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Research Article

ANTIPROLIFERATIVE ACTIVITY OF RECOMBINANT HUMAN INTERFERON ALPHA2B ON ESTROGEN POSITIVE HUMAN BREAST CANCER MCF-7 CELL LINE

Ratih Asmana Ningrum*, Popi Hadi Wisnuwardhani, Adi Santoso
Neng Herawati

Research Center for Biotechnology, Indonesian Institute of Sciences
Jalan Raya Bogor km 46
Cibinong, West Java, Indonesia, 16911

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*Corresponding author
Ratih Asmana Ningrum

Email:
ratih.asmana.ningrum@lipi.go.id

ABSTRACT

Indonesia is the top three countries with hepatitis and moreover has 40.000 cancer mortalities per year. Interferon alpha 2b (IFNα-2b) is a therapeutic standard for cancer and hepatitis B/C treatments. We developed recombinant human interferon alpha 2b (rhIFNα-2b) in methilotropic yeast *Pichia pastoris X-33.* The protein was produced as extracellular protein with 24 kDa in size. This research was aimed to characterize the protein based on its amino acid sequence and to study its antiproliferative activity on MCF-7 cell line. Amino acid sequencing by using MALDI TOF TOF mass spectrometry with trypsin as proteolytic enzyme identified protein as hIFNα-2b with 33% of amino acid coverage. The antiproliferative activity was determined by 3-[4.5-dimethyltiazol-2il]-2.5-diphenylltetrazolium bromide (MTT) assay. Based on several studies about the synergistic activity of rhIFNα-2b with other anticancer drugs, we combined our rhIFNα-2b with tamoxifen (tmx). The growth percentage of the cells after being treated with 1μM of tmx at various concentrations of rhIFNα-2b was compared with that of untreated cell. This study showed that the antiproliferative activity was dose-dependently. Cell viability assay with calcein and ethidium bromide-based staining by fluorescence microscope confirmed that the rhIFNα-2b had ability to inhibit proliferation of human breast cancer cell line MCF-7.

Keywords: human interferon alpha 2b, *Pichia pastoris*, antiproliferative and MCF-7

INTRODUCTION

hIFNα-2b is a glycoprotein that consist of 166 amino acids with O-glycosylated threonine at position 106. It has two disulfide bonds that are formed by cysteines at position 1 and 98 as well as 29 and 138. hIFNα-2b has been used as therapeutic protein on several cancer treatments. These include melanoma, renal cell carcinoma, AIDS related Kaposi's sarcoma (KS), follicular lymphoma, hairy cell leukemia, and chronic myelogenous leukemia (CML). Its antiproliferative activity on cancer cells covers direct and indirect activities. Direct activities are growth inhibition by cell cycle arrest, apoptosis, or differentiation. Indirect activities are activation of immune cells such as T cells and natural killer cells, inhibition of vascularization (antiangiogenesis), and induction of cytokines (Wang *et al.*, 2002; Bekisz, 2010; Jonash and Haluska, 2000).

The antiproliferative activity is initiated by activation of JAK STAT pathway which interference to MAPK pathway that control a variety of processes in the cell, such as proliferation, differentiation, survival, and apoptosis. hIFNα-2b can inhibit extracellular signal-regulated kinase (ERK) mitogen ERK kinase (MEK) pathway as a group of MAPK (Sarkar, 2003; Bekisz, 2010; Ningrum, 2014). rhIFNα-2b as antiproliverative protein is applied as monotherapy or combination therapy with other cancer drugs, such as vinblastine, 5-fluorouracil, tamoxifen, or interleukin-2 (Jonash and Halusca, 2000). The synergistic antitumor effects of a combination of interferon and tamoxifen on estrogen

receptor-positive and receptor- negative human tumor cell lines have been reported by Lindner and Borden (1997). Sequential treatment of MCF-7 cells in vitro with tamoxifen followed by IFN-α resulted in growth inhibition, suggesting that tamoxifen modulated the anticellular response to IFN-α.

We developed rhIFNα-2b in methilotropic yeast *Pichia pastoris*. The wildtype and Mut$^+$ X-33 strain was used as a host. Other reports used GS115 strain which has mutation in the histidinol dehydrogenase gene (his4) that prevents it to synthesize histidine. So, It allow the selection of expression vectors containing HIS4 upon transformation (Shi *et al.,* 2007; Ghosalkar *et al.,* 2008; Li *et al.,* 2007). Our rhIFNα-2b was produced as extracellular protein with 24.05 kDa in size containing polyhistidine tag and c-myc epitope in its C terminus. The yield on optimized condition was 28 mg/L (OD600= 2.45). The characterization has been performed based on molecular weight by using SDS PAGE and specific antibody recognition by Western blotting methods (Ningrum, 2013; Santoso, 2013; Herawati, 2014). This research was purposed to characterize the rhIFNα-2b based on amino acid sequence by using mass spectrometry MALDI TOF TOF and to study its in vitro antiproliferative activity. We studied the activity of our rhIFNα-2b in combination with tamoxifen on esterogen reseptor-positive MCF-7 cell line as a model.

MATERIAL AND METHODS

Clone, media and strain

Clone number 12 (methylotrophic yeast *Pichia pastoris* strain X-33 harboring hifnα-2b ORF) that has been selected by using 2000μg/mL of zeocin from our previous study was used (Ningrum *et al.,* 2013). BMGY (1% yeast extract, 2% peptone, 100mm potassium phosphate ph 6, 1.34% YNB, 1% glycerol) and BMMY (1% yeast extract, 2% peptone, 1.34% YNB and 0.5% methanol) were used in protein overproduction.

Overproduction, purification and characterization of rhIFNα-2b

Single colony was grown overnight in 50mL BMGY medium at 30°C and 250rpm until OD_{600}= 2-6. The culture was centrifuged at 1500 x g for 5min. The pellet was resuspended in 100 ml BMMY medium (OD_{600} = 1.0, the media was containing 0.5% methanol as inducer). The methanol induction was repeated at 24h of cultivation time. Harvesting was performed by centrifugation at 1500 x g for 5min at room temperature to collect the supernatant. Phenylmethylsulfonyl fluoride 1mM was added and used as protease inhibitor. Ultrafiltration was carried out by using Minimate™ TFF system (PALL) with molecular weight cut off was 10kDa. 100mL of supernatant was concentrated into 10mL. The concentrated fraction then purified by AKTA purifier 10 system using His trap column (GE Healthcare). 10mM of sodium phosphate containing 20mM imidazol and 500mM sodium chloride was used as washing buffer. Elution buffer composition was 10mM of sodium phosphate, 500mM imidazol and 500mM sodium chloride. Purified protein was characterized by SDS PAGE. Its concentration was quantified based on bicinchoninic acid assay (BCA protein kit, Pierce) by using various concentrations (25 to 2000μg/mL) of bovine serum albumin as standard. The purple colored product was read at 562nm. The SDS PAGE band slices of protein were dried and characterized based on amino acid sequence of rhIFNα-2b was performed by using MALDI TOF TOF (Proteomic Inc). The peptides were derived by trypsine cleavage.

Antiproliferation assay

Cell cultivation and treatments

MCF-7 cells (from mammalian cell culture laboratory, Indonesian Institutes of Sciences) were thawed and washed with 9 mL of DMEM medium containing penicillin (100units/mL) and streptomycin (100 mg/mL). Cells were grown in the same medium containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. After 90% of confluency, the cells were washed with phosphate buffer saline (1.15 g Na2HPO4; 0.2g KH2PO4; 8g NaCl and 0.2g KCl perliter, pH 7.2) and detached with 500μL of trypsin-EDTA(0.25% trypsin in 0.53mM EDTA) at 37°C for 5min. The cells were transferred into a 96 well (3000 cell/well) or 24 well plate (15.000/ well) for further study. The cells were grown overnight in DMEM media containing penicillin-

streptomycin with 5% FBS, washed with 100 µL of PBS and treated by 1 µM tamoxifen (Merck) overnight. Further treatment was applied by various concentration of hIFNα-2b for 5 days. We used rhIFNα-2b standard to validate the assay. The treatment conditions were based on a report by Lindner and Borden (1997) with some modifications.

MTT Assay

Treated cells in 96 well plates were washed twice with 100µL of PBS. 100µL of DMEM with 5% FBS containing MTT (with final concentration 0.5mg/mL) was added to each well. Cells were then incubated for 3h and the medium was discarded. Formazan crystals formed at the bottom of the well were dissolved in 100µL of SDS 10%. The cells were incubated for overnight. The reaction was stopped by 0.01 M HCl and dissolved formazan was measured at 570nm. The percentage of viable cell was compared to control (untreated cells). The experiments were done in triplicates in three dependent experiments (Septisetyani *et al.,* 2014). The statistical analysis was performed by student T test.

Viability Staining

Treated cells in 24 well plates were washed twice with 1mL of PBS and stained with 500µL of 2µM calcein AM and 4µM ethidium bromide. The cells were incubated at room temperature for 45min and observed under fluorescence microscope (Life technologies) at 485nm of excitation wavelenght. The experiments were done in triplicates in three dependent experiments.

RESULTS AND DISCUSSIONS

Overproduction, Purification and Characterization of rhIFNα-2b

Overproduction was performed by using optimal condition from previous research (Herawati, 2014). Molecular weight characterization of concentrated supernatant by SDS PAGE showed a protein band at 24kDa as its theoretical size. The different size of our protein compared to the native form was due to the presence of polyhistidine tag and c-myc epitope sequences at C-terminus. Purification of the rhIFNα-2b protein was performed based on interaction between Ni-NTA resin and polyhistidine tag at C-terminus of the protein. The impurities were removed by using 20mM of histide analog imidazole and 500mM of sodium chloride as previously reported by Bornhorst and Falke (2000). The chromatogram showed absorbance peak at elution fractions (C3 to C5) that corresponding to purified rhIFNα2b (Figure 1). The unpurified and purified rhIFNα-2b was shown in figure 2.

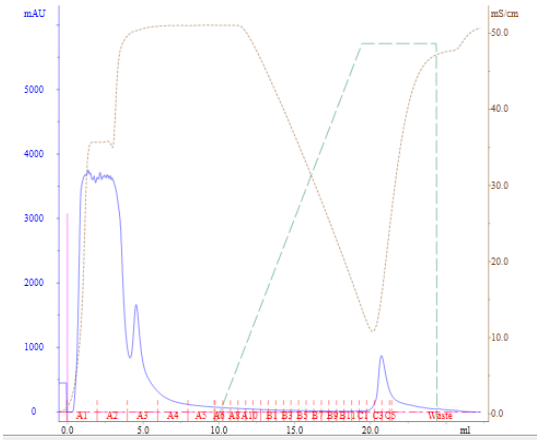
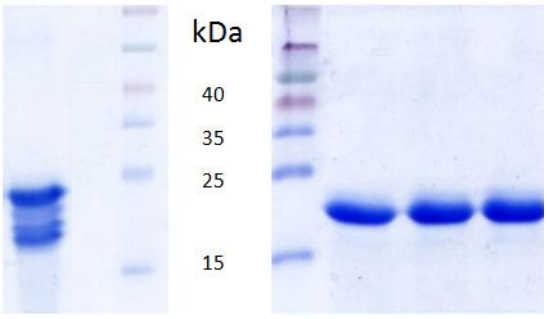


Figure 1. Purification of rhIFNα2b. A1-A10= flow through fractions; B1-B11= washing fractions and C1 to C5= elution fractions



A B

Figure 2. Characterization of rhIFNα-2b : A. Unpurified ; B. Purified

Protein identity characterization was performed by amino acid sequence determination. Trypsin was used as protease enzyme on MALDI TOF TOF mass spectrometry analysis. Peptide summary report identified the protein as hIFNα-2b. There were five peptide fragments which matched with hIFNα-2b sequence: K.EDSILAVR.K;

CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVL HEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMK EDSILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE

Figure 3. Amino acid sequence determination by MALDI TOF TOF and proteolytic cleavage site of trypsin in hIFNα-2b by using peptide cutter (Expasy). The identified residues were shown in red.

R.ITLYLKEK.K; R.SFSLSTNLQESLR.S; R.HDFGFPQEEFGNQFQK.A and K.DRHDFGFPQEEFGNQFQK.A. These fragments covered 55 amino acid residues or 33% of the hIFNα-2b sequence. Based on trypsin cleavage sites analysis by using Expasy peptide cutter software, there were 20 sites of trypsin cleavage in the hIFNα-2b sequence (Figure 3). Lower amino acid coverage found in this research may be caused by impurities in the sample. This research used one dimensional electrophoresis on sample preparation. According to Gonnet *et al* (2003), this kind of preparation may lead to more than one protein per gel bands so the suppression effect can occur and causing poor coverage of protein sequence.

Antiproliferation assay

There were many cancer drugs that have been previously applied in combination with hIFNα2-b in cancer treatments, such as leucovorin, vinblastine, 5-fluorouracil, tamoxifen, interleukin-2, retinoic acid or folinic acid (Taylor *et al.,* 1992; Jonash and Haluska, 2000; Lee *et al.,* 1992; Lindner *et al.,* 1997; Bernhard *et al.,* 1992). Tamoxifen is cell-permeable and reversible inhibitor of protein kinase C. It induces apoptosis in human malignant glioma cell lines and inhibits prostate cancer cell growth by induction of p21 protein. Tamoxifen is a potent synthetic anti-estrogenic agent. It is cytostatic for estrogen-dependent cell line. Estradiol binds to estrogen receptor (ER) in ER positive cell and induces

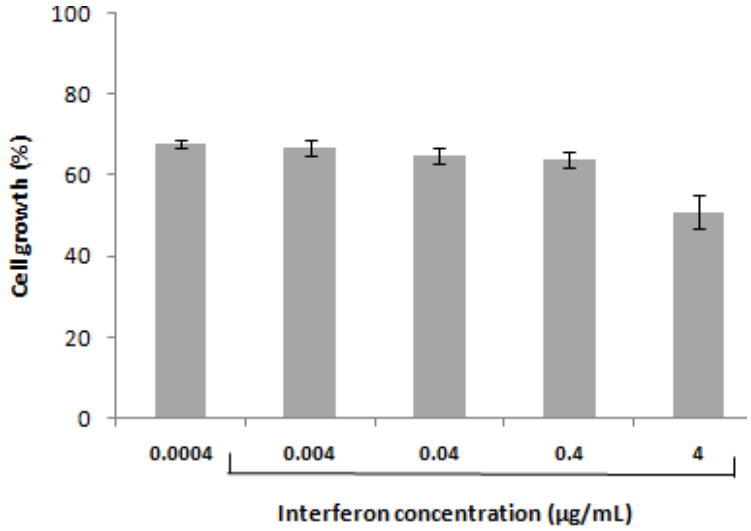


Figure 4. MCF-7 cell line growth inhibition by rhIFNα-2b (P <0.0001).

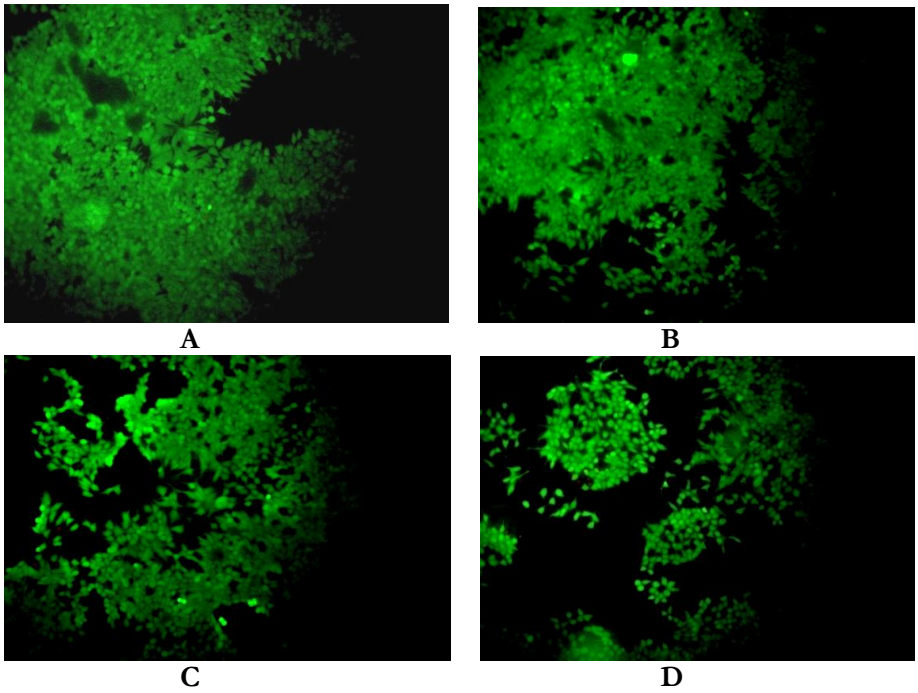


Figure 5. Calcein AM and Ethidium bromide-based staining of MCF-7. A=untreated, B, C, D= treated by tmx 1 µM, rhIFNα-2b standard and rhIFNα-2b in sequence.

transcription to promote growth. Tamoxifen competes with estrogen for binding to the receptor, so growth inhibition occurs (Lippman and Bolan, 1975). MCF-7 is human breast adenocarcinoma and ER positive cell line. We used MCF-7 as a model based on several studies which report that IFN-α has inhibitory effect on its proliferation and adhesion (Maemura *et al.,* 1999; Robinson *et al.,* 1990; Porta, 2004; Tiwari *et al.,* 1991 ; Lindner *et al.,* 1997).

Antiproliferative activity was performed as reported by Lindner and Borden (1997) with some modifications. Sequential treatment of

MCF-7 cell with tmx and rhIFNα-2b was performed. According to previous study, tmx can modulate the anticellular response to rhIFNα2-rather than the opposite. Since the IC50 is 100μM and to make sure that it has less cytostatic effect, as much as 1μM of tmx was applied (Lindner and Borden, 1997). After 5 days of treatment with rhIFNα-2b, the viable cells were undirectly counted by using MTT assay. Figure 4 showed that growth inhibition of MCF-7 cell line was dose dependent and the highest inhibition was achieved at 4μg/mL. This result was in line with previous publication which reported activity of rhIFNα2-b on HepG2. This study used rhIFNα-2b that produced in *E. coli* BL21 (DE3) as mentioned in our previous work (Ningrum *et al.,* 2012). Most publications on biological activity of rhIFNα2-b determination were used viral cytophatic inhibition as the observed parameter. (Srivasta *et al.,* 2005; Valente *et al.,* 2005; Neves *et al.,* 2004; Essafi *et al.,* 2007).

Antiproliferative activity of our rhIFNα-2b was also studied by using calcein AM-Ethidium bromide III double staining method. The assay was founded on the measurement of two recognized parameter of living cells, intracellular esterase activity and plasma membrane integrity. Calcein AM is nonfluorescent cell-permeant that can be enzimatically converted into intensely fluorescent calcein (Papadopoulos *et al.,* 1994). The polyanionic dye calcein is retained within the living cells producing green fluorescence. Ethidium bromide III is excluded by intact plasma membrane of live cells and is 40% brighter than Ethidium bromide I (Papadopoulos *et al.,* 1994). In this study, the highest concentration of rhIFNα-2b was applied to treat MCF-7 cells and the result was compared with the effect of 1μM tmx in treated and untreated cells. As a positive control, rhIFNα-2b protein obtained from Biovision was used. The result showed that untreated cells had higher growth than the treated ones. As mentioned earlier, the treatment of tmx at 1μM of concentration did not give any significant growth inhibition. Our rhIFNα-2b had comparable ability to inhibit MCF-7 growth (Figure 5). This result strongly indicated that our rhIFNα-2b was biologically active. This preliminary study must be confirmed with other methods, such as cell cycle analysis by flowcytometry or gene expression by real time PCR.

Mechanism of action of hIFNα2b in influencing the growth of a variety of cells occurs through the JAK-STAT signal transduction. JAK-STAT pathway associated with the MAPK pathway as a major pathway in cell proliferation. Proteins that play a role in MAPK is the protein G and three protein kinases, namely MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. MAPKKK can phosphorylate and activate the MAPK protein kinase (MAPKK), and MAPKK can activate MAPK. hIFNα2b can inhibit extracellular signal-regulated kinase pathways (ERK) mitogen ERK kinase (MEK) that belong to the MAPK pathway (Chiariello *et al.,* 2000; Kolch, 2000; Romerio and Zella, 2002). Study of ERK inhibition of MEK by hIFNα2b been widely reported. Several publications reported that the inhibition of proliferation occurred because hIFNα2b induced PP2A enzyme that inactivated the ERK and MEK proteins. hIFNα2b also regulates the docking protein that inhibits the interaction of MEK or ERK by MEK interactions with other kinases. Inhibition of protein will decrease the activity of cyclin-dependent kinase (CDK-2 and CDK-4) and lower protein expression of cyclin D and E which involve in cell proliferation. The Inhibition also increases p21 Waf1 and p27 kip1 as an inhibitor of cell division and decreases protein phosphorylation RB / p105 (Romerio *et al.,* 2000; Romerio and Zella, 2002; Steelman, 2004). The antiproliferative activity of rhIFNα2b also occurs by apoptosis mechanism. There are two major pathways of apoptosis through the receptor family of tumor necrosis factor alpha (TNF-α) and cytochrome c release by mitochondria. Both of these pathways activating caspase signaling cascade that results in DNA fragmentation and cell death (Bekisz *et al.,* 2010; Bazhanova, 2003; Ningrum, 2014).

CONCLUSIONS

The rhIFNα-2b that used in combination with antiestrogenic tamoxifen had antiproliferative activity. It's activity on

estrogen positive human breast cancer MCF-7 cell line was dose dependently.

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