



FINAL REPORT

Project Title: Development of novel dual Insulin-like Growth Factor-1 Receptor (IGF-1R)/Epidermal Growth Factor Receptor (EGFR) inhibitors for treatment of Ewing's sarcoma

Project Number: SFA09-25

1. Date project was initiated: June 1, 2009
2. Period covered by this report: From June 1, 2009 To December 31, 2010
3. Publications, Abstracts, and Presentations:
 - a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry must include the author(s), article title, journal [book, editors(s), publisher, volume number, page number(s), and date.]
 - (1) Lay Press: N/A
 - (2) Peer-Reviewed Scientific Journals: N/A
 - (3) Invited Articles: N/A
 - (4) Abstracts: N/A
 - b. List presentations made during the last year (international, national, local societies, etc.). Use an asterisk (*) if presentation produced a manuscript.
N/A
4. Provide a brief list of keywords: (limit to 20 words)

IGF-1R, EGFR, receptor, inhibitor, dual inhibitor, synthesis, inhibitory activity, assay.

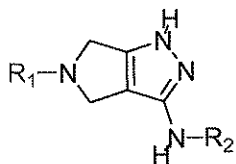
- Summarize the progress during the period of this report and its impact on your plans for the remainder of the project. Include a summary of the progress toward the achievement of the originally stated aims and list the significant results:

Specific Aim 1. Synthesize a focused library of dual inhibitors of the Insulin-like Growth Factor-1 Receptor (IGF-1R) and Epidermal Growth Factor Receptor (EGFR) for the treatment of Ewing's sarcoma. We will synthesize a library of compounds based on the structural core and novel modeling based on Maybridge building blocks and Geom selection.

Specific Aim 2. Determine the *in vitro* inhibitory activity and selectivity of the novel dual IGF-1R/EGFR inhibitors. We hypothesize that simultaneous targeting of the two receptor tyrosine kinases, IGF-1R and EGFR, will be an efficient treatment for Ewing's sarcoma (EWS). We will assess the *in vitro* inhibitory activity of the selected compounds on IGF-1R and EGFR and evaluate *in vitro* antitumor activity of these inhibitors in EWS cell lines (tumor cell viability, cell cycle, apoptosis)

Preliminary results: An initial search was conducted via PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) to identify compounds with $\geq 60\%$ similarity to those six compounds in order to generate a list of $\sim 3M$ searchable compounds. Duplicates were removed and then a rough filter for drug-like properties was used to reduce this to 633,308 molecules. A Topomer search was completed with a cutoff of 200 (default is 185) to get a list of 1,375 topomerically similar molecules to the original six structures. These molecules were docked into crystal structures for IGF-1R (2oj9), EGFR (1m17), and Insulin Receptor (1gag). The KNIME v1.3.5 workflow software was utilized to select those compounds with highest IGF-1R/EGFR activity and selectivity over IR. Only two of 14 molecules matching the cutoff criteria appeared to have the expected IGF-1R binding mode, but both of those molecules were covered by patents, either specifically for IGF-1R or on other kinases, greatly reducing the novelty of such compounds. Surprisingly, though, one of the compounds, with structural core of 1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-amine seemed to lack coverage for any kinase with an arrangement of the sulfonamide and amide groups opposite to those identified in a recent patent application. Therefore, we decided to generate a virtual library based on this novel arrangement and screen the library to determine if we could identify potential dual inhibitors of IGF-1R/EGFR that would be specific over Insulin Receptor. A virtual library of 141,432 (213 sulfonyl chlorides X 664 aliphatic/aromatic acids – **Figure 1**) was generated by Legion and filtered by applying drug-like criteria to give a final library of 94,098 structures.

Figure 1. IGF-1R/EGFR Structural core



Type	R1	R2
Carboxylic Acid	664	
Sulfonyl Chloride		213

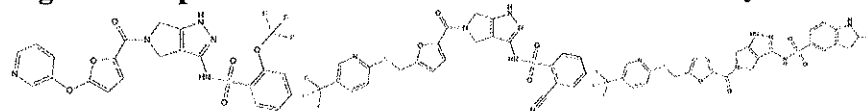
A Surflex docking job was initiated with "Screen" options to identify the Top 10% structures. This initial selection was followed by a more rigorous docking with "Geom" option and selection of Top 20% (2% overall). These 1,882 structures were docked into EGFR and IR and processed via a KNIME v1.3.5 workflow to identify the top six compounds based on the scoring and selectivity. The highest ranked inhibitor docked into the IGF-1R structure is shown in **Figure 2**. The top ranked six inhibitor structures are presented in **Figure 3**.

Figure 2. Proposed structure of the highest ranked inhibitor in IGF-1R

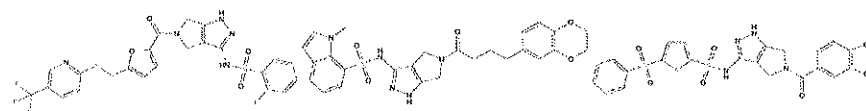


Image generated with Sybyl-X 1.2, Tripos, Inc., 1699 South Hanley Road, St. Louis, Missouri, 63144, USA.

Figure 3. Top 6 structures from IGF-1R/EGFR library



Rank: 1	Rank: 2	Rank: 3
title: IGFR4_852180_39_3_103008_51_1	title: IGFR4_238742_86_4_69360_26_5	title: IGFR4_238742_86_4_199328_31_9
IGFR Score: 11.1	IGFR Score: 11.84	IGFR Score: 11.91
Selectivity: 5.22	Selectivity: 5.75	Selectivity: 5.0
QPlogS: -5.644	QPlogS: -6.793	QPlogS: -4.914
R1: 852180-39-3	R1: 238742-86-4	R1: 238742-86-4
R2: 103008-51-1	R2: 69360-26-5	R2: 199328-31-9



Rank: 4	Rank: 5	Rank: 6
title: IGFR4_238742_86_4_2905_21_7	title: IGFR4_14939_93_6_941716_95_6	title: IGFR4_94_53_1_166964_37_0
IGFR Score: 11.66	IGFR Score: 10.5	IGFR Score: 11.36
Selectivity: 4.455	Selectivity: 4.035	Selectivity: 4.88
QPlogS: -5.361	QPlogS: -5.549	QPlogS: -4.88
R1: 238742-86-4	R1: 14939-93-6	R1: 94-53-1
R2: 2905-21-7	R2: 941716-95-6	R2: 166964-37-0

*Reports due by 31 July 2010

Specific Aim 1. Synthesize a focused library of dual inhibitors of the Insulin-like Growth Factor-1 Receptor (IGF-1R) and Epidermal Growth Factor Receptor (EGFR). Synthesis of the compound library followed the scheme outlined in **Figure 4**. The core starting material, 1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-amine scaffold, (**6**), was synthesized from the commercial *N*-(2-cyanoethyl)glycine (**1**). Treating **1** with sulfuric acid and methanol gave methyl ester **2**, which in the next step was protected with *tert*-butoxycarbonyl to give **3**. 4-Oxo-pyrrolidine-3-carbonitrile (**4**) was obtained when **3** reacted with sodium methoxide. Cyclization step 5 proved to be problematic, although reported literature was followed to synthesize this scaffold. After trying different reaction conditions, the desired 1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-amine (**6**) scaffold was obtained. Treatment of **6** with a solution of ethyl chloroformate in THF and diisopropylethyl amine in THF provided a mixture of compounds (**7**, **8**, and **9**). X-ray crystal structure confirmed the synthesis of the desired compound **9** as the major reaction product (**Figure 5**).

Figure 4. Synthetic scheme for the potential dual IGF-1R/EGFR inhibitors library.

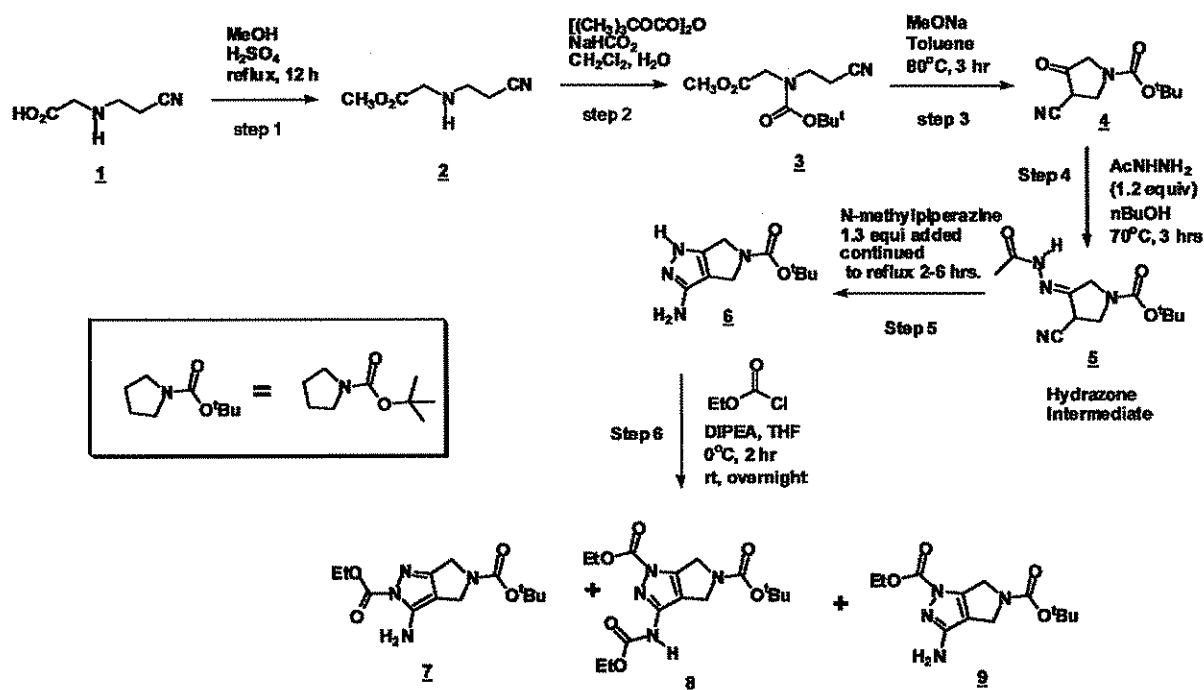
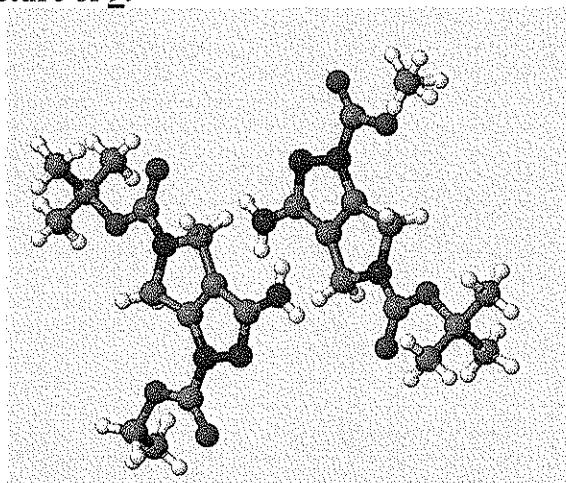
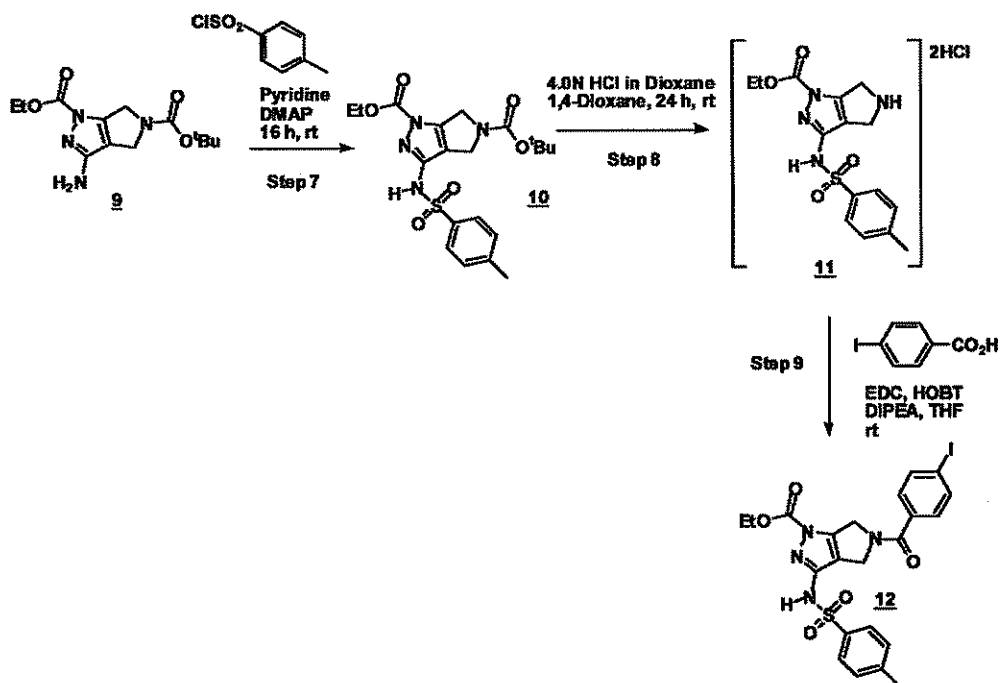


Figure 5. X-ray crystal structure of **9**.



The *first round of molecular diversity* was introduced by derivatization of the free amine with different substituted sulfonyl chlorides. In order to effect the *second round of molecular diversity* we had first to deprotect the N-Boc by treatment with a solution of 4.0N hydrochloric acid in dioxane as shown in *step 8*. The resulting ring nitrogen at position 5 can now be treated with the appropriately substituted carboxylic acid as shown in *step 9*. **Figure 6** emphasizes the molecular diversity introduced on scaffold **9** and the synthetic scheme for the first test compound (**12**). This final compound is not one of the designed inhibitors but it was the test compound that proves the feasibility of the reaction pathway. Following the same reaction scheme the highest ranked inhibitor from our newly designed library was successfully synthesized. Following the same synthetic pathway (**Figures 4 and 6**), the syntheses of the other 5 inhibitors from the top inhibitors list (**Figure 3**) are in progress.

Figure 6. Molecular diversity of scaffold **9**.

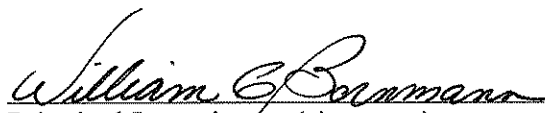


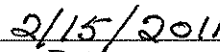
6. In layperson's terms, summarize the progress during the period of this report.

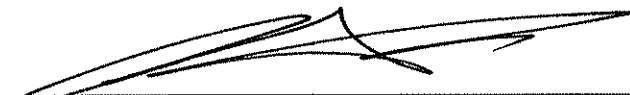
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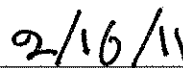
Explain any medical significance or implications of your results to date:

We hypothesized that simultaneous targeting of the two receptor tyrosine kinases will be an efficient treatment for EWS. The newly designed dual inhibitors will be assessed for their inhibitory activity. The EWS cell lines (RDES, SKES, T71) will be incubated in 96 well plates in triplicate. Inhibitors will be added at incremental concentrations from 0 to 10 micromolar. Viability will be assessed by MTS assay (Promega). Interesting compounds will then be assessed by flow cytometric assay for TUNEL and cell cycle simultaneously. Compounds with promising activity will then be used at the predetermined active concentration against EWS cell lines in vitro and protein lysates collected. Lysates will then be analyzed by western blot analysis to qualitatively determine the activity of the inhibitors against the targets and downstream signaling molecules. We will evaluate for changes in IGF-1R, p-IGF-1REGFR, p-EGFR, MapK, p-MapK, AKT, p-AKT, PKC, p-PKC in treated and untreated cells.


Principal Investigator (signature)


Date


Department Chair (signature)


Date