

Title: FUROXAN AND BENZOFUROXAN DERIVATIVES ACTIVE AGAINST *MYCOBACTERIUM TUBERCULOSIS*: ACTIVITY AND MECHANISM OF ACTION STUDIES

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Abstract:

Recent data from the WHO points to 1.5 million deaths from tuberculosis (TB) in 2013 and 9 million new cases. The emergence of drug-resistant strains is worrying and places critical importance on the development of new compounds to treat the disease. In this context we developed and tested 15 new molecules: benzofuroxan and furoxan derivatives linked to an isoniazid (INH) moiety. Activity against strains susceptible and resistant to current first-line drugs and cytotoxicity against VERO cells were determined by resazurin-based assays. Microarray experiments were performed after 4hrs of treatment using the Agilent Expression Two-Color System. Kinetic assays employing *M. smegmatis* and ethidium bromide (EtBr) were performed to evaluate the ability of these new compounds to inhibit efflux pumps. Activity based on nitric oxide (NO) release was evaluated with diaminofluorescein diacetate (DAF-FM DA). Activity against *M. tuberculosis* H₃₇Rv was promising with MIC₉₀ values between 1.02 and 20.62µM. Susceptibility testing of clinical isolates resistant to INH and rifampicin yielded MIC₉₀ values as low as 3.09, 4.96 and 7.00µM, ruling out a mechanism-of-action (MOA) dependent on the INH moiety. After IC₅₀ measurements, 5 compounds presented acceptable safety patterns (SI≥10.00) and were chosen for further mechanistic studies. Assays evaluating EtBr accumulation inside *M. smegmatis* in the presence of the compounds showed insignificant accumulation compared to verapamil and CCCP, known efflux pump inhibitors (EPI) used as positive controls. These results eliminated the idea that the compounds would act as EPI (as previously suggested). The hypothesis of activity based on NO release was ruled out in the assay with DAF-FM DA, in which fluorescence emitted using the compounds was similar to the negative controls (DMSO), in contrast to the results with the positive control NO donor (DETA/NO). Microarray results were surprising showing a general effect in genes encoding ribosomal proteins even at an early time-point with 39 genes being overexpressed with treatment. Similar effects have been previously described for aminoglycosides, which inhibit protein synthesis. Currently, resistant mutants are being selected for whole genome sequencing in an attempt to elucidate the MOA of furoxan compounds. Considering these results, benzofuroxan and furoxan derivatives appear as excellent candidates for new compounds to treat TB with a possible novel MOA, inhibiting protein synthesis.

Keywords: tuberculosis, mechanism-of-action, microarray, resistance.

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