Lung cancer is the leading cause of cancer-related deaths, accounting for nearly a quarter of all cancer-related deaths. Non-small cell lung cancer (NSCLC) represents >85% of all lung cancers. Current treatments for NSCLC are dependent on platinum-doublet therapy, however, addition of a third chemotherapeutic agent does not significantly improve survival. Thus, treatment of NSCLC has reached a therapeutic plateau. Recently, NSCLC, and other cancers, have been shown to be reliant on enzymatic activities and metabolites involved in the folate cycle. Targeting the folate cycle has been an important strategy for chemotherapy for over 60 years, including the drugs methotrexate and 5-fluorouracil. Serine hydroxymethyltransferase (SHMT) and methylenetetrahydrofolate dehydrogenase (MTHFD) are two enzymes NSCLC relies on the in folate cycle. The mitochondrial isoforms of these enzymes (SHMT2 and MTHFD2, respectively) significantly correlate with poor prognosis in lung cancer. Previous studies showed that SHMT2 and MTHFD2 support rapid cancer cell proliferation, and knockdown of these enzymes reduces proliferation. Thus, this evidence supports SHMT2 and MTHFD2 as novel anti-cancer targets. However, no selective small molecule inhibitors exist for either of these folate-dependent enzymes. To identify such compounds, we developed a novel SHMT2-MTHFD coupled assay and screened ~110,000 compounds. This assay couples the SHMT2 reaction to the E. coli isoform of MTHFD (ecMTHFD), allowing us to screen for inhibitors of both enzymes simultaneously. Using this assay, we have identified inhibitors of both SHMT2 and ecMTHFD. Our hits will be studied and optimized for activity against the human isoforms of SHMT2 and MTHFD2. Thus far, our hits have been explored using differential scanning fluorimetry and docking studies. Co-crystal structure determination of SHMT2 and hMTHFD2 in complex with our respective hit compounds is in progress, albeit at different stages. After optimization of our hit compounds, we will evaluate the in vitro and in vivo efficacy in NSCLC models. The successful completion of this research will result in the identification of novel, potent compounds that are active against SHMT2 or hMTHFD2 in vitro as well as characterization of their potential anticancer properties.