

Kinase inhibitors of the IGF-1R as a potential therapeutic agent for rheumatoid arthritis

メタデータ	言語: English 出版者: 公開日: 2017-03-20 キーワード (Ja): キーワード (En): 作成者: 津島, 浩 メールアドレス: 所属:
URL	https://jair.repo.nii.ac.jp/records/2001949

Kinase inhibitors of the IGF-1R as a potential therapeutic agent for rheumatoid arthritis

Hiroshi Tsushima^{1,2}, Shinji Morimoto^{1,3,*}, Maki Fujishiro¹, Yuko Yoshida¹, Kunihiro Hayakawa¹, Takuya Hirai^{1,2}, Tomoko Miyashita^{1,2}, Keigo Ikeda^{1,3}, Ken Yamaji², Kenji Takamori¹, Yoshinari Takasaki², Iwao Sekigawa^{1,3}, Naoto Tamura²

¹Institute for Environment and Gender Specific Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan

²Department of Internal Medicine and Rheumatology, School of Medicine, Juntendo University, 2-1-1 Hongo, Bunkyo, Tokyo 113-8421, Japan

³Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan

***Corresponding author:** Shinji Morimoto, MD, PhD

Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, 2-1-1 Tomioka, Urayasu, Chiba 279-0021, Japan

Tel: 81-4-7353-3111 Fax: 81-4-7381-5054 Email: morimoto@juntendo.ac.jp

Kinase inhibitors of the IGF-1R as a potential therapeutic agent for rheumatoid arthritis

Abstract

We have previously shown that the inhibition of connective tissue growth factor (CTGF) is a potential therapeutic strategy against rheumatoid arthritis (RA). CTGF consists of four distinct modules, including the insulin-like growth factor binding protein (IGFBP). In serum, insulin-like growth factors (IGFs) bind IGFBPs, interact with the IGF-1 receptor (IGF-1R), and regulate anabolic effects and bone metabolism. We investigated the correlation between IGF-1 and the pathogenesis of RA, and the inhibitory effect on osteoclastogenesis and angiogenesis o

f the kinase inhibitor of the IGF-1R, NVP-AEW541, against pathogenesis of RA *in vitro*. Cell proliferation was evaluated by cell count and immunoblotting. The expression of IGF-1 and IGF-1R was evaluated by RT-PCR. Osteoclastogenesis was evaluated using tartrate-resistant acid phosphatase staining, a bone resorption assay, and osteoclast-specific enzyme production. Angiogenesis was evaluated by a tube formation assay using human umbilical vein endothelial cells (HUVECs). The proliferation of MH7A cells was found to be inhibited in the presence of NVP-AEW541, and the phosphorylation of extracellular signal-regulated

kinase (ERK) and Akt was downregulated in MH7A cells. IGF-1 and IGF-1R mRNA expression levels were upregulated during formation of M-colony stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL)-mediated osteoclast formation. Moreover, osteoclastogenesis was suppressed in the presence of NVP-AEW541. The formation of the tubular network was enhanced by IGF-1, and this effect was neutralized by NVP-AEW541. Our findings suggest that NVP-AEW541 may be utilized as a potential therapeutic agent in the treatment of RA.

Running head: Kinase inhibitors of the IGF-1R for RA

Key Words: Rheumatoid arthritis, Insulin-like growth factor system, IGF-1R tyrosine kinase inhibitor, Small molecular-weight compound, Osteoclastogenesis

Disclosure statement: The authors report no conflicts of interest. This study supported by in part by a Grant-in-Aide () from MEXT (Ministry of Education, Culture, Sports, Science and Technology) Supported Program for the Strategic Research Foundation at Private University [grant number S1311011].

Biographical note:

Hiroshi Tsusima is a graduate student of the Institute for Environment and Gender Specific Medicine. His research interests focus on clinical immunology of Rheumatology.

Shinji Morimoto is an associate professor of the Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital. His works focus on clinical immunology of Rheumatology, especially Systemic Lupus Erythematosus. He also teaches graduate students at the Institute for Environment and Gender Specific Medicine.

Maki Fujishito is a technician of the the Institute for Environment and Gender Specific Medicine. She is a expert of immunologic experiment.

Yuko Yoshida is a postdoctoral fellow of the the Institute for Environment and Gender Specific Medicine. Her research interests focus on epigenetics on atopic disease.

Kunihiro Hayakawa is a postdoctoral fellow of the Institute for Environment and Gender Specific Medicine. Former, he worked at department of pathology, International Medical Center of Japan. Now His works focus on relation between lipid and immunology.

Takuya Hirai is a graduate student of the Institute for Environment and Gender Specific Medicine. His research interests focus on clinical immunology of Rheumatology ,especially pathogenesis on relation between microRNA and myositis.

Tomoko Miyashita is an assistant professor of the Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital. Her research interests focus on clinical feature of lupus nephritis.

Keigo Ikeda is an assistant professor of the Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital. His research interests focus on clinical immunology of Rheumatology, especially the role of janus kinase inhibitor on lupus model mice.

Ken Yamaji is an associate professor of the Department of Internal Medicine and Rheumatology, Juntendo University Hospital. He is a chief of plasma exchange section.

Kenji Takamori is a director of the Institute for Environment and Gender Specific Medicine. His specialty is biochemistry and dermatology.

Yoshinari Takasaki is a director of Juntendo University Koshigaya Hospital. His major is Rheumatology. His research interests focus on the role of anti-nucleolar antibody on rheumatic disease.

Iwao Sekigawa is a professor of the Department of Internal Medicine and Rheumatology, Juntendo University Urayasu Hospital, and vice-president of the Institute for Environment and Gender Specific Medicine. His research interests focus on epigenetics of Rheumatic

disease.

Naoto Tamura is an associate professor of the Department of Internal Medicine and Rheumatology, Juntendo University Hospital. His research interests focus on clinical immunology, especially of rheumatoid arthritis and spondylarthritis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial proliferation, pannus with aberrant angiogenesis, and joint deformity. We previously showed that inhibition of connective tissue growth factor (CTGF) may serve as a potential strategy against the pathogenesis of RA [1]. Furthermore, we demonstrated that CTGF promotes articular damage, and that its blockade may represent a novel therapeutic strategy against RA [2-4]. CTGF comprises four homologous modules: insulin-like growth factor (IGF)–binding protein (IGFBP), von Willebrand factor type C repeat, thrombospondin type 1 repeat, and the C-terminal [5].

IGFBP, one of the modules of CTGF, binds IGFs to regulate their bioavailability and transport [6]. IGFBPs are known to modulate the actions of IGFs in circulation and within the immediate extracellular environment [7], and their activity is considered to be involved in the

pathogenesis of RA [8, 9]. The IGF-1 receptor (IGF-IR) consists of two α subunits and β subunits; IGF-1 interacts with the α subunits and induces the phosphorylation of the intracellular β subunits. The IGF-IR signaling pathway involves the activation of phosphatidylinositol-3 kinase (PI3K). A second well-known pathway involves the activation of extracellular-regulated kinase (ERK)-1 and ERK-2, which are isoforms of the mitogen-activated protein kinase (MAPK) family and Akt signaling [10]. Increasing evidence suggests that IGF-IR additionally signals via the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway[11].

A number of studies have shown that the inhibition of the IGF-1/IGF-1R pathway may play a critical role in the suppression of malignant tumors [12, 13]. Several clinical trials in the field of oncology were carried out to investigate the therapeutic effects of monoclonal antibodies against IGF-1R [14]. The inhibitory effect of the pyrrolo[2,3-d]pyrimidine derivative NVP-AEW541, which is a IGF-1R tyrosine kinase inhibitor, against IGF-1 signaling has been reported. This molecule has additionally been shown to exert suppressive effects in cancer models [15, 16].

Recently, we demonstrate important functions of the IGF system in the development of synovitis in patients with RA [17]. Our report suggested that the IGF system plays significant

roles in the pathogenesis of RA, especially through aberrant activation of osteoclasts and disturbance of cartilage tissue homeostasis, thus resulting in articular destruction. In addition, the IGF system plays significant roles in acceleration of angiogenesis of RA synoviocytes [17].

On the basis of these findings, we investigated the correlation between the IGF-1 pathway and the inhibitory effect of NVP-AEW541 on RA *in vitro*. This work suggests that inhibition of the IGF system by NVP-AEW541 is a potential therapeutic strategy against RA.

Materials and Methods

Materials and cell lines

Female DBA/1J mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Mice were 8 weeks old at the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee. Recombinant mouse macrophage colony-stimulating factor (rmM-CSF) and receptor activator of NF- κ B ligand (rmRANKL) were obtained from R&D Systems (Minneapolis, MN, USA) and Wako Pure Chemical Industries (Chuo-ku, Osaka, Japan), respectively.

MH7A synovial fibroblast cells isolated from the knee joint of a patient with RA were

obtained from Riken Cell Bank, Ibaraki, Japan. Human umbilical vein endothelial cells (HUVECs) were purchased from LONZA (Walkersville, MD, USA). Cells were used for analysis from the 3rd passage onwards.

Pyrrrolo[2,3-d]pyrimidine derivative, NVP-AEW541, which is a IGF-1 receptor tyrosine kinase inhibitor, was obtained from Adooq Bioscience (Irvine, CA, USA). The original stock solution of this compound was dissolved in dimethyl sulfoxide to obtain a 10 mM solution, which was stored at -20°C.

Cell proliferation and inhibition assay

MH7A cells were plated at a density of 1×10^5 cells on 6-well plates in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) medium plus 10% FBS. After incubating overnight, the medium was replaced with fresh medium containing 1% FBS, with or without NVP-AEW541 (500 nM or 5 μ M). Twenty-four and 48 hours later, cells were detached using 0.25% trypsin EDTA (Nacalai Tesque, Inc. Nakagyo-ku, Kyoto, Japan). Finally, the total cell counts were measured using a TC20 Automated Cell Counter (Bio-Rad, Hercules, California, USA).

Immunoblotting

MH7A cells (4×10^5) were seeded on 12-well plates and incubated overnight. In order to

exclude the effects of growth factors, the medium was changed from 10% to 0.1% FBS for 2 hours. Then medium was replaced with serum-free medium, and NVP-AEW541 (1 μ M or 5 μ M) was added 20 minutes before stimulation with IGF-1 (50 ng/ml). These plates were washed with ice-cold PBS and cells were collected using RIPA buffer containing 1% phosphatase inhibitor (Thermo Fisher Scientific, Yokohama, Kanagawa, Japan) and 2% proteinase inhibitor. For western blotting assay, cells were stimulated with recombinant IGF-1 for 30 minutes. Lysates were mixed with 2 \times gel sample buffer and stored at -80°C until use. The equivalent of 10 μ g of total lysate protein was loaded onto each lane of 12.5% SDS-PAGE gels, separated by electrophoresis, and then transferred to nitrocellulose membranes using Semi-Dry Trans-Blot apparatus (Bio-Rad). The primary antibodies used for these studies were mouse anti-ERK1/2 (L34F12, 1 : 2,000), mouse anti-phospho-ERK1/2 (D1314.4E, 1 : 2,000), mouse anti-Akt1 (2H10, 1 : 1,000), mouse anti-phospho-Akt (S473, 1 : 1000) purchased from Cell Signaling Technology Japan (Chiyoda-ku, Tokyo, Japan), and mouse anti- β -actin (A4551, 1 : 10,000) from Sigma-Aldrich. Secondary antibodies conjugated to horseradish peroxidase were rabbit anti-mouse IgG obtained from Agilent Technologies Japan (Hachioji, Tokyo, Japan).

Osteoclast differentiation

Female DBA1/J mice were sacrificed and bone marrows were removed from femora, crushed and passed through 40 μ m pore nylon mesh. Then, 5×10^5 cells were cultured in alpha minimum essential medium (Invitrogen, Grand Island, NY, USA) with 10% FBS, and stimulated with rmMCSF (50 ng/ml), rmRANKL (100 ng/ml); next, various concentrations of NVP-AEW541 were added. Medium was replaced every other day. Cells were stained for tartrate-resistant acid phosphatase (TRAP) expression using trap staining kit (COSMO BIO, Tokyo, Japan). The number of TRAP-positive multi-nucleated (nucleus \geq 10) cells in each well was counted as osteoclasts at 40 \times magnification by light microscopy (Leica, Tokyo Japan).

Total RNA extraction and real-time RT-PCR

Bone marrow cells from DBA1/J mice were differentiated into osteoclasts on 24-well plates as described above. Total RNA was isolated from these cells using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript RT reagent kit (Takara, Shiga, Japan) with 1 μ g of total RNA. Quantitative PCR was performed in a 10- μ L reaction, with cDNA using the SYBR Premix Ex Taq kit (Takara); IGF-1, IGF-1R, matrix metalloproteinase-9 (MMP9), cathepsin K (CTSK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels

were quantified. To quantify the expression of transcripts, sample loading was monitored and the levels were normalized relative to the expression of GAPDH transcripts. The primers sequences were as shown in Table 1. The amplification protocol was as follows: 95°C for 5 sec (one cycle), 95°C for 5 sec and 60°C for 30 seconds for each gene (45 cycles), 95°C for 5 sec, 60°C for 30 sec and 95°C for 15 sec (one cycle). Relative quantification of these transcripts was carried out using the delta delta Ct (threshold cycle) comparative method.

HUVEC tube formation assay

HUVECs were cultured with EGM-2 BulletKit set (Lonza), containing 0.1% R3-IGF-1, 0.1% vascular endothelial growth factor (VEGF), 0.4% fibroblast growth factor (FGF)-2, 0.1% epidermal growth factor (EGF), 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100, and 2% FBS. Growth factor-reduced Matrigel matrix (Corning, Tewksbury, MA, USA) was diluted two-fold with growth factor-free EGM-2 medium. Then, with or without recombinant human IGF-1 (rhIGF-1, 100 ng/ml, R&D, USA), NVP-AEW541 (1 μ M) were adjusted in each condition, and 30 μ l of the matrix was placed in the 96-well plate. After solidification, 50 μ l of HUVECs (4×10^5 cells/ml, serum free) was added to each well and incubated at 37°C overnight. The numbers of junctions were counted for each well using a light microscope at 40 \times magnification, and the average of the values was used for

comparison.

Statistical analysis

The results are presented as means \pm SD for each sample. The statistical significance of differences was determined by Student's two-tailed t-test in two groups. For determining statistical differences between multiple groups, one-way ANOVA with repeated measures followed by Dunnett's t-test was used; p values < 0.05 were considered to represent statistical significance.

Results

Suppressive effect of NVP-AEW541 in MH7A cell proliferation

Firstly, we investigated whether the addition of NVP-AEW541 resulted in suppression of cell proliferation, which is a well-known effect of IGF-1. Our findings indicated that prolonged incubation of MH7A cells in the presence of NVP-AEW541 suppressed cell proliferation in a dose-dependent manner (Figure 1).

Inhibition of downstream signaling of IGF-1R by NVP-AEW541 in MH7A cells

Secondly, to confirm the inhibition of IGF-1/IGF-1R signaling by NVP-AEW541 in MH7A cells, we performed western blot analysis. The binding of IGF-1 to its receptor IGF-1R

activates PI3K/Akt or MAPK/ERK pathways, causing substantial anabolic changes such as like anti-apoptotic effects, and cell proliferation and differentiation [10]. As expected, IGF-1 stimulation elicited the phosphorylation of Akt and ERK, which was neutralized by addition of NVP-AEW541 (Figure 2).

Prevention of M-CSF/RANKL-mediated osteoclastogenesis by NVP-AEW541

Next, we examined mouse bone marrow cells from DBA/1J mice to the osteoclast formation model. Aberrant osteoclastogenesis is a major pathogenesis of RA [18]. IGF-1 is one of the important hormones facilitating osteoclast formation [11]. In this experiment, mRNA expression levels of IGF-1 IGF-1R, CTSK and MMP-9 in these cells were increased during M-CSF/RANKL-mediated osteoclastogenesis (Figure 3A). Furthermore, osteoclast formation was decreased by addition of NVP-AEW541. Its inhibitory effect was enhanced in dose dependent manner (Figure 3B).

HUVEC tube formation assay for investigating the correlation between angiogenesis and IGF-1 as well as the effects of NVP-AEW541

Lastly, in order to examine the correlation between IGF-1 and angiogenesis, we performed tube-formation assay using HUVECs. Intra-articular pathology of patients with active RA reveals that their synovial tissue undergoes inflammatory and proliferative changes, pannus

formation, and angiogenesis [19, 20]. As expected, angiogenesis was increased by the addition of IGF-1, and this effect was neutralized by NVP-AEW541 (Figure 4).

Discussion

Previous reports reveal that IGF-1 levels in synovial fluid are significantly higher in patients with RA than in those with osteoarthritis [21]. Other studies have shown that IgGs from patients with RA activate IL-16, RANTES expression, and T cell chemoattraction through IGF-1R in synovial fibroblasts [22]. We showed that the proliferation of MH7A cells, which are derived from a human RA synovial fibroblast cell line, was inhibited by the addition of the IGF-1R kinase inhibitor, NVP-AEW541. Therefore, in addition to immunosuppressive effects, the inhibition of synovial proliferation by the blockade of IGF-1R in RA patients may suppress joint inflammation.

It is well known that IGF-1 stimulation through IGF-1R stimulates PI3K and MAPK signaling. IGF-1 is one of the most potent activators of Akt phosphorylation [23]. In this report, we showed that p-ERK and p-Akt were upregulated in the presence of IGF-1, and this effect was neutralized by NVP-AEW541. Previous reports have shown that the inhibition of IGF-1R signaling by NVP-AEW541 suppresses the phosphorylation of IGF-1R, ERK, and

the Akt pathway [15] [24]. These data were consistent with our findings in the present study using MH7A cells. However, Tazzari et al. showed that NVP-AEW541 did not affect the phosphorylation of ERK signaling [16]. The discrepancy between these results may be attributed to the different concentrations of tyrosine kinase inhibitor used in the studies.

We have previously shown that IGF-1 and IGFBP3 enhance osteoclastogenesis, and that the anti-IGF-1R monoclonal antibody (mAb) neutralizes this effect in human peripheral blood mononuclear cells (PBMCs) [17]. However, according to the manufacturer's protocol, mAb does not exhibit cross-reactivity with mouse antibodies. Therefore, we examined the inhibitory effect of NVP-AEW541 against osteoclastogenesis in mouse bone marrow cells in vitro. IGF-1 is a non-TNF superfamily growth factor that plays an important role in bone remodeling [25]. We showed that IGF-1 and IGF-1R mRNA expression levels were upregulated during osteoclastogenesis and that NVP-AEW541 inhibited this differentiation in a dose-dependent manner. It is considered that blockade of IGF-1 signaling delays bone deformity; however, IGF-1 plays an important role in bone growth [7]. Further studies are required in order to elucidate the mechanisms by which the blockade of IGF-1R by NVP-AEW541 affects bone metabolism.

Abnormal synovial proliferation, invasion of lymphocytes, and pannus formation in

association with angiogenesis have been observed in patients with RA [18]. During this process, the pannus invades cartilage and bone, eventually causing the destruction of these structures. In our previous report, it was shown, via HUVEC tube formation assay, that IGF-1 and IGFBP3 enhance angiogenesis. However, it has recently been reported that IGFBP3 exerts these effects without requiring interaction with IGF-1 [24]. Accordingly, in the present study, we investigated whether IGF-1 alone aggravates angiogenesis without IGFBP3. As a result, we showed that angiogenesis was enhanced by high levels of IGF-1, and that this was inhibited by NVP-AEW541.

ZSTK474, a selective PI3K inhibitor, has been previously utilized to clarify the correlation between the PI3K pathway and RA [26]. In the report, ZSTK474 suppressed cell proliferation of T cells, B cells, and synovial cells *in vitro*. Moreover, this inhibitor suppressed arthritis in the collagen-induced arthritis mouse model [26]. As mentioned above, NVP-AEW541 strongly inhibited the PI3K/Akt pathway. The inhibition of IGF-1R phosphorylation is also expected to regulate RA activity *in vivo*.

IGF-1 has been implicated in the pathophysiology of numerous human cancers. Several studies have shown that high concentrations of circulating IGF-1 represent risk factors for some cancers [27]. IGF-1R plays important roles in cancer cell signaling and is therefore an

attractive therapeutic target; various monoclonal antibodies and TKIs against IGF-1R have been investigated for their potential utility in the treatment of solid tumors [28]. Recently, the first-in-human phase I trial of KW-2450, an oral TKI with IGF-1R and insulin receptor sensitivity, was conducted in patients with solid tumors [29]. We expect that the therapeutic potential of NVP-AEW541 will be similarly tested in clinical trials.

In conclusion, these results indicate that aberrant IGF-I production plays a role in abnormal osteoclastic activation and angiogenesis in RA. And, the inhibition of the IGF system signaling pathway by NVP-AEW541 has beneficial effects in the treatment of RA. Originally, NVP-AEW541 was developed as a cancer therapeutic agent. Our data indicate that NVP-AEW541 has potential for drug repositioning as a treatment for RA. *In vivo* studies involving the administration of NVP-AEW541 to mouse models of RA are anticipated in order to develop effective and novel therapeutic strategies for the treatment of this disease.

Acknowledgments

We are grateful to Ms. Fumi Kawabata and Ms. Kazumi Goto for providing secretarial assistance.

References

1. Sekigawa I, Yanagida M, Iwabuchi K, et al. Protein biomarker analysis by mass spectrometry in patients with rheumatoid arthritis receiving anti-tumor necrosis factor-alpha antibody therapy. *Clin Exp Rheumatol.* 2008;26:261-267.
2. Miyashita T, Morimoto S, Fujishiro M, et al. Inhibition of each module of connective tissue growth factor as a potential therapeutic target for rheumatoid arthritis. *Autoimmunity.* 2016;49:109-114.
3. Nozawa K, Fujishiro M, Kawasaki M, et al. Connective tissue growth factor promotes articular damage by increased osteoclastogenesis in patients with rheumatoid arthritis. *Arthritis Res Ther.* 2009;11:R174.
4. Nozawa K, Fujishiro M, Kawasaki M, et al. Inhibition of connective tissue growth factor ameliorates disease in a murine model of rheumatoid arthritis. *Arthritis Rheum.* 2013;65:1477-1486.
5. Takigawa M, Nakanishi T, Kubota S, et al. Role of CTGF/HCS24/ecogenin in skeletal growth control. *J Cell Physiol.* 2003;194:256-266.
6. Collett-Solberg PF, Cohen P. Genetics, chemistry, and function of the

IGF/IGFBP system. *Endocrine*. 2000;12:121-136.

7. Kawai M, Rosen CJ. The insulin-like growth factor system in bone: basic and clinical implications. *Endocrinol Metab Clin North Am*. 2012;41:323-333.

8. Guvakova MA. Insulin-like growth factors control cell migration in health and disease. *Int J Biochem Cell Biol*. 2007;39:890-909.

9. Heemskerk VH, Daemen MA, Buurman WA. Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine Growth Factor Rev*. 1999;10:5-14.

10. Subramaniam S, Shahani N, Strelau J, et al. Insulin-like growth factor 1 inhibits extracellular signal-regulated kinase to promote neuronal survival via the phosphatidylinositol 3-kinase/protein kinase A/c-Raf pathway. *J Neurosci*. 2005;25:2838-2852.

11. Giustina A, Mazziotti G, Canalis E. Growth hormone, insulin-like growth factors, and the skeleton. *Endocr Rev*. 2008;29:535-559.

12. Beltran PJ, Mitchell P, Chung YA, et al. AMG 479, a fully human anti-insulin-like growth factor receptor type I monoclonal antibody, inhibits the growth and survival of pancreatic carcinoma cells. *Mol Cancer Ther*.

2009;8:1095-1105.

13. Hailey J, Maxwell E, Koukouras K, et al. Neutralizing anti-insulin-like growth factor receptor 1 antibodies inhibit receptor function and induce receptor degradation in tumor cells. *Mol Cancer Ther.* 2002;1:1349-1353.

14. Chugh R, Griffith KA, Davis EJ, et al. Doxorubicin plus the IGF-1R antibody cixutumumab in soft tissue sarcoma: a phase I study using the TITE-CRM model. *Ann Oncol.* 2015;26:1459-1464.

15. Garcia-Echeverria C, Pearson MA, Marti A, et al. In vivo antitumor activity of NVP-AEW541-A novel, potent, and selective inhibitor of the IGF-IR kinase. *Cancer Cell.* 2004;5:231-239.

16. Bao XH, Takaoka M, Hao HF, et al. Esophageal cancer exhibits resistance a novel IGF-1R inhibitor NVP-AEW541 with maintained RAS-MAPK activity. *Anticancer Res.* 2012;32:2827-2834.

17. Suzuki S, Morimoto S, Fujishiro M, et al. Inhibition of the insulin-like growth factor system is a potential therapy for rheumatoid arthritis. *Autoimmunity.* 2015;48:251-258.18. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. *Cell.* 1996;85(3):307-10.

19. La Cava A, Alviggi C, Matarese G. Unraveling the multiple roles of leptin in inflammation and autoimmunity. *J Mol Med (Berl)*. 2004;82:4-11.
20. Salvi R, Peclat V, So A, et al. Enhanced expression of genes involved in coagulation and fibrinolysis in murine arthritis. *Arthritis Res*. 2000;2:504-512.
21. Matsumoto T, Gargosky SE, Iwasaki K, et al. Identification and characterization of insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and IGFBP proteases in human synovial fluid. *J Clin Endocrinol Metab*. 1996;81:150-155.
22. Pritchard J, Tsui S, Horst N, et al. Synovial fibroblasts from patients with rheumatoid arthritis, like fibroblasts from Graves' disease, express high levels of IL-16 when treated with Igs against insulin-like growth factor-1 receptor. *J Immunol*. 2004;173:3564-3569.
23. Walsh PT, Smith LM, O'Connor R. Insulin-like growth factor-1 activates Akt and Jun N-terminal kinases (JNKs) in promoting the survival of T lymphocytes. *Immunology*. 2002;107:461-471.
24. Bielen A, Box G, Perryman L, et al. Dependence of Wilms tumor cells on signaling through insulin-like growth factor 1 in an orthotopic xenograft model

targetable by specific receptor inhibition. *Proc Natl Acad Sci U S A.* 2012;109:E1267-276.

25. Sabokbar A, Mahoney DJ, Hemingway F, et al. Non-canonical (RANKL-independent) pathways of osteoclast differentiation and their role in musculoskeletal diseases. *Clin Rev Allergy Immunol.* 2016;51:16-26.

26. Toyama S, Tamura N, Haruta K, et al. Inhibitory effects of ZSTK474, a novel phosphoinositide 3-kinase inhibitor, on osteoclasts and collagen-induced arthritis in mice. *Arthritis Res Ther.* 2010;12:R92.

27. Miller BS, Yee D. Type I insulin-like growth factor receptor as a therapeutic target in cancer. *Cancer Res.* 2005;65:10123-10127.

28. Chitnis MM, Yuen JS, Protheroe AS, et al. The type 1 insulin-like growth factor receptor pathway. *Clin Cancer Res.* 2008;14(6):6364-6370.

29. Schwartz GK, Dickson MA, LoRusso PM, et al. Preclinical and first-in-human phase I studies of KW-2450, an oral tyrosine kinase inhibitor with insulin-like growth factor receptor-1/insulin receptor selectivity. *Cancer Sci.* 2016;107:499-506.

Figure Captions

Figure 1. NVP-AEW541 suppressed cell proliferation of MH7A cells.

MH7A cells (1×10^5) were cultured in medium containing 10% FBS. After incubation overnight, medium was replaced with fresh medium containing 1% FBS, and various concentrations of NVP-AEW541 were added. Cells were incubated for 24-48 hours. Bars = SD, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

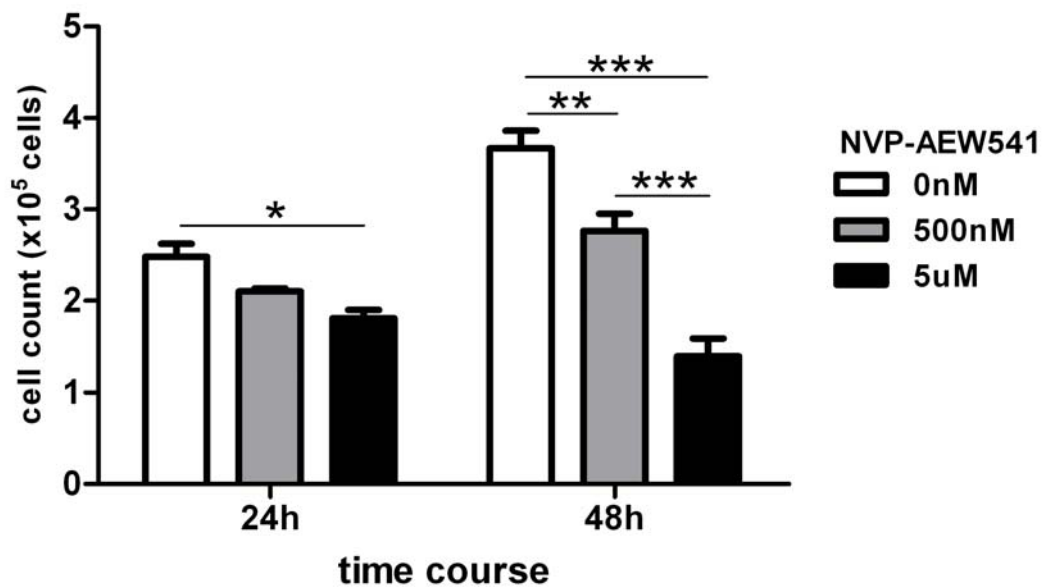


Figure 2. Inhibition of IGF-1R signaling by NVP-AEW541 in MH7A cells.

MH7A cells were stimulated with IGF-1 and NVP-AEW541. ERK1/2 and Akt phosphorylation and total protein levels were determined by western blot.

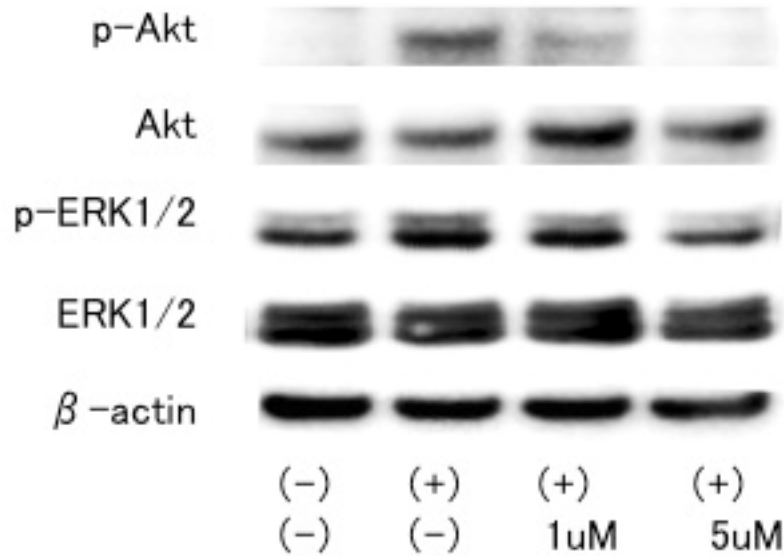


Figure 3. Effect of IGF-1/IGF-1R and inhibitory effect of NVP-AEW541 on osteoclastogenesis.

(A): Comparison of gene expression during osteoclastogenesis. Mice bone marrow cells were stimulated with rmM-CSF and rmRANKL to induce osteoclast formation. Total RNA was extracted 48 and 96 hours after the initiation of incubation. Expression of IGF-1 and IGF-1 receptor were upregulated during osteoclastogenesis. GAPDH expression was analyzed as a reference. (B): Inhibitory effects of NVP-AEW541 on rmM-CSF/rmRANKL-mediated osteoclastogenesis; numbers of TRAP-positive cells (nucleus ≥ 10) were decreased by the inhibition of IGF-1R phosphorylation. Bars = SD, * $p < 0.05$; ** $p < 0.01$.

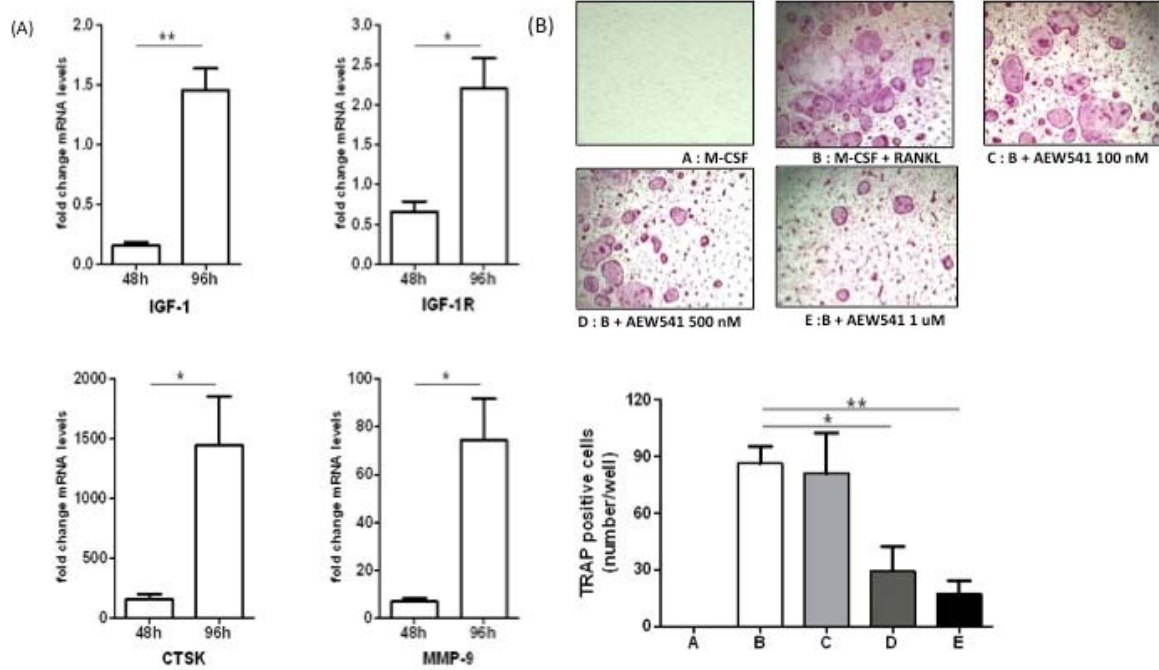


Figure 4. NVP-AEW541 inhibits angiogenesis in *in vitro* models.

HUVECs were plated on growth factor reduced-Matrigel with or without IGF-1 and NVP-AEW541. After incubation for 12 hours, the number of cells exhibiting tube formation was determined. Bars = SD, * $p < 0.05$.

