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Abstract The use of small molecule inhibitors of cellular processes is a powerful approach to understanding gene function that complements the genetic approach. We have designed a high throughput screen to identify new inhibitors of eukaryotic protein synthesis. We used a bicistronic mRNA reporter to multiplex our assay and simultaneously screen for inhibitors of cap-dependent initiation, internal initiation and translation elongation/termination. Functional screening of >90 000 compounds in an *in vitro* translation reaction identified 36 inhibitors, 14 of which are known inhibitors of translation and 18 of which are nucleic acid-binding ligands. Our results indicate that intercalators constitute a large class of protein synthesis inhibitors. Four non-intercalating compounds were identified, three of which block elongation and one of which inhibits initiation. The novel inhibitor of initiation affects 5? end-mediated initiation, as well as translation initiated from picornaviral IRESs, but does not significantly affect internal initiation from the hepatitis C virus 5?-untranslated region. This compound should be useful for delineating differences in mechanism of initiation among IRESs.