 doses over 10 days, the maximum tolerated dose reported in a WSU-WM SCID xenograft model [4], or vehicle alone (n=9). (R+)XK469 was provided by the Developmental Therapeutics Program (DTP) NCI/NIH. It was dissolved in 1% NaHCO_3 , diluted with PBS and filter sterilized. Animals were sacrificed after 10 days and tumors removed for evaluation. Animals were treated according to NIH animal care and use guidelines and protocols approved by the University of Chicago Institutional Animal Care and Use Committee. Statistical analysis was conducted utilizing a student's t-test comparing all percentage results in control and treatment groups.

Results

(R+)XK469 inhibits neuroblastoma cell cycle progression

MTT assay revealed proliferation inhibition with IC_{50} of 4 μ M, 29 μ M and 57 μ M in NBL-W-N, SMS-KCNR and LA1-55n cell lines, respectively (Figure 1A). (R+)XK469 also induced G₂/M cell cycle arrest (Figures 1B-D). Following treatment with (R+)XK469, the percentage of G₂/M arrested cells increased in LA1-55n and SMS-KCNR cells by 17.8 ± 1.27 – fold (p = 0.006) and 3.22 ± 0.869 – fold (p = 0.005), respectively. Cell cycle arrest was not detected following treatment of the NBL-W-N cells with (R+)XK469, suggesting that decreased proliferation seen in this cell line is likely due to an increase in apoptosis.

Treatment with (R+)XK469 inhibits neuroblastoma tumor growth *in vivo*

After ten days of treatment the average tumor size was 328 mm³ in experimental animals compared to 2203 mm³ in controls (p=0.009) (Figure 2).

Discussion

Outcome remains poor in children with high-risk neuroblastoma despite multi-modality treatments, and there is no known curative therapy for relapsed high-risk disease [5]. Novel agents are being tested in pediatric Phase I and II studies, and some lead to responses or prolonged stable disease in small patient cohorts. Our patient was enrolled on an institutional Phase I study designed to test (R+)XK469 in adult patients with recurrent solid tumors. The study was amended to allow minors who were 14 years or older or weighed greater than 45 Kg with relapsed solid tumors to be treated at 80% of adult MTD. Based on prolonged disease stabilization in this patient, we conducted laboratory studies to evaluate anti-neuroblastoma activity of (R+)XK469. We found that (R+)XK469 inhibited neuroblast proliferation, caused G2 cell cycle arrest and significantly inhibited neuroblastoma xenograft growth. These preclinical results, along with the response seen in our patient, suggest that (R+)XK469 may be effective in the treatment of high-risk neuroblastoma. However, due to a lack of clinical response in adults, (R+)XK469 is not being further developed.

In vitro, (R+)XK469 inhibits topoisomerase II β [6], causes G₂ arrest by inhibiting cyclin B1 ubiquitination [7], and p53 activation [8]. It causes cell death via an autophagy pathway in L1210 leukemia cells [9], and apoptosis through caspase 3 activation, and up-regulation of p53-dependent proteins including Bax, p21 and Gadd 45 [8,10] in several cancer cell lines. Preclinical *in vivo* studies have demonstrated (R+)XK469 activity against various murine models of adult cancers [11].

Phase 1 studies show that (R+)XK469 has a longer half-life in humans than in rats (63 hours vs 13.2 hours) [12], and efficacious doses are well-tolerated in humans when given once over a 21 day period [12]. Prolonged disease stabilization has been seen in patients with AML (n=5) and squamous cell carcinoma (n=1) [2,12]. One patient with metastatic nasopharyngeal carcinoma had a partial response and complete remission was reported in one AML patient [2,12].

Myelosuppression is the most commonly encountered toxicity [2,12], and our patient primarily experienced prolonged neutropenia and thrombocytopenia. With (R+)XK469 dose reduction, 28 days was required for count recovery. It is likely that the intensive multi-modality primary treatment that this patient received may have compromised her bone marrow function, and contributed to severity of myelosuppression.

Although this patient eventually progressed, her disease remained stable with a good quality of life for 14 months with (R+)XK469 monotherapy. Her participation in a phase I study initially limited to adult patients was facilitated by a unique collaboration between adult and pediatric oncology programs at our institution. Although we were only able to enroll one pediatric patient before study closure, this patient's clinical response, combined with promising preliminary *in vitro* and *in vivo* data suggest that clinical trials with agents that have similar mechanisms of action are warranted. There are likely other experimental agents effective in pediatric cancers that may not be fully evaluated because of lack of efficacy in adult diseases.

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Figure 1.

Figure 2.