Label-Free Screening of Drug–Protein Interactions by Time-Resolved Fourier Transform Infrared Spectroscopic Assays Exemplified by Ras Interactions

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Time-resolved Fourier transform infrared (FT-IR) spectroscopy can reveal molecular details of protein interactions. Analysis of difference spectra selects the absorptions of respective protein groups involved in an interaction against the background of the whole sample. By comparison of the same difference spectrum with and without a small molecule, one can determine whether the small molecule interferes with the protein or not. Usually a marker band of a specific residue of the protein is monitored. Here, we show three different time-resolved FT-IR assays detecting interactions of potential small molecules for molecular therapy with the GTPase Ras as an example for small GTPase binding proteins. Ras regulates signal transduction processes through a switching mechanism, cycling between an active "on" GTP-bound form and an inactive "off" GDP-bound state. Molecular defects in Ras can impair the ability of Ras and the Ras–RasGAP complex to hydrolyze GTP, contributing to uncontrolled cell growth and cancer. Oncogenic mutated Ras is found in about 30% of all cancer cells. We show in vitro assays, indicating (I) the shift of Ras into its "off" conformation, which inhibits the Ras pathway; (II) down-regulation of Ras signaling by changes in the Ras–Raf effector interaction; and (III) down-regulation of Ras signaling pathway by catalyzing GTP hydrolysis. Since almost all molecules have characteristic marker bands in the infrared, time-resolved FT-IR spectroscopy can be used label-free. No artificial nucleotides that could influence the interaction are needed. Both, sample preparation and evaluation can be automated in order to allow for high-throughput screening.

Index Headings: **Time-resolved Fourier transform infrared spectroscopy; FT-IR spectroscopy; Label-free; Screening; GTPase; Protein interactions; Metal macrocycles; Anticancer activity; High throughput.**

INTRODUCTION

The screening for the interaction of small molecules with proteins for molecular therapy is usually carried out by indirect methods by means of artificial labels or reporter systems. Often, the protein and/or the small molecule are labeled, e.g., by a fluorescent molecule. In these cases one can never rule out an influence of the usually bulky label on the result. On the other hand, time-resolved Fourier transform infrared (FT-IR) spectroscopy can monitor interactions of small molecules with proteins label-free.[1] Further, most reporter systems provide only very limited information on the interaction, whereas time-resolved FT-IR can often specify the type of interaction. Absorption bands can be assigned by isotopic labeling or site-directed mutagenesis and thus the interaction can be assigned to specific groups.[2] This information can be of aid in developing drugs that interact specifically with a protein in a molecular therapy.

GTPases are molecular switches that maintain cellular growth control by cycling between the active GTP-bound state ("on" state) and the inactive GDP-bound state ("off" state).[3] In the activated GTP-bound form, Ras interacts with multiple effector proteins involved in cell proliferation, cell migration, and other important processes.[4] Numerous studies of Ras interaction with regulatory proteins such as GTPase activating proteins (GAP)[5] and guanine nucleotide exchange factors (GEF)[5] or downstream effectors[6] were conducted with different biophysical methods, including X-ray,[7,8] nuclear magnetic resonance (NMR),[9] and time-resolved FT-IR[10–12] in order to understand the molecular mechanism, which is of high importance for medicinal chemistry because Ras is the most frequently mutated oncogene in both solid tumors and haematological neoplasias.[13] Remarkable progress in understanding the mechanisms of Ras regulation and signal transduction has provided targets for rational drug design approaches to block or possibly revert the aberrant function of Ras signaling in human cancer. As a result, a large number of different inhibitors have been developed to target oncogenic Ras itself or the components upstream and downstream of Ras in tumors, i.e., (1) interfering RNAs, (2) membrane association of Ras, (3) Ras activation by GEFs, (4) Ras–effector interaction, (5) GTPase reaction of Ras, and (6) multiple signaling pathways.[14–18] Phosphate chelating agents such as zinc cyclen can down-regulate signaling by shifting the equilibrium of Ras–GppNHp towards the "off" conformation.[19] Further, molecular modeling had predicted polyamines as potential candidates to interfere with the effector binding domain of Ras.[17]

In the present study, we show that changes in time-resolved FT-IR spectra can be used as assays in screening for small molecules that possibly prevent movement to the "on" state of Ras and/or accelerate GTP hydrolysis. Both would down-

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