**Introduction:** The mitogen-activated protein kinase (MAPK) signaling pathway is activated by signals from growth factor receptors, and it plays a crucial role in the cell proliferation. Raf-1, which is an essential serine/threonine kinase, is a downstream effector of the central signal transduction mediator Ras in the MAPK signaling pathway, and the therapeutic targeting of Raf-1 are undergoing clinical evaluation on some human malignancies. We consider that the antitumor activity will be demonstrated on human sarcomas by Raf-1 kinase inhibition and MAPK signaling inhibition. We examined the expression of Raf-1 and the existence of MAPK signaling pathway in human osteosarcoma cell lines, and the inhibitory effect of Raf-1 kinase inhibitor and MEK1/2 inhibitor on the cell proliferation.

**Materials and Methods:** Cell lines and reagent.

3 human osteosarcoma cell lines (KTHOS, MG63 and KHOS) were used in this study. All cell lines were grown in culture medium consisting of Dulbecco’s Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). The cell lines were routinely maintained at 37°C in a humidified 5% CO2 atmosphere. GW5074, a specific Raf-1 kinase inhibitor, and S0126, a selective MEK1/2 inhibitor, was purchased from Sigma-Aldrich.

mRNA expression of Raf-1.

Total RNAs were eluted by selective binding to a silica-gel-based membrane using an RNeasy Mini Kit® (QIAGEN Inc., Valencia, CA). Reverse transcription of RNA into cDNA was performed by using Reverse Transcription System (Promega, Madison, WI). Raf-1 and GAPDH mRNA expression were examined by reverse transcription (RT) PCR. After PCR amplification, 8-μl aliquots of the PCR products were electrophoresed in a 2% agarose gel, followed by ethidium bromide dye.

The inhibitory effect of GW5074 and S0126.

The cell proliferation was assayed using the MTS assay (CellTiter 96® Aqueous One Solution Cell Proliferation Assay; Promega, Madison, WI). Cells were seeded in 96-well cell culture plates. After 48 hours (h), the medium was refreshed containing GW5074 or U0126 in the indicated concentrations. After 24, 48, 72h, the optical density was measured. The percent viability of each well was calculated. The percent viability of each well was calculated.

Western blotting.

Cells were pretreated for 60 min with 1% FBS containing GW5074 in the indicated concentrations before stimulation with or without 10 ng/ml PDGF for 10 min. Whole cell lysates were collected for protein content, and cell lysates were separated by SDS polyacrylamide gel electrophoresis under reducing conditions. Then gels were electrophoretically transferred to PVDF membrane, and immunoblotted with anti-Raf-1 antibody (Upstate Biotechnology, Lake Placid, NY) and anti-phospho-Raf-1 antibody (Upstate Biotechnology). Bound antibodies were detected using the ECL plus western blotting detection system (GE Healthcare Bio-Sciences, Piscataway, NJ).

Results: mRNA expression of Raf-1. The Raf-1 mRNA was expressed in all osteosarcoma (Image 1). The effect of GW5074 and S0126. GW5074 and U0126 inhibited the cell proliferation of all 3 cell lines in a dose- and time-dependent manner. 10μM GW5074 inhibited the cell proliferation of KTHOS, at the percent viability of 50% or less (Image 2).

![KTHOS VS MG63 VS KHOS](image1)

10μM S0126 inhibited the cell proliferation of KHOS at the percent viability of 50% or less (Image 3).

Expression of Raf-1 and phospho-Raf-1 kinases.

Western blotting analysis revealed that not only Raf-1 but phospho-Raf-1 were expressed in all cell lines under the normal condition. Phosphorylation of Raf-1 were increased by PDGF stimulation, and 10μM GW5074 decreased phosphorylation of Raf-1 in all cell lines.

Discussion: The MAPK pathway is very important as a target of the molecular targeting therapy. In our study, Raf-1 kinase inhibitor and MEK1/2 inhibitor showed a dose- and time- dependent inhibitory effect on the cell proliferation. GW5074 decreased the phosphorylation of Raf-1 in a dose-dependent manner. These results suggest that GW5074 may be a selective inhibitor of Raf-1 kinase in human osteosarcoma, and MAPK signaling pathway exists and plays an important role in osteosarcoma cells. Although further studies are needed to explore the mechanisms for the inhibitory effect on cell proliferation in human osteosarcoma cells. Raf-1 kinase inhibitor and MAPKs signaling inhibitor will be a potent chemotherapeutic agent for human osteosarcomas.

References: