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# Engineering small molecule specificity in nearly identical cellular environments

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**Abstract**—Methotrexate (MTX), an inhibitor of dihydrofolate reductase, was tethered to an FKBP12 ligand (SLF), and the resulting bifunctional molecule (MTXSLF) potently inhibits either enzyme but not both simultaneously. MTXSLF is cytotoxic to fibroblasts derived from FKBP12-null mice but is detoxified 40-fold by FKBP12 in wild-type fibroblasts. These studies demonstrate that non-target proteins in an otherwise identical genetic background can be used to predictably regulate the biological activity of synthetic molecules.


Cell-permeable small molecules that perturb the functions of proteins have proven utility as therapeutic agents as well as probes of basic biological processes.[1] In certain therapeutic cases a lack of specificity can be valuable, as inhibition of several related proteins may contribute to the desired outcome.[2] The issue of specificity is more pressing when these probes are used as tools for basic science. When interpreting the results of studies using cell-permeable probes, one would like to be confident that the observed biological responses are directly related to the putative target(s) of a given probe. Wortmannin has been widely used to probe PI3K function, and SB 203580 has similarly been used as an inhibitor of p38 MAP kinase. However, several recent studies have shown that both of these molecules are more promiscuous than previously believed.[3–5]

In addition to their roles as probes of basic biological processes, many drugs take advantage of differences in the expression patterns of target proteins to achieve the desired therapeutic effects. This is particularly true for antimicrobial, antiviral, and anticancer agents. For example, β-lactam antibiotics inhibit transpeptidase enzymes to achieve their antimicrobial activity, and the lack of similar enzymatic targets in mammals renders these antibiotics quite selective. Similarly, the thymidine kinase enzyme of herpes simplex virus activates the prodrug, acyclovir, in infected cells to achieve antiviral selectivity. However, the therapeutic window is typically much smaller when the target protein is similarly expressed in both target and non-target cells.

Recently there has been increased research emphasis on modulating the effects of small molecules through covalent linkage of two ligands to create bifunctional molecules.[6] This strategy has been used to block β-amyloid protein aggregation with potential for treating neurodegenerative disease.[7] Tethering a traditional anticancer agent to a ligand for the estrogen receptor provided a bifunctional molecule that is selectively toxic to cells expressing the estrogen receptor.[8] There is a clear opportunity for further development of molecules whose activities are regulated by the cellular environment. A subtle example of this approach would be one molecule that displays different activity in two populations of cells that differ only in the expression of a single gene. An elegant theoretical framework for this concept has been put forward by Varshavsky.[9]

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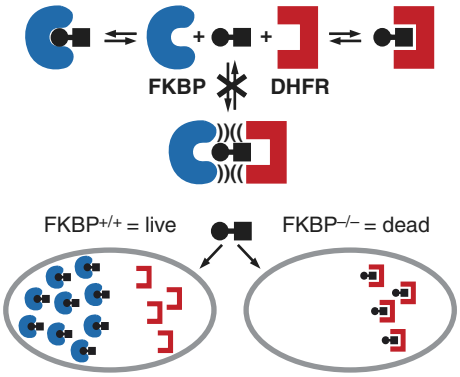


**Scheme 1.** Selective detoxification of MTXSLF binding either FKBP12 or DHFR but not both enzymes simultaneously.

With improved selectivity as the goal, we sought to take advantage of differences in the expression patterns of non-target proteins to predictably modulate the biological activity of synthetic molecules. Methotrexate (MTX), a dihydrofolate reductase inhibitor that is an anti-inflammatory and anti-tumor drug, was closely tethered to a synthetic ligand for FKBP12 (SLF).[10] This bifunctional molecule, MTXSLF, can potently inhibit either enzyme but not both simultaneously due to unfavorable protein–protein interactions that destabilize the ternary complex (Scheme 1).

Previous studies showed that MTXSLF is cytotoxic toward the malaria parasite, *Plasmodium falciparum*, but relatively nontoxic toward human cells due to higher expression levels of the human FKBP as well as the tighter affinity of the FKBP-binding half of MTXSLF for human FKBP relative to parasite FKBP.[10] However, malaria parasites and human cells differ in many respects, some of which might contribute to the selective toxicity that is observed.

We hypothesized that because murine FKBP is 97% identical to the human FKBP amino acid sequence (Fig. S1) and expressed at similar cellular concentrations,[11] we could use murine cells to observe selective detoxification in a more biologically relevant comparison. In the present study, we show that the context-dependent cytotoxicity of MTXSLF is robust in two different murine cell lines that differ only in the presence or absence of the FKBP12 gene.

Mouse embryonic fibroblast (MEF) cell lines were derived from wild-type mice as well as from mice in which both alleles of the FKBP12 gene were disrupted using homologous recombination.[12] Immunoblotting cell lysates using antibodies against FKBP12 showed that FKBP12 is undetectable in the FKBP-null cells (Fig. 1a). The MTT assay was used to determine the sensitivity of each cell to various concentrations of DHFR inhibitors.[13,14] Both cell lines are sensitive to MTX with $IC_{50}$ values of 180 and 120 nM for the wild-type and FKBP-null cells, respectively (Fig. 1b and c). The bifunctional DHFR inhibitor, MTXSLF, is quite cytotoxic to FKBP-null cells ($IC_{50}$ = 78 nM) but is only modestly toxic toward wild-type MEFs ($IC_{50}$ = 3200 nM) (Fig. 1b and c).

The attenuation in activity is due to the expression of FKBP12 as competition with FK506-M, a high-affinity FKBP12 ligand, restores the majority of the cytotoxicity ($IC_{50}$ = 550 nM). FK506-M alone has no effect on MEF viability. The magnitude of detoxification is perhaps the most important quantitative measure of the selectivity of the bifunctional molecule, and we find that MTXSLF is over 40-fold more toxic to FKBP-null MEFs.

This study demonstrates that selective biological activity can be predictably engineered in nearly identical genetic backgrounds. With the growing abundance of genomic and proteomic information from genome sequencing, microarrays, and other proteomic studies, strategies in which small molecules are engineered to display context-dependent activity may increasingly influence basic biological research as well as human disease.

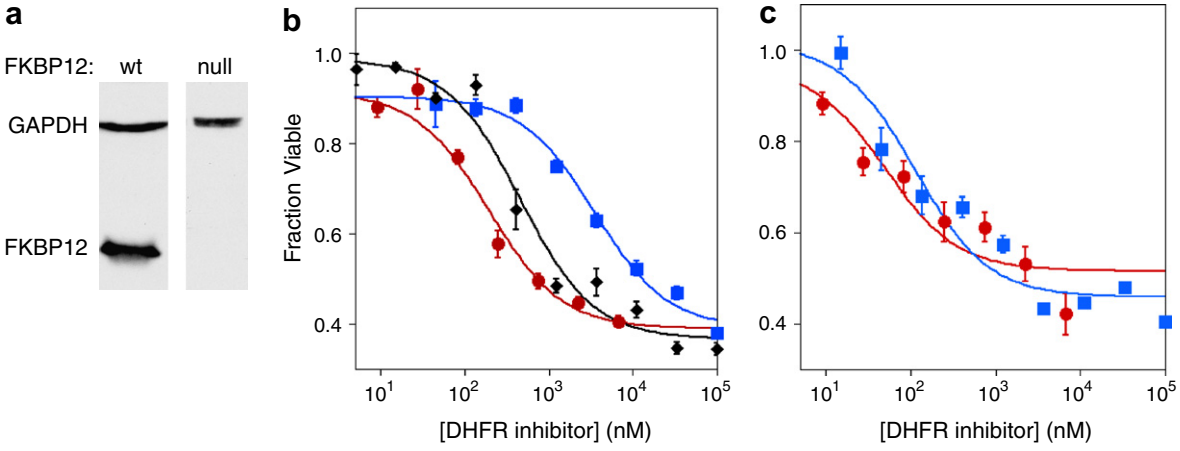


**Figure 1.** Selective detoxification in MEFs based on the presence or absence of FKBP12. (a) Immunoblots against FKBP12 for lysates of wild-type and FKBP12$^{-/-}$ MEFs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the loading control. (b) Wild-type MEFs were treated with various concentrations (four replicates) of MTX (circle), MTXSLF (square), or MTXSLF in the presence of 5 μM FK506-M (diamond), and cell viability was quantitated using the MTT assay. (c) MEFs derived from FKBP-null mice were treated with various concentrations of MTX (circle) or MTXSLF (square), and cell viability was measured as in (b). Error bars represent the standard deviation.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.012.

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14. Supporting information available: (1) MEF cell creation; (2) Western blot technique; (3) cytotoxicity assay; (4) $IC_{50}$ calculations.