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Copper-CX-5461: A novel liposomal formulation for a small molecule rRNA synthesis inhibitor

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ABSTRACT

CX-5461 is currently in Phase I/II clinical trials for advanced hematologic malignancies and triple negative or BRCA-deficient breast cancer. The compound is currently administered to patients intravenously (i.v.) at low pH (3.5) due to solubility challenges. Reliance of low pH to enhance solubility of CX-5461 can adversely impact pharmacokinetics, biodistribution and therapeutic potential. We have addressed this solubility issue through a formulation method that relies on the interactions between CX-5461 and copper. Copper binds CX-5461 through the nitrogens of the pyrazine ring. Here, we describe synthesizing this copper-complexed CX-5461 (Cu(CX-5461)) within liposomes. CX-5461 was added to copper-containing liposomes and incubated at 60 °C for 30 min. The pharmacokinetics of CX-5461 was assessed in mice following a single i.v. injection at 30 mg/kg. Efficacy studies were completed in multiple subcutaneous mouse xenografts as well as in a bone marrow engraftment model of acute myeloid leukemia (AML). The novel Cu(CX-5461) formulation was stable at pH 7.4 and exhibited increased plasma circulation longevity, increasing the total exposure to CX5461 by an order of magnitude. Cu (CX-5461) was more active than CX-5461 in AML models *in vivo*. In HCT116-B46 and Capan-1 solid tumour models that are BRCA-deficient, the Cu(CX-5461) formulation engendered activity that was comparable to that of the low pH CX-5461 formulation. We have generated the first Cu(CX-5461) formulation suitable for i.v. administration that is more efficacious than the existing low-pH formulation in pre-clinical models of AML. The Cu(CX-5461) formulation may serve as an alternative formulation for CX-5461 in BRCA-deficient cancers.

1. Introduction

Ribosome biogenesis is a fundamental cellular process that controls the production of ribosomes, the protein factories of the cell [1, 2]. Uncontrolled cancer cell proliferation in both hematological and solid tumours is supported by increased ribosomal RNA (rRNA) synthesis through increased activity of RNA polymerase I (Pol I). This is required to meet the abnormally high demand for protein synthesis in rapidly growing cells as proteins make up 80–90% of a cell's dry mass [3–6]. In fact, deregulated rRNA synthesis is a result of altered expression of many commonly known oncogenes and tumour suppressors such as ERK, MYC, PTEN, and p53 [2, 3, 7, 8]. It can be concluded that rRNA synthesis may be one of the most important therapeutic targets in cancer cells.

While some of the most commonly used anticancer drugs (*e.g.* cisplatin and 5-fluorouracil) are known to have secondary mechanisms of action that lead to inhibition of rRNA synthesis, there is currently no approved drug that directly targets Pol I transcription [3, 9]. CX-5461 is a novel compound that selectively inhibits RNA Pol I by disrupting the interaction between the SL1 protein and the rDNA promoter, which is vital to the recruitment and formation of a multiprotein complex with RNA Pol I [10, 11]. It is believed that in hematological malignancies, inhibition of rRNA transcription by CX-5461 induces nucleolar stress, activating wild-type p53 which ultimately causes cancer cells to undergo apoptosis while normal cells are spared [11]. CX-5461 is also very potent against solid tumours, with half-maximal inhibitory concentrations (IC_{50}) in the nanomolar range against most cancer cell lines [10]. This potent activity is not p53-dependent [10]. Perhaps most

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interestingly, recent studies demonstrated that CX-5461 is particularly potent against BRCA-deficient cancers [12]. In this context, Xu et al. discovered that CX-5461 is acting primarily as a DNA damaging agent through stabilization of G-quadruplexes.

CX-5461 is currently in Phase I clinical trial in Australia for patients with advanced hematologic malignancies [13, 14] and is being assessed in a Phase I/II clinical trial in triple negative or BRCA-deficient breast cancer in Canada (NCT02719977). Thus far, most published pre-clinical studies associated with this compound focused on subcutaneous xenograft models with CX-5461 given either orally or intraperitoneally (i.p.). Although the compound is orally bioavailable (24% in mice) [15], CX-5461 is being administered intravenously in the human clinical trial. CX-5461 is a sparingly soluble compound and in order to develop a formulation suitable for intravenous applications the drugs was solubilised in 50 mM sodium phosphate (pH 3.5). Such low pH phosphate buffers can engender acute toxicities due to irritation while being administered [16]. Furthermore, the injected compound may precipitate when equilibrated to physiological pH. While the compound is extremely potent *in vitro*, activity *in vivo* when given orally or i.p. is associated with either high dosages (125 mg/kg weekly) [17] or frequent dosing (25 mg/kg, 5 days a week) [15], which could be associated with toxicity issues as well as unnecessarily high exposure to the compound as a result of formulation issues. The presence of an electron-deficient carbonyl group of the amide within the structure of CX-5461 (Fig. 1) also suggests potential instability of the compound under physiological conditions. We believe that the solubility and stability issues can be addressed by reformulating CX-5461. We have demonstrated that CX-5461 coordinates with Cu^{2+} or Zn^{2+} *via* a bidentate binding motif [18]. The complexes did not have any effect on the anticancer activity of CX-5461, but the coordination with the metal ions enhanced the ligand's solubility at physiological pH [18]. To investigate the utility of Cu(CX-5461) *in vivo*, we have developed a novel formulation reliant on the synthesis of the copper complex within liposomes (14). The resulting liposomal Cu(CX-5461) formulation (referred hereafter as Cu(CX-5461)) demonstrated superior efficacy in subcutaneous and intravenous models of AML and comparable therapeutic activity as CX-5461 in BRCA-deficient xenografts.

2. Materials and methods

2.1. Lipids and chemicals

CX-5461 was purchased from Selleck Chemicals (Houston, TX). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol (Chol) and Sephadex G-50 were obtained from Sigma-Aldrich (St. Louis, MO). Sucrose and chloroform were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Tritiated [^{3}H] cholesteryl hexadecyl ether (CHE) was obtained from PerkinElmer (Waltham, MA). All other chemicals were purchased from Sigma-Aldrich as analytical grade reagents.

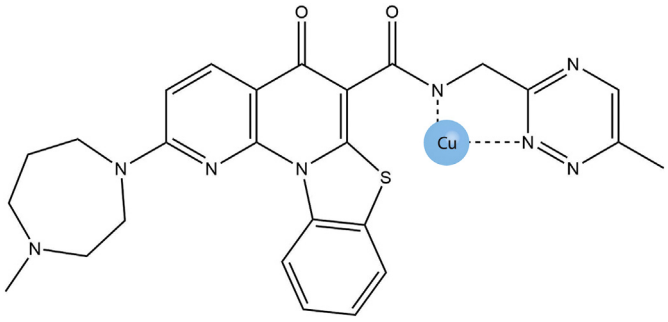

Fig. 1. Cu(CX-5461) can be synthesized in lipid-based nano-scale reaction vessels. The structure of CX-5461 along with the previously proposed coordination of Cu(II) ions is shown.

2.2. Mouse strains

RAG2-M, NOD-scid gamma (NSG), and NOD-congenic (NRG) mice, which harbor *Rag1*^{null} and *IL2rγ*^{null} mutations, were obtained from the British Columbia Cancer Agency Joint Animal Facility breeding colony and maintained in a pathogen-free environment, housed in groups of four. NRG breeders were purchased from The Jackson Laboratory (Bar Harbor, ME) and replenished regularly. All animal studies were completed under A14-0290, an animal care protocol approved by the Institutional Animal Care Committee (IACC). The IACC for studies conducted at BC Cancer's Vancouver Research Centre is managed and run through the University of British Columbia. The studies completed are in accordance to the Canadian Council of Animal Care (CCAC) and Dr. Bally's group is meeting ARRIVE guidelines.

2.3. Cell culture and reagents

MV-4-11 biphenotypic B myelomonocytic leukemia cells were purchased from ATCC (Manassas, VA) and maintained at 37 °C and 5% CO_2 in IMDM (Life Technologies, Burlington, ON) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 2 mM L-glutamine (Life Technologies). MV-4-11 cells have a doubling time of approximately 18–20 h and only cells that were cultured within the first 20 doubling times were used. Wild-type HCT116 human colorectal carcinoma cells (HCT116-WT) were purchased from ATCC. HCT116 BRCA2 knockout cells (HCT116-B46) were generously provided by Dr. Sam Aparicio's laboratory at the BC Cancer Agency. The knockout cell line was originally created by Dr. Carlos Caldas' laboratory (University of Cambridge). Capan-1 pancreatic ductal adenocarcinoma cell line was obtained from ATCC. Both HCT116 cell lines and Capan-1 cells were cultured in McCoy's 5A medium (Stemcell Technologies (Vancouver, BC) supplemented with 10% FBS and 2 mM L-glutamine. All cells used for *in vivo* studies were tested mycoplasma negative. For adherent cell lines, only cells between passages #3 and #10 with 80–90% confluence were harvested for *in vivo* studies.

2.4. Preparation of liposomes

DSPC/Chol (55:45; mol:mol) solutions were prepared by dissolving the lipids at the desired ratio in chloroform. [^{3}H]-CHE was added as a non-exchangeable and non-metabolized radioactive lipid tracer [19]. The organic solvent was evaporated under a stream of nitrogen gas and the lipid films produced were dried under vacuum to remove any residual solvent. The dried lipid films were rehydrated with 300 mM $CuSO_4$ at 65 °C and the resulting suspension was subjected to five freeze-thaw cycles in liquid nitrogen followed by 10 extrusions through two-stacked 80 nm polycarbonate filters (Evonik Transffera Nanosciences, Vancouver) at 65 °C. Un-encapsulated copper was removed by passing the liposome solution down a Sephadex G-50 size exclusion column equilibrated with SHE (300 mM Sucrose, 20 mM HEPES, and 15 mM EDTA, pH 7.4) buffer. The external buffer was then exchanged by size exclusion chromatography (SEC) into 50 mM sodium phosphate (pH 3.5).

2.5. Synthesis of Cu(CX-5461) in liposomes

CX-5461 was solubilised in 50 mM sodium phosphate (pH adjusted to 3.5 with 3% phosphoric acid) at 5 mg/mL and filter-sterilized through a 0.22 μm filter (EMD Millipore, Billerica, MA). The pre-solubilised drug was then added to the copper containing liposomes such that the final CX-5461 to liposomal lipid ratio was 0.2 (mole ratio). The solutions was incubated for 30 min at 60 °C to facilitate movement of CX-5461 across the lipid bilayer where it could then react with the entrapped copper to form Cu(CX-5461); a reaction that could be detected easily by a change in the optical properties of the solution (appearance of blue colour consistent with an increase in absorbance at