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Research article

The novel HSP90 inhibitor NVP-AUY922 shows synergistic anti-leukemic activity with cytarabine *in vivo*

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ABSTRACT

HSP90 is a molecular chaperone essential for stability, activity and intracellular sorting of many proteins, including oncoproteins, such as tyrosine kinases, transcription factors and cell cycle regulatory proteins. Therefore, inhibitors of HSP90 are being investigated for their potential as anti-cancer drugs. Here we show that the HSP90 inhibitor NVP-AUY922 induced degradation of the fusion oncoprotein FOP2-FGFR1 in a human acute myeloid leukemia (AML) cell line, KG-1a. Concordantly, downstream signaling cascades, such as STAT1, STAT3 and PLCγ were abrogated. At concentrations that caused FOP2-FGFR1 degradation and signaling abrogation, NVP-AUY922 treatment caused significant cell death and inhibition of proliferation of KG-1a cells *in vitro*. In an animal model for AML, NVP-AUY922 administrated alone showed no anti-leukemic activity. However, when NVP-AUY922 was administered in combination with cytarabine, the two compounds showed significant synergistic anti-leukemic activity *in vivo*. Thus NVP-AUY922 and cytarabine combination therapy might be a prospective strategy for AML treatment.

1. Introduction

Heat shock protein 90 (HSP90) belongs to a family of molecular chaperones with at least three other members, HSP27, HSP40, and HSP70, all under transcriptional regulation of the transcription factor heat shock factor 1 (HSF1). HSP90 is critically involved in mediating the stability and function of proteins crucial for cell survival. It has key roles in folding of nascent polypeptides as well as folding and assembly of multimeric protein complexes [1]. It is involved in the regulation of stability and activation of a vast of signaling proteins (http://www.picard.ch/downloads/Hsp90interactors.pdf). Inhibition of HSP90 leads to misfolding, ubiquitination and subsequent proteosomal degradation of client proteins [2,3]. HSP90 clients that have been transformed to oncoproteins due to genetic aberrations have been shown to require permanent assistance of HSP90, due to the impaired stability of such proteins compared to their wild-type counterparts [4,5]. Moreover, in neoplastic cells the expression of HSPs is often increased and this is considered as a stress phenotype of malignant diseases. Therefore, HSP90 is considered to be an important co-factor for the development and progression of the malignant phenotype and a potential drug target for anti-cancer therapy [1,6].

Chromosomal translocation of the *fibroblast growth factor receptor (FGFR)* genes is found in several different types of cancer, including glioblastoma and myeloproliferative syndrome/stem cell leukemia/lymphoma syndrome (EMS/SCLL) [7–10]. EMS/SCLL, a condition, which normally within a year after diagnosis transforms into acute myelogenous leukemia and/or lymphoma, is a result of reciprocal chromosomal translocations of the *FGFR1* gene, generating permanently active tyrosine kinase fusion proteins that constitutively stimulate cell proliferation and survival. At least 11 fusion partners of the *FGFR1* gene holding these properties have been identified in patients [11]. KG-1a, a cell line developed from a human myeloid leukemia [12], possesses a chromosomal rearrangement of the *FGFR1* gene and the *FGFR1OP2* gene (FGFR1 Oncogene Partner 2; FOP2). The *FGFR1OP2* gene encodes an unrelated protein of unknown function comprising four putative coiled-coil domains. The fusion protein (FOP2-FGFR1) consists of the N-terminal part of FOP2, comprising 132 amino acids,

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including coiled-coil domains, while the FGFR1 part of the protein comprise 394 amino acids including the entire tyrosine kinase domain [13]. The chimeric gene encodes a 60 kDa soluble fusion protein localized in the cytosol, and possibly in the nucleoplasm. The tyrosine kinase domain of FOP2-FGFR1 is constitutively active. The coiled coil domain in FOP2-FGFR1 results in dimerization/oligomerization and is presumably the reason for the constant tyrosine kinase activity [13]. FOP2-FGFR1 is critically involved in the pathogenesis of EMS/SCLL [14–16]. Therefore the KG-1a cell line, the only FOP2-FGFR1 driven cell line available, provides a unique model for studying oncogenic signaling mediated by FGFR fusions proteins as well as EMS/SCLL derived leukemias/neoplasms.

Patients with 8p11 myeloproliferative syndrome, including those harboring the FOP2-FGFR1 fusion, have a poor prognosis in spite of aggressive chemotherapy, with only few patients achieving long-term clinical remission after stem cell transplantation [9]. Studies using the KG-1a cell line have shown that pharmacological inhibition or depletion of FOP2-FGFR1 by specific siRNA results in impaired phosphorylation of several key signaling proteins such as STAT1 and STAT3 as well as growth of the KG-1a cells, and also induced apoptosis [14]. Previously, we have shown that two natural HSP90-inhibitors geldanamycin and radicicol and also the geldanamycin-derived less toxic, synthetic analog, 17-AAG (tanespimycin) impaired the stability of the oncogenic fusion protein FOP2-FGFR1 in KG-1a cells, and indicated that the oncoprotein was addicted to HSP90 for its stability [17]. However, 17-AAG as well as alvespimycin (17-DMAG, a water-soluble analog of tanespimycin) still possesses clinical disadvantages, including toxicity and formulation challenges [18–21]. At present, clinical trials of around 15 different HSP90 inhibitors are being conducted in various cancers including acute lymphoblastic leukemia (ALL), non-small-cell lung cancer (NSCLC), gastrointestinal stromal tumor (GIST), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL) (http://www.clinicaltrails.org), [6,22–25].

NVP-AUY922 is a second generation synthetic, small molecule HSP90 inhibitor with improved physiochemical and pharmacological properties. Just as geldanamycin and radicicol, NVP-AUY922 inhibits the ATP-ase activity of HSP90 by interacting with the ATP binding site at the N-terminal part of the protein. NVP-AUY922 has shown promising anti-tumor effects in prostate, breast, gastric and non-small cell lung cancer *in vivo* [26–31].

Cytarabine (ara-C) is a nucleoside analog chemotherapeutic agent. It that has been an important anti-cancer drug in several hematologic malignances including CLL and AML as well as some solid tumors over the last four decades [48]. AML is a heterogeneous malignant disease able to develop several protective mechanisms against cytarabine. Therefore a cure of such cancer is still elusive.

Here we show that NVP-AUY922 impairs the stability of FOP2-FGFR1 and therefore inhibits proliferation of KG-1a cells and cause cell death *in vitro*. Moreover, combined treatment of cytarabine and NVP-AUY922 shows synergistic anti-leukemic activity *in vivo*.

2. Materials and methods

2.1. Chemicals and reagents

The mesylate salt of NVP-AUY922 was provided by NOVARTIS (Basel, CH). Dimethyl sulfoxide (DMSO) was from Sigma-Aldrich. Mini-PROTEAN® TGX™ precast gels and Trans-Blot® Turbo 0.2 μm PVDF™ was purchased from BIO RAD. Amersham™ ECL™ Prime Western Blotting Detection Reagent (RPN 2232) was from GE Healthcare, Cytarabine™ was from Pfizer, NY, US.

2.2. Antibodies

The primary antibodies used in this study are listed with their catalog number indicated in parentheses: rabbit anti-FGFR1 (2144) were from Epitomics, mouse anti-phospho-FGFR (3476), rabbit anti-phospho-STAT1 (Y701) (9171), rabbit anti-phospho-FGFR (Y653/654) (3471), mouse anti-STAT1 (9176), rabbit anti-STAT3 (9132) and rabbit anti-phopho-STAT3 (Tyr705) (9131) were from Cell Signaling, mouse anti HSP70 (ab-6535) and mouse anti-GAPDH-HRP (ab-9482) were from Abcam, rabbit anti-Raf1 (sc133), rabbit anti-phospho PLCγ (Y783) (sc-12943-R), and mouse anti-PLCγ (sc-7290) were from Santa Cruz Biotechnology. Secondary antibodies were from Jackson Immuno-Research Laboratories.

2.3. Cell lines

The KG-1a cell line was obtained from ATCC. The KG-1a cells were grown in RPMI-1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. The cells were cultured in 5% CO_2 at 37 °C. The cells were seeded one day prior to the experiment.

2.4. Immunoblotting

Cells were lysed in 50 μl 2x SDS sample buffer and boiled at 96 °C for 10 min before separated by SDS-PAGE and transferred on to a PVDF-membrane. The membrane was incubated with primary antibodies followed by secondary antibodies (HRP-conjugated) and protein-bands were detected using Amersham™ ECL™ Prime Western Blotting Detection Reagent or Super Signal West Dura Chemiluminescent Reagent (Thermo) and developed on a BIO RAD Molecular Imager ChemiDoc XRS+ system.

2.5. Cell viability test

50000 KG-1a cells were treated with 1000 nM NVP-AUY922 or DMSO (control). The cell viability was measured 55 and 80 h after treatment using the Countess™ automated cell counter from Invitrogen™, analyzing both the number of viable as well as dead cells on the basis of trypan blue staining. For statistical analysis, a *t*-test was performed for three individual experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

2.6. Animals and systemic AML model

Male and female NOD/SCID gamma mice, bred at the nude rodent facility at the Norwegian Radium Hospital were used. The animals were maintained under specific pathogen-free conditions, and food and water were supplied ad libitum. The animal studies here included were performed according to specific protocols approved by the animal care and use committee at the Norwegian Radium Hospital, Oslo University Hospital, in compliance with the National Committee for Animal Experiments and the Federation of Laboratory Animal Science Associations (FELASA) guidelines on animal welfare.

For the establishment of systemic AML in mice, 1 million KG-1a cells suspended in serum free RPMI 1640 cell growth medium, were injected into the lateral tail vein. Three days after inoculation of tumor cells, the animals were divided into groups and treated with either 5% Glucose (control), cytarabine or/and NVP-AUY922 at indicated doses and schedules. Treatment schedules for NVP-AUY922 were derived from a previous study [29]. Cytarabine and NVP-AUY922 were dissolved in 5% glucose, to obtain final injection volumes of 0.1 ml/10 g. Controls were administered intravenously, NVP-AUY922 was administrated intravenously or given as intraperitoneal injections, whereas cytarabine was given as

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