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Determination of cellular uptake and intracellular levels of *Cenersen* (Aezee[®], EL625), a *p53* Antisense Oligonucleotide in Acute Myeloid Leukemia Cells

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Abstract

TP53 encodes for tumor protein *p53*. The suppression of *p53* protein results in interruption of DNA repair mechanisms in dividing malignant cells thereby increasing the DNA damage and activating *p53*-independent mechanisms of apoptosis. This ultimately may translate into enhanced cytotoxic effects of standard chemotherapy. Based on this rationale, *Cenersen* a phosphorothioate oligonucleotide antisense to *p53*-mRNA was synthesized and tested in clinical trials for patients with acute myeloid leukemia (AML). An important component of *Cenersen* clinical development is to develop a sensitive and specific method to quantify plasma and intracellular levels of *Cenersen* in different biologic matrices in order to determine tissue and intracellular distribution of the parent compound and its metabolites. Ultimately, this will allow us to determine pharmacokinetic and pharmacodynamic relationship for dose-effect correlation and design effective regimen to be rapidly translate into the clinic. An ELISA-based assay was adapted for assay development and validation of *Cenersen* in mouse plasma and cell lysate. Cellular uptake of *Cenersen* was studied in MV4-11 and KASUMI-1 AML cell lines. Real-time RT-PCR was used to measure *P53*-mRNA expression changes in treated cells. The assay had a limit of quantification of 35pmol/L in mouse plasma. Within-day and between-day precision of <15% and accuracy nearly 100% were observed in a linear range of 10-2000pmol/L ($R^2=0.99$) in AML cell lysate. The selectivity of this assay examined as cross-reactivity with its 3' N-1, 3' N-2-metabolites, was 16.8% and 0.4%, respectively, and with its mismatch and the scramble oligonucleotides was 0.06% and 0.4% respectively. *Cenersen* was stable in mouse plasma up to 8 hrs at 37°C°. When exposed to 0.1-1µmol/L *Cenersen*, MV4-11 and KASUMI-1 cells showed intracellular concentration in the range of 9.97-45.34nmol/mg protein and 0.1-2.1nmol/mg protein, respectively. Successful downregulation of *p53*-mRNA expression was observed in *Cenersen* treated cells. This ELISA-based assay was applicable to plasma and intracellular concentration measurement of *Cenersen*. Assessment of achievable concentration of *Cenersen* in different biologic matrices will be useful

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to elucidate the biological and clinical activity of this promising drug and define its recommended dose in future clinical trials.

Keywords

Cenersen; p53 Antisense; Acute Myeloid Leukemia (AML); enzyme-linked immunosorbent assay (ELISA); Intracellular uptake

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous hematologic malignancy characterized by block of differentiation and rapid proliferation of leukemic blasts, which then accumulate in bone marrow and blood leading to hematopoietic failure [1]. Today still most patients with AML die because of refractoriness or relapse after initial treatment [1, 2]. Therefore, novel therapeutic approaches are needed to improve outcome of these patients.

Antisense oligonucleotides are short, synthetic DNA molecules that have sequences complementary to their target mRNAs. Antisense oligonucleotides hybridize to their mRNA complements, preventing the sequence of the target gene being converted into a protein, thereby blocking the gene expression [3, 4]. Antisense therapy is a gene-targeting strategy that has been actively examined in clinical trials in the last several years, and because it aims at specific target may also hold promise for incorporation to personalized treatment strategies. *P53* is a tumor suppressor gene whose expression is induced by DNA damage. Once expressed, the p53 protein acts as a transcription co-factor and induces upregulation of *p53*-dependent genes, synthesis of cell cycle checkpoint proteins, cell arrest in G1, and eventually apoptosis [5]. *P53* is found most frequently to be mutated in human cancer [6]. Although known as a tumor suppressor gene, it is thought that the mutant forms of *P53* have a dominant oncogene features [7, 8]. Mutations in one allele of the *P53* tumor-suppressor gene have been widely reported in cancer [5], and participate in aberrant proliferation, survival and mechanisms of treatment resistance of malignant cells.

Although counterintuitive, the use of antisense to *p53* such as *Cenersen* appears to have clinical benefit in cancer. The rationale behind the development of this compound is based on the experimental evidence that total suppression of p53 protein in malignant cells results in interruption of partially active DNA repair and an increase in DNA damage. This ultimately activates p53-independent mechanisms of apoptosis, which culminates cell death. As a result, *Cenersen* sensitizes AML cells, to low levels of DNA-damaging agents, including chemotherapeutic agents used at doses that have minimal or no effect on leukemia cells in the absence of *Cenersen*. Wild-type, as well as *p53*-mutated melanoma cell lines treated with *p53* antisense oligonucleotides, showed significant decrease in growth arrest and subsequently massive cell death [9]. Increased ionizing radiation induced apoptosis was observed in MCF7 breast cancer cells, when treated with antisense oligonucleotide targeting *P53* [10]. Antisense against *P53* was also found to be selectively cytotoxic to primary myeloid blasts and therefore may be therapeutically useful in AML [11]. Indeed, *Cenersen* appears to increase the cytotoxic effect of chemotherapy due to increase in p53-independent apoptotic pathways activated by the unrepaired chemotherapy-dependent DNA damage.

Based on these preclinical data, phase I and II trials for the *p53* antisense *Cenersen* were conducted in AML and chronic lymphocytic leukemia (CLL) and concluded that *p53* antisense in combination with chemotherapy is feasible and showed preliminarily encouraging clinical responses rates with acceptable toxicity [12-14]. Unfortunately, none of these trials with *Cenersen* includes successful pharmacokinetic and correlative studies, possibly because of the lack of suitable analytical methods. In this study, we established and validated a novel assay to measure plasma, urine and intracellular concentration of *Cenersen* *in vivo*. At the same time, we investigated the activity of *Cenersen* in AML cell lines in order to allow for potential pharmacokinetics and pharmacodynamics (PK/PD) correlation.

2. Materials and Methods

2.1. Reagents

Cenersen was kindly provided by Eleos Inc.; the specific nucleotide sequence is 5'-d[P-Thio](CCCTG CTCCC CCCTG GCTCC)- 3. Both the 3' -end (3' N-1, 3' N-2, 3' N-3) and the 5' - end (5' N-1) putative metabolites of *Cenersen*, the mismatch and the scramble were purchased from Integrated DNA Technologies (Coralville, IA, USA). All probes used in the assays here were custom-synthesized by Integrated DNA Technologies. The purity and identity of each oligomer were verified by HPLC/UV/MS (ion trap mass spectrometer model LCQ, Finnigan, San Jose, CA, USA).

2.2. Cell culture and drug treatment

KASUMI-1, MV4-11, and K562 AML cell lines were grown in RPMI medium supplemented with 10% fetal bovine serum or 20% fetal bovine serum (KASUMI-1) at 37°C. AML cells were incubated with *Cenersen* or PBS as a negative control. Treated cells were harvested at 24, 48, 72 hours.

2.3. A Two-step hybridization-ligation enzyme-linked immunosorbant assay (ELISA) Assay

We have adopted and optimized a previously reported hybridization-based fluorescence ELISA for antisense oligonucleotides developed in our laboratory [15] for the measurement of *Cenersen*. Briefly, the antisense hybridizes to a capture template with an overhang 3' end, which in turn hybridizes to a detection 3' digoxigenin-probe, which is then ligated to the analyte. The addition of antidigoxigenin-alkaline phosphatase (AP) and the Attophos substrate generates a measurable fluorescence intensity that is proportional to the quantity of the analyte.

2.4. Real-time reverse transcription–polymerase chain reaction to measure pre and post-*Cenersen* treatment *P53* messenger RNA levels

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) for *P53* was performed on an ABI 7900. TaqMan Universal Master Mix, primers, and labeled probes were used according to the manufacturer's procedure (Applied Biosystems, Foster City CA), using *18S* RNA as an endogenous control. Mean threshold cycle (Ct) values were calculated by SDS 2.3 software (Applied Biosystems) to determine fold differences according to the manufacturer's instructions.

3. Results

3.1. Validation of the ELISA of *Cenersen* in plasma and cell extracts

Mouse plasma spiked with 2000 pmol/L *Cenersen* was diluted with 10% mouse plasma to establish standard curve dilutions ranged from 10 to 2000 pmol/L. The limit of detection (LOD) of the assay (defined as 3 times the standard deviation of noise level) was found to be 10 pmol/L and the limit of quantification (LOQ) (defined as 10 times the standard deviation of noise level) was found to be 35 pmol/L. A linear calibration curves of the fluorescence signal versus concentration within the range 10 to 200 pmol/L ($R^2 = 0.99$) and within the range 200-2000 pmol/L ($R^2 = 0.99$) were obtained (Fig.1). The mean within-day precision coefficients of variation (CVs) of the assay in mouse plasma at 50, 200, 500, 1000, 2000 pmol/L were found to be 12.86%, 9.94%, 4.79%, 15.07%, 10.33%, with corresponding accuracy values of 99.05%, 99.9%, 100.5%, 101.4%, 99.6%, respectively. The between days CVs of assay were found to be 23.9%, 16.7%, 7.01%, 14.86%, 15.57% for the 50, 200, 500, 1000, 2000 pmol/L standards, respectively with the corresponding accuracy values of 83.54%, 89.59%, 96.46%, 92.72%, 89.31% of the nominal concentrations.

K562 cell lysate spiked with 2000 pmol/L *Cenersen* was diluted with 10% cell lysate to establish standard curve dilutions ranged from 10 to 2000 pmol/L. Linear calibration curves of the fluorescence signal versus concentration ($R^2 = 0.99$) within the range 10 to 200 pmol/L ($R^2 = 0.99$) and within the range 200-2000 pmol/L ($R^2 = 0.99$) were obtained using K562 cell lysate (Fig.1). The mean within-day precision coefficients of variation (CVs) of the assay in cell lysate at 50, 100, 500, 1000, 2000 pmol/L were found to be 8.5%, 13%, 11.77%, 16.49%, 12.9%, with corresponding accuracy values of 112.52%, 117.99%, 111.33%, 113.9%, 101.99%, respectively. The between days CVs of assay were found to be 8.18%, 14.19%, 11.67%, 8.27%, 7.72% for the 50, 200, 500, 1000, 2000 pmol/L standards, respectively with the corresponding accuracy values of 115.05%, 106.71%, 112.19%, 103.71%, 98.08% of the nominal concentrations. When known concentrations of *Cenersen* were added to cell lysate, the limits of detection were found to be 6 pmol/L and the limits of quantification were 33 pmol/L.

3.2. The specificity of the assay

Although metabolism study for *Cenersen* has not been performed as yet, we expect that, similar to other antisense compounds, the 3'-end chain of *Cenersen* may be subjected to 3'-exonucleases to generate chain-shortened metabolites and less likely by 5'-exonucleases [16], both *in vitro* and *in vivo*. These potential metabolites may interfere with the quantification of *Cenersen*. Therefore, the cross-activity of *Cenersen* and its putative 3'-N-1, 3'-N-2, 3'-N-3, and 5' N-1 metabolites was tested in mouse plasma. We measured the fluorescence signal generated by four putative *Cenersen* metabolites (3' N-1, 3N-2, 3N-3, 5' N-1), mismatch and scramble and found The cross-reactivity of 3' N-1, N-2, and 5' N-1 were 16.78 % and 0.44 % and 87.11 %, respectively, and <0.1% with the 3' N-3, the mismatch and the scramble *Cenersen* (Fig.2).

3.3. The Stability of *Cenersen* in mouse plasma

We measured the stability of *Cenersen* in mouse plasma following incubation at 4 °C, 25 °C and 37 °C for 4, 8, 24 hours. Data showed that *Cenersen* is stable up to 8 hours at 4, 25, and 37 °C. However, the fluorescence decreased to almost 20 % at 24 hours compared with the initial level ($p < 0.001$). This significant decline in *Cenersen* stability at 24 hours was found in all incubation temperature (Fig. 3).

3.4. *Cenersen* intracellular concentration measurements

The intracellular concentrations were measured using our optimized highly sensitive ELISA. *Cenersen* intracellular concentrations were measured in MV4-11 and KASUMI-1 cells after exposure to 0.1, 0.5, 1 μ M *Cenersen* for 24 and 48 hours. At 24 hours of incubation, MV4-11 cells exhibited the following intracellular concentrations 9.9, 26.9, and 47.2 nM/mg protein when treated with 0.1, 0.5, 1 μ M, respectively (Fig.4). Similar intracellular concentrations were obtained at 48 hours following treatment. Lower intracellular concentrations were instead found in KASUMI-1 cells treated with the same *Cenersen* concentrations. At 24 hours of incubation, KASUMI-1 cells showed the following intracellular concentrations 0.1, 1.15 and 2.11 nmol/mg protein and did not significantly change at 48 hours.

3.5. *p53* mRNA expression in AML cell lines treated with *Cenersen*

To confirm the biological function of *Cenersen in vitro* we treated AML cells with different concentrations of *Cenersen* and evaluated the downregulation of *P53* mRNA. In MV4-11 cells, approximately 30% down-regulation of *P53* mRNA was observed at 24 and 48 hours following 5 μ M *Cenersen* exposure compared to untreated cells ($P = 0.01$ and $P = 0.004$ respectively). Under the same conditions, approximately 50% reduction of *P53* mRNA levels was attained in K562 cells compared to untreated ($P < 0.001$, $P = 0.001$ at 24 and 48 hours, respectively) (Fig 5).

4. Discussion

Cenersen is a phosphorothioate antisense oligonucleotide complementary to 20 bases within exon 10 of the *p53* mRNA. Following cellular uptake, *Cenersen* binds to *p53* mRNA which undergoes subsequent cleavage by ribonuclease H (RNase H) at the site to which *Cenersen* binds [17, 18]. Clinical evaluation of pharmacokinetic activities of *Cenersen* and its relationship with efficacy, toxicity require a specific, sensitive, and accurate quantification method in biological matrices. We developed and validated a sensitive and specific ELISA assay to measure the intracellular levels of *Cenersen*. Our method represents the first quantification assay for *Cenersen* and is ultra-sensitive with high precision and accuracy. We also successfully applied this assay to measure intracellular concentration of *Cenersen* in AML cell lines.

While this assay is not selective toward 5' metabolites; it has high sensitivity and selectivity toward 3'-end and detects the sequence that reaches to a single nucleotide resolution with a little or no cross-reactivity with the putative 3'-end metabolites. This method is applicable for pharmacokinetic-pharmacodynamic studies, since 5'-end metabolism is considered a

minor pathway [15, 19]. The ultrasensitivity of the ELISA assays allows accurate characterization of pharmacokinetics of *Cenersen*, such as the half-lives and clearance values. We conclude that this method provides a specific and valuable tool for the pharmacologic study and clinical development of *Cenersen* for future trials.

In initial phase I clinical trial, 16 patients with either refractory AML or advanced myelodysplastic syndromes were given *Cenersen* at doses of 0.05 to 0.25 mg/kg/h for 10 days by continuous intravenous infusion. The study has demonstrated the feasibility of *Cenersen* administration with limited toxicity [20]. However, the study failed to correlate *Cenersen* plasma concentration with clinically significant morphological response similar to what has been previously reported *in vitro* when similar concentrations were used. The study related this discrepancy to the difference levels of oxygen between the bone marrow and the *in vitro* long-term culture. Hence, according to the study, high *in vitro* oxygen levels have shown to promote and amplify the apoptotic activity of *Cenersen* [20]. Nevertheless, the study has utilized both liquid scintillation counting and a high-performance liquid chromatography as quantification methods for their pharmacokinetic analysis on blood and urine samples. These conventional approaches have several limitations when applied to clinical evaluation of pharmacokinetic behaviors of antisense drugs. Low sensitivity [limit of quantification (LOQ) >10 nM] and/or lack of selectivity toward metabolites limit their ability to fully determine the plasma pharmacokinetics and/or measure intracellular drug concentrations. This results in erroneous assessment of the pharmacokinetics (PK)/pharmacodynamics (PD) relationship following drug administration [15, 21]. Moreover, these methods require extensive sample processing prior to analysis and this may lead to less accuracy caused by matrix effects. In a phase II randomized study, *Cenersen* was co-administered with idarubicin either alone or in combination with cytarabine as salvage regimen in refractory and relapsed AML patients [13]. The results of this study concluded that *Cenersen* is well tolerated when combined with other chemotherapeutic agents and may have clinical efficacy [13]. *Cenersen* was also tested in a phase II study in combination with Fludarabine, Cyclophosphamide, and Rituximab in high risk chronic lymphocytic leukemia (CLL) patients (refractory patients with del 17p13 or somatic mutation of *TP53*). This combination showed preliminarily promising clinical responses rates with tolerable toxicity [14]. However, all these clinical trials lacked pharmacokinetic and pharmacodynamic correlative studies. Therefore, whether the inadequacy or the uncertainty of clinical benefit for *Cenersen* in AML was due to pharmacokinetic limitation or other factors is still unknown. Hence, it is crucial to determine whether a detectable intracellular amount of drug can be achieved, and to establish a correlation between intracellular levels and their biological effects. The assay we are reporting here has been successfully applied to measurement of antisense compounds investigated in clinical trials (G3139 [22], GTI-2040, and microRNAs [23]). Therefore we expect that utilizing this method for quantification of *Cenersen* levels in future clinical trials will provide better pharmacokinetic analysis to determine this drug efficacy, toxicity and ultimately dose-response relationship.

5. Conclusions

We developed and validated a sensitive and specific ELISA assay to measure the intracellular levels of *Cenersen*. This ELISA-based assay was applicable to plasma and

intracellular concentration measurement of *Cenersen*. Assessment of achievable concentration of *Cenersen* in different biologic matrices will be useful to elucidate the biological and clinical activity of this promising drug and define its recommended dose in future clinical trials.

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Abbreviations

AML	acute myeloid leukemia
AP	alkaline phosphatase
CV	coefficient of variation
ELISA	enzyme-linked immunosorbent assay
LOQ	lower limit of quantification
LOD	limit of detection
mRNA	messenger ribonucleic acid
PD	pharmacodynamics
PK	pharmacokinetics
RNase H	ribonuclease H

Highlights

- An ELISA-based assay was developed for clinical investigation of *Cenersen*.
- The developed assay was validated in mouse plasma and cell lysate.
- Cellular uptake of *Cenersen* was studied in MV4-11 and KASUMI-1 AML cell lines.
- The stability of *Cenersen* was evaluated in mouse plasma.
- Successful downregulation of *p53*-mRNA expression was observed in *Cenersen* treated cells.

Fig.1. Validation of the ELISA-based assay of Cenersen
a, representative standard curve of *Cenersen* in mouse plasma. *b*, representative standard curve of *Cenersen* in cell lysate. Each concentration was run in duplicates, and average was used for linear regression analysis. The mean fluorescence signal was plotted against *Cenersen* concentrations (pM).

Fig.2. Concentration-response curves of *Cenersen* and its possible 3' and 5' metabolites
Cenersen and oligomers at the 3' and 5' end ranging from 10 to 100,000 pmol/L were added into mouse blank plasma, and dose-response curves were constructed by nonlinear regression analysis since the curve is out of the linear range.

Fig.3. Cenersen Stability in mouse plasma

1nmol/L of *Cenersen* were added to mouse plasma and incubated at 4°C, 25°C, 37°C for 4, 8 and 24 hours. Each condition was run in duplicates, and the average was plotted.

Fig.4. Cenersen intracellular concentrations

MV4-11 and KASUMI-1 cells were treated with 0.1, 0.5, 1 $\mu\text{mol/L}$ *Cenersen* for 24 hours (gray) and 48 hours (black). Cells were then washed with PBS and lysed. *Cenersen* intracellular concentrations were measured by the validated ELISA and plotted against *Cenersen* concentrations used for cell treatment.

Fig.5. P53 mRNA expression in AML cells treated with *Cenersen*

Decrease in *P53* mRNA expression levels measured by qRT-PCR in MV4-11 cells (white) and K562 cells (black) at 24 and 48 hours post-treatment with 5μmol/L *Cenersen*.