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13. ABSTRACT (Maximum 200 Words)

The constitutive activation of Stat3 is frequently detected in human breast cancer cell lines as well as in clinical breast cancer specimens and may play an important role in oncogenesis of breast carcinoma. Activated Stat3 may participate in oncogenesis by stimulating cell proliferation, promoting tumor angiogenesis, and resisting to apoptosis. Hence, Stat3 represents an attractive target for cancer therapy. In this study, of the nearly 429,000 compounds screened by virtual database screening, chemical samples of top 100 compounds identified as candidate small molecule inhibitors of Stat3 were evaluated using Stat3-dependent luciferase reporter as well as other cell-based assays. Through serial functional evaluation based on our established cell-based assays, one compound, termed STA-21, was identified as matching our selection criteria best. Further investigation demonstrated that STA-21 inhibits Stat3 DNA binding activity, Stat3 dimerization, and Stat3-dependent luciferase activitiy. Moreover, STA-21 reduces the survival of breast carcinoma cells with constitutive Stat3 signaling but has minimal effect on the cells in which constitutive Stat3 signaling is absent. Together, these results demonstrate that STA-21 inhibits breast cancer cells that express constitutive active Stat3. STA-21 may have a therapeutic potential to be developed as a new class of anti-cancer drug for the treatment of human breast cancer with activated Stat3.

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Introduction:

Signal transducers and activators of transcription 3 (Stat3) are activated in response to cytokines and growth factors. JAKs, Src, and epidermal growth factor receptor (EGFR) are Stat3 upstream regulators. The main domains of Stat3 protein include the tetramerization and leucine zipper at the N-terminus, the DNA binding domain, and the SH2 transactivation domain at the carboxy-terminal end. The SH2 region is responsible for the binding of Stat3 to the tyrosine-phosphorylated receptors and for the dimerization which is necessary for DNA binding and gene expression. Stat3 is activated by phosphorylation at Tyrosine 705 (Y-705), which leads to dimer formation, nuclear translocation, recognition of Stat3-specific DNA binding elements, and activation of target gene transcription.

The constitutive activation of Stat3 is frequently detected in breast carcinoma cell lines but not in normal breast epithelial cells. It has been reported that approximately 60 percent of breast tumors contain persistently activated Stat3. *Stat3* gene has been classified as a proto-oncogene because in its activated form it can mediate oncogenic transformation in cultured cells and tumor formation in nude mice. Stat3 may participate in oncogenesis by stimulating cell proliferation, promoting angiogenesis, and conferring resistance to apoptosis induced by conventional therapies. The possible downstream targets of Stat3 include the anti-apoptotic molecules Bcl-2, survivin, Mcl-1, and Bcl-X_L, the cell-cycle regulators cyclin D1 and c-myc, and VEGF, an inducer of tumor angiogenesis. Activated Stat3 signaling directly contributes to malignant progression of cancer. Stat3 oncogenic function acts through the pro-survival proteins such as survivin, Mcl-1, Bcl-2, and Bcl-X_L and results in inhibition of apoptosis. Blockade of Stat3 signaling inhibits cancer cell growth, indicating that Stat3 plays a role in the survival or growth of tumor cells.

Since Stat3 is frequently activated in breast cancer, it represents an attractive target for the development of new anti-cancer therapy in the treatment of breast cancer. In one approach, peptide-based Stat3 inhibitors were designed to target the Stat3 SH2 domain and were effective in blocking the Stat3 function *in vitro*. In another approach, compounds have been used to inhibit Stat3 upstream regulators Janus kinases (JAKs), especially JAK2. In our opinion, direct inhibition of Stat3 using a drug-like, non-peptide small molecule has several advantages, including blocking all the activity mediated by Stat3 activation and compounds with better cell permeability, and better *in vivo* stability and bio-availability.

Based on a high-resolution three-dimensional crystal structure of the Stat3ß homodimer, the SH2 domain is critical for Stat3 dimerization, which is a decisive event for the activation of Stat3. Therefore, we hypothesize that a small molecule that binds to the Stat3 SH2 domain may directly block Stat3 dimerization and its activity. In this study, we report the discovery of a Stat3 small molecule inhibitor through virtual database screening.

RESULTS

Discovery of STA-21 through Structure Based Virtual Database Screening.

The three-dimensional structure of Stat3ß homodimer shows that the dimerization of Stat3

occurs between two SH2 domains (Figure 1A). These two SH2 domains are hinged together by a

loop segment (from alanine 702 to phenyalanine 716) from each monomer. The phosphoryl

tyrosine 705 (pY-705), critical for the biological function of Stat3, locates right on this loop

segment and binds, together with several adjacent amino acid residues (leucine 706, threonine

708, and phenyalanine 710), to a cavity on the SH2 domain of the other monomer. The targeted

region defined in our virtual screening covers where the pY705-Phe710 peptide segment binds.

We hypothesize that a small molecule that binds to this region will compete with the pY705-

containing peptide, consequently blocking the dimerization of Stat3.

Fig. 1. Schematic diagrams of modeling of structure based virtual database screening. (*A*) SH2 domain dimerization interface of the STAT3ß protein. Structure is based on PDB entry 1BG1. Two SH2 domains are colored differently. Circled region indicates the target PTR binding site used in our virtual screening study. *(B)* Predicted binding model of STA-21 to the STAT3ß SH2 domain. STA-21 is rendered by a ball-and-stick model. Molecular surface of the STAT3ß SH2 domain is colored with the electrostatic potentials: red for the most positively charged regions, blue for the most negatively charged regions. (*C*) Specific hydrogen bonds formed between the STAT3ß SH2 domain and STA-21. The binding model was predicted by the DOCK program. Only the residues that form hydrogen bonds with STA-21 are shown in explicit atomic models. (*D*) Chemical structure of STA-21. Figures 1A-1C were generated by using the Sybyl program.
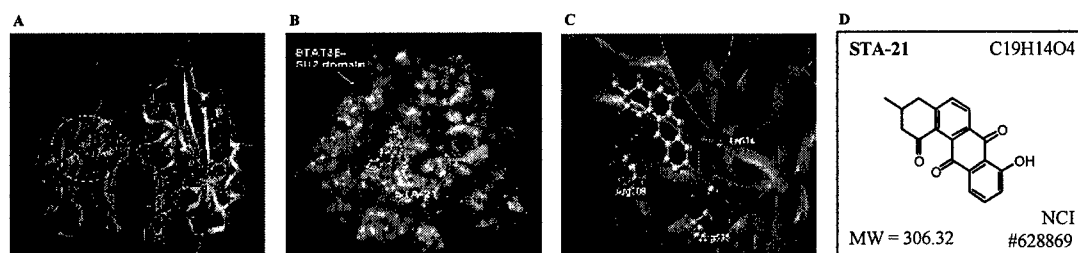

With the aid of structure-based virtual screening, we narrowed our interests from a total of

429,000 compounds to 200 top candidate compounds and were able to obtain the chemical

samples for 100 compounds. We first tested these 100 compounds using an *in vitro* cell luciferase

assay. Of the 100 compounds tested, the most promising compound is STA-21 (NSC 628869) obtained from NCI. STA-21, a natural product extract, is a deoxytetrangomycin, an angucycline antibiotic with a molecular weight of 306 (Figure 1D). The binding mode of STA-21 to Stat3 was predicted by the DOCK program and refined by structural optimization using the AMBER force field implemented in the Sybyl software. The refined model, shown in Figure 1B and 1C, predicts that STA-21 binds at the same site where the pY-705 containing peptide binds and forms a number of hydrogen bonds with nearby residues, including Arg595, Arg609, and Ile634 (Figure 1C).

STA-21 Inhibited Stat3-dependent Luciferase Reporter Activity in Carcinoma Cells with Constitutive Stat3 Signaling. The selected inhibitors were evaluated using a Stat3 luciferase reporter system. Both MDA-MB-435s breast carcinoma cells and Caov-3 ovarian carcinoma cells express constitutively activated Stat3. We established cloned cells from these two cell lines by stable transfection of a Stat3-dependent luciferase reporter, pLucTKS3. As a result of persistently activated Stat3, both cell clones showed high luciferase activity. Of the 100 small molecules tested, STA-21 showed a remarkably inhibitory effect on Stat3 induced luciferase activity in Caov-3 cloned cells (Fig. 2A). The inhibition of STA-21 on Stat3 activation was further confirmed using MDA-MB-435s cloned cells stably transfected with pLucTKS3 (Fig. 2B). For MDA-MB-435s cloned cells, after the exposure to 20 of μM STA-21 for 48h, luciferase activity was decreased more than five-fold (Fig. 2B). In contrast, STA-21 did not affect luciferase activity in the clones transfected with SV40 luciferase reporter that had no Stat3 binding site (Fig. 2B), indicating that STA-21 does not reduce luciferase activity in the absence of Stat3. As controls, we also listed several compounds that did not possess Stat3 inhibitory function, as shown in Figure 2A.

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Fig. 2. **STA-21** inhibits Stat3 dependent luciferase activity in cancer cells. (*A*) The clone from Caov-3 carcinoma cells stably transfected with pLucTKS3 Stat3-dependent luciferase reporter was used for initial Stat3 small molecule inhibitor screening. The cloned cells were treated with 20 μM of STA-21 as well as other small molecule compounds for 48h, then the cells were harvested for luciferase activity analysis. (*B*) The clones from MDA-MB-435s cells stably transfected with pLucTKS3 Stat3-dependent luciferase reporter or SV40 luciferase reporter were treated with 20 μM of STA-21 for 48h. Luciferase activity was measure by using a Promega luciferase kit according to manufacturer's instructions. The results were based on the means and standard deviations from three separate experiments.
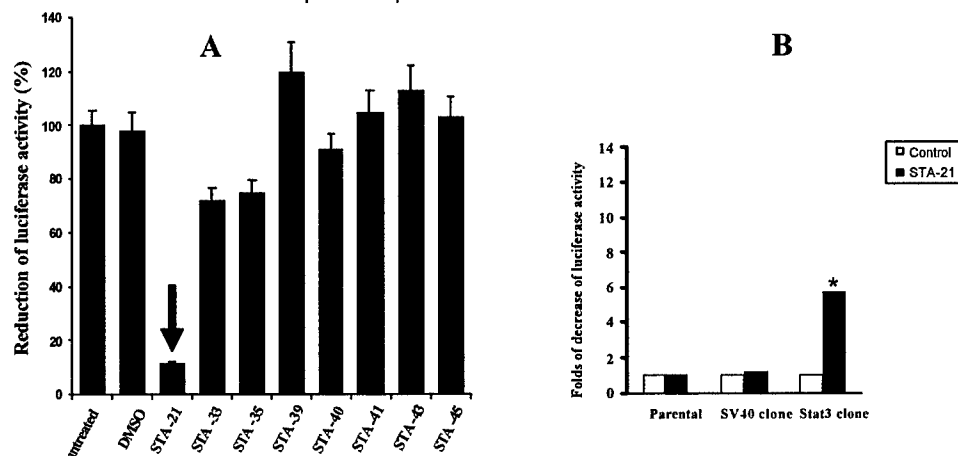
STA-21 Inhibited Stat3 But Not Its Upstream Regulators. We further examined whether or not STA-21 could reduce Stat3 DNA binding activity. In MDA-MB-435s breast carcinoma cells with constitutive Stat3 signaling, high Stat3 DNA binding activity was observed (Fig. 3A). In contrast, MCF10A and TERT breast cells without constitutive Stat3 signaling did not show Stat3 DNA binding activity (data not shown). STA-21 inhibited Stat3 DNA binding activity (Fig. 3A) but did not inhibit Stat1 and Stat5 DNA binding activity in MDA-MB-435s cells (Fig. 3A). STA-21 also inhibited downstream anti-apoptotic factors and cell cycle regulator Cyclin D1 in breast carcinoma cells with constitutive Stat3 signaling (Fig. 3B). Interestingly, the phosphorylation of Stat3 upstream regulators JAK2 (P-JAK2), Src (P-Src), EGFR (P-EGFR), and of itself (P-Stat3 at Y-705) were not affected by STA-21 (Fig. 3B). Nor did STA-21 affect the phosphorylation of

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AKT (P-AKT) and ERK (P-ERK) (Fig. 3B). Taken together, our data suggest that the inhibition of Stat3 activity by STA-21 is specific.

Fig. 3. **STA-21** inhibits Stat3 DNA binding activity and Stat3-regulated anti-apoptotic factors. (A) MDA-MB-435s cell nucleus extract was incubated with 30 μM of STA-21 for 30 min at room temperature, and then incubated with r-32P-ATP labeled consensus binding sequence for 20 min at room temperature. The reaction mixtures were resolved on 8% polyacylamide gel. (B) The lysates from MDA-MB-468 cells treated with indicated concentrations of STA-21 for 48h were resolved on 10% SDS-PAGE, then immunoblotted with antibodies as indicated.
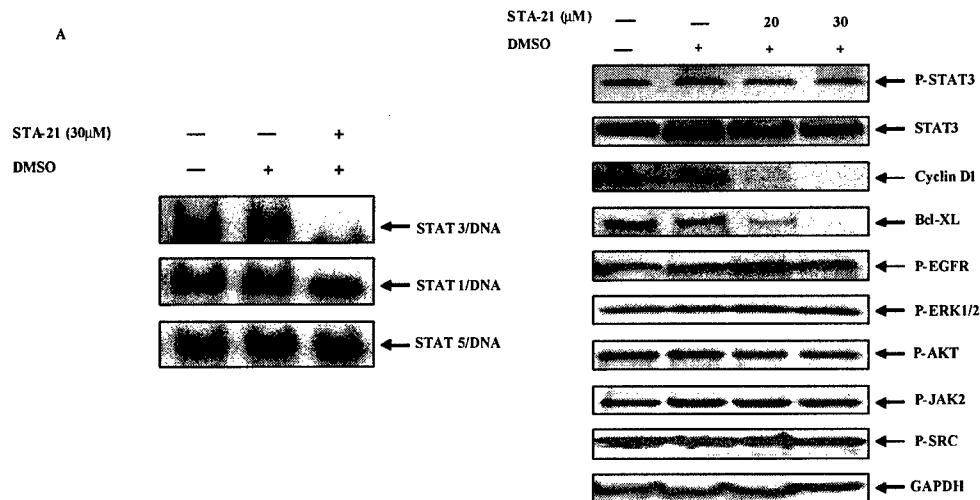
STA-21 Significantly Inhibited the Growth and Survival of Breast Carcinoma Cells with Constitutive Stat3.

Since STA-21 inhibits Stat3-dependent luciferase and DNA binding activities, we next examined whether STA-21 inhibited the growth and survival of breast cancer cells with constitutive Stat3 signaling. After the cells were exposed to STA-21, STA-21 showed remarkable inhibition of the survival of the breast carcinoma cells, MDA-MB-231, MDA-MB-435s, and MDA-MB-468 cells that are present in cells with persistently activated Stat3 (Fig. 4A). In comparison, STA-21 had minimal inhibitory effect on MCF7, MDA-MB-453 breast carcinoma cells, and human skin fibroblasts (HSF), which have no constitutive Stat3 signaling (Fig. 4B). Combined with the data from a cell viability assay (MTT, data not shown), STA-21 specifically

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inhibited the growth and survival of breast cancer cells in which constitutively active Stat3 is

present.

Fig. 4. STA-21 inhibits the survival of breast carcinoma cells with constitutive Stat3 signaling but not in cells having no constitutive Stat3 signaling. (*A*) The phosphorylation of Stat3 at Y-705 in different cell lines. (*B*) The cell lines were treated with STA-21 at concentrations as indicated for 48h, and then cells were harvested and analyzed for the Sub-G1 profile that indicated apoptotic cells on a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA). The results were based on the averages and standard deviations from three separate experiments.
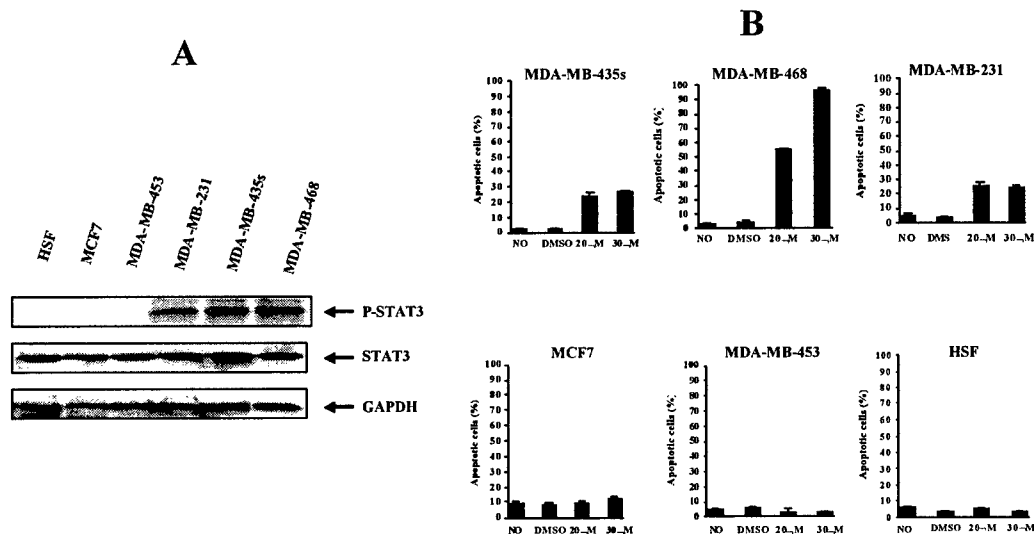
Stat3 Nucleus Translocation and Protein Dimerization were Abrogated by STA-21 in MDA-MB-435s Breast Carcinoma Cells.

The plasmids pCMV-Stat3-Flag and pCMV-Stat3-HA for expression of Stat3-Flag and Stat3-HA tagged proteins were co-transfected into MDA-MB-435s breast carcinoma cells. The nucleus translocation of Stat3-Flag and Stat3-HA proteins was blocked by STA-21 (Fig. 5). The cells were treated with 20 µM of STA-21 for 24h, then immunostained with anti-rabbit HA IgG-FITC (green) and/or anti-mouse Flag IgG-Rhodamine (red). When using separate FITC and Rhodamine staining, STA-21 seemed to inhibit Stat3-Flag or Stat3-HA nucleus translocation, as shown in Fig. 5E and 5F. In the cells without STA-21 treatment, strong orange staining was observed in the nucleus using combined FITC and

Rhodamine staining, indicating that Stat3-Flag and Stat3-HA tagged proteins co-localized into the nucleus and two colors merged to become orange (Fig. 5D). However, when treated with 20 μM of STA-21, much weaker orange staining was seen in the whole cell (Fig. 5G), suggesting that STA-21 blocked nucleus translocation of Stat3-Flag and Stat3-HA proteins. As a control, DMSO showed no effect on the control cells (data not shown).

Fig. 5. STA-21 inhibits Stat3 translocation and dimerization in breast carcinoma cells. The MDA-MB-435s cells co-transfected with pCMV-Stat3-Flag and pCMV-Stat3-HA plasmid were exposed to 20 μM of STA-21 compound for 24h, then fixed with 100% methanol. Subsequently, the fixed cells were stained with anti-HA (rabbit, Santa Cruz Biotechnology) or/and anti-Flag (mouse, Sigma) antibodies, and secondary anti-rabbit IgG-FITC or/and anti-mouse IgG-Rhodamine antibodies were added. The cells were observed with a fluorescence microscope. (A) Untransfected and untreated MDA-MB-435s cells. (B and E) The cells co-transfected by pCMV-Stat3-Flag and pCMV-Stat3-HA plasmids were immunostained with anti-flag IgG- Rhodamine. (B) Untreated cells and (E) STA-21 treated cells. (C and F) The transfected cells were immunostained with anti-HA IgG-FITC. (C) Untreated cells and (F) STA-21 treated cells. (D and G) The transfected cells were co-immunostained with both anti-HA IgG-FITC and anti-flag IgG-Rhodamine. (D) Untreated cells showed bright orange color. (G) STA-21 treated cells showed dark and nuclear orange and separate green and red color.
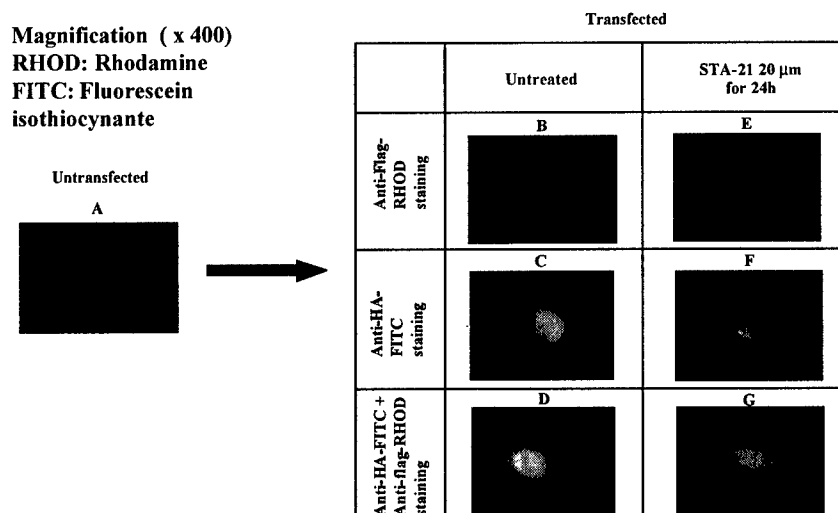
We also investigated the effect of STA-21 on Stat3 *in vivo* dimerization in breast cancer MDA-MB-435s cells. After the exposure to 20 μM of STA-21 for 24h, the cells were harvested and lysates from the cells expressing Stat3-Flag and Stat3-HA tagged proteins were immunoprecipitated with an anti-Flag or anti-HA antibody, respectively. The immunoprecipitated

reaction mixtures were resolved on a 10% SDS-PAGE and immunoblotted with an anti-HA, anti-Flag, or anti-Stat3 antibodies, respectively. The results showed that STA-21 abrogated Stat3 dimerization between Stat3-Flag and Stat3-HA proteins in the cells tested (Fig. 6).

Figure 6. The MDA-MB-435s cells were co-transfected with pCMV-Stat3-Flag and pCMV-Sat3-HA plasmids and exposed to 20 µM of STA-21 compound for 24h, then cell lysates were immunoprecipitated with anti-HA or anti-Flag antibodies, respectively as described previously. After resolving on 10% SDS-PAGE and then immunoblotted with anti-HA, anti-Flag or anti-Sat3 antibodies as described above.
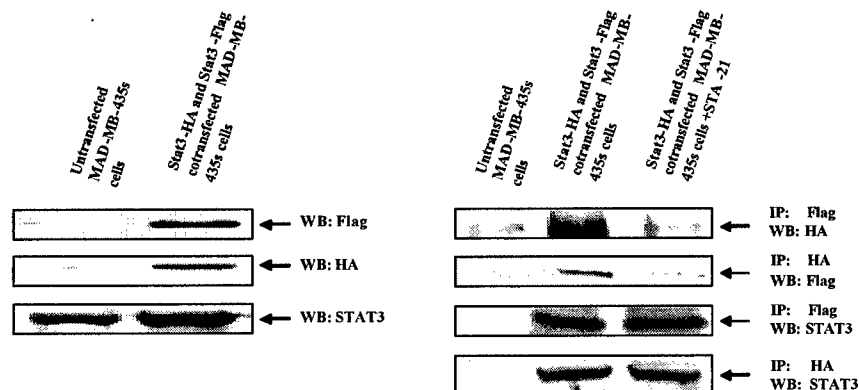
Since the dimerization of Stat3 is a decisive event for its activation, blocking the dimerization and nucleus translocation of Stat3 using cell-permeable, non-peptide small molecules is a very attractive approach for the inhibition of the Stat3 signaling pathway in breast cancer. In this study, we used a computational screening approach to identify potential small-molecule inhibitors of Stat3 from a database of 429,000 small molecules. Of the 100 candidate compounds tested, STA-21 showed remarkable inhibition of Stat3 dimerization, DNA binding, and nucleus translocation as well as the Stat3-regulated anti-apoptotic factors such as Bcl-X_L and Cyclin D1. STA-21 selectively inhibited the growth and survival of breast carcinoma cells with constitutively active Stat3 and had minimal toxicity to the cells without Stat3 signaling. Based on these results,

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we conclude tentatively that inhibition of Stat3 by STA-21 in the cells with persistent Stat3 signaling results in cell death due to the irreversible disruption of the Stat3 survival pathway that the cells mainly depend on. STA-21 represents a novel lead compound that specifically inhibits the Stat-3 activity.

Key Research Accomplishments:

The constitutive activation of Stat3 is frequently detected in human breast cancer cell lines as well as in clinical breast cancer specimens and may play an important role in oncogenesis of breast carcinoma. Activated Stat3 may participate in oncogenesis by stimulating cell proliferation, promoting tumor angiogenesis, and resisting to apoptosis. Hence, Stat3 represents an attractive target for cancer therapy. In this study, of the nearly 429,000 compounds screened by virtual database screening, chemical samples of top 100 compounds identified as candidate small molecule inhibitors of Stat3 were evaluated using Stat3-dependent luciferase reporter as well as other cell-based assays. Through serial functional evaluation based on our established cell-based assays, one compound, termed STA-21, was identified as matching our selection criteria best. Further investigation demonstrated that STA-21 inhibits Stat3 DNA binding activity, Stat3 dimerization, and Stat3-dependent luciferase activitiy. Moreover, STA-21 reduces the survival of breast carcinoma cells with constitutive Stat3 signaling but has minimal effect on the cells in which constitutive Stat3 signaling is absent. Together, these results demonstrate that STA-21 inhibits breast cancer cells that express constitutive active Stat3. STA-21 may have a therapeutic potential to be developed as a new class of anti-cancer drug for the treatment of human breast cancer with activated Stat3.

Reportable Outcomes:

1. Hui Song, Renxiao Wang, Shaomeng Wang, and Jiayuh Lin, A low-molecular-weight compound discovered through virtual database screening inhibits Stat3 function in breast cancer cells, *Proceedings of the National Academy of Sciences, USA*, 2005 (in press).

2. A patent application has been filed on the novel class of Stat3 inhibitors we have discovered, as supported by this DOD CONCEPT grant. DOD funding was acknowledged in the patent application.

Conclusions:

Supported by this DOD CONCEPT grant, we have discovered a novel small-molecule inhibitor (termed STA-21) of Stat-3 and have performed detailed in studies to characterize its molecular mechanism of action in human breast cancer cells. Our results demonstrate that STA-21 inhibits breast cancer cells that express constitutive active Stat3. STA-21 may have a therapeutic potential to be developed as a new class of anti-cancer drug for the treatment of human breast cancer with activated Stat3.