ELETTRA PROPOSAL DESCRIPTION
Proposal number: 20095114
Title: Fourier Transform InfraRed microscopic analysis of single leukaemic cell.

Proposer: Giuseppe BELLISOLA
Beamline(s): SISSI
Required shifts: 18
Objectives: To identify and validate IR features of apoptosis in the spectrum of single K562 leukaemic cells treated with the drug STI571 and to obtain a panel of FT-IR absorbance spectra representative of apoptotic processes in single cells.

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1.Background

Fourier Transform InfraRed Microspectroscopy (microFT-IR) is a non-invasive, reagent- and label-free, highly selective and sensitive, and easily automatable analytical technique giving information on the nature and spatial distribution of biochemical components within a biological sample. It is promising to be an objective, high throughput technique, for instance to test drugs in "ex vivo" cell models such as the human K562 erythro-leukaemic cells and/or to rapidly identify drug-resistant/-sensitive blast cells in the peripheral blood of patients with leukaemia. During the previous beamtime (proposal number 2008232) we optimized the preparation of analytical samples and we identified the best instrumental conditions to acquire with a single-channel MCT detector average FT-IR spectra from 50x50 μm squared spot areas (~15 cells) using Globar as well as FT-IR spectra of single, isolated cells from 20x20 μm squared spot area illuminated with a Synchrotron Radiation IR beam. Early and significant changes were identified in the average spectra of K562 cells treated with drugs that interfere with intracellular phosphonetworks. An IR marker of protein phosphorylation was identified in the interval of frequencies between 1000 and 900 cm^{-1} and cross-validated by complementary biochemical information. Also unsupervised Hierarchical Cluster Analysis (HCA) applied to spectral region from 1290 to 900 cm^{-1} confirmed the validity of results interpretation since the average FT-IR absorbance spectra of replicates were grouped according to the three K562 cell clones and to the type of drug utilized in "ex vivo" experiments. Unfortunately, the low efficiency of the last gold mirror mounted at the time of the beamtime at SISSI@Elettra did not allow to analyze the spectra of single cells in great detail and compare the IR signals below ~1100 cm^{-1}. Nevertheless, HCA in the region of amides (1690-1480 cm^{-1}) clustered the spectra of single cells in two groups roughly corresponding to the IR spectral profiles of living and of apoptotic cells [1-3] that we related to drug-resistance and drug-sensitivity of K562 cells and of blast cells from a patient with acute leukaemia, respectively (see Figure 1). A paper with the results of experiments summarized in the report of previous proposal is in editing.

2.Motivation for the present proposal

Many anti-leukaemia drugs, included the selective inhibitor of BCR-ABL tyrosine kinase phosphorylation STI571 (Imatinib mesylate) [4], induce or restore apoptosis in blast cells. Therefore, we need to associate IR features of biochemical changes induced in the spectrum of single K562 cells by STI571 and apoptotic events which involve time-dependent biochemical and structural changes in lipids, proteins, and nucleic acids molecules. With respect to the previous proposal, we will expose cells to drug for longer times in order to identify drug-induced IR features of apoptosis in the spectra of single cells. Since beamline performances below 1100 cm^{-1} have been significantly improved by the replacement of the last mirror of SISSI biobranch in March 2009, we expect to analyze in greater detail also the so called "fingerpriniting region" (1290-900 cm^{-1}) reflecting vibrations of functional groups characterizing nucleic acids, phospholipids, sugars and phosphorylated proteins in the spectrum of single cell. It is very important to have spectra of single cells representative of apoptotic events at different time-points, since they can be used as reference spectra, for instance to identify drug-sensitive/-resistant blasts in patients with leukaemia, as well as to test their sensitivity to drugs, by combining FT-IR absorbance spectroscopy and HCA. To distinguish the spectral profile of one drug-resistant blast among hundred surrounding drug-sensitive blasts, IR features obtained in the FT-IR absorbance spectra of single cells must be validated by complementary biochemical and morphological analysis performed on single cells and related to specific events of cell apoptosis. Therefore, we ask for the possibility of another beamtime in order to complete the previous experiment, to analyze a high number of single cell spectra, and to validate IR profiles of apoptosis in the spectra of single cells.

3.Experimental plan

We will utilize cells from three K562 cell clones stably transfected with the empty vector (CTRL mock), or with full-length human PTPRγ cDNA (FLPTPRγ), or with full-length human D198A mutated PTPRγ cDNA (FLD198APTPRγ) and differing for the expression and the activity of receptor-like Protein Tyrosine Phosphatase gamma (PTPRγ). In order to reduce inter-individual variability, we will synchronize cell cycle to G0/G1 phase by growing K562 cells in the essential RPMI 1640 medium without foetal bovine serum for 24 hours. Then cells will be plated at a cell density of $5x10^6$cells/well and grown suspended in the complete medium restored with 10% bovine serum. Aliquots of 5μM STI571 will be added to logarithmically growing cells to three replicates of each K562 clone that will be separately incubated for 20, 60, 120 and 240 minutes, respectively. At the end of incubation, cells and untreated controls will be fixed for 30 minutes in 1% formalin buffered in PBS, washed with pure water to eliminate PBS, deposited and dried on ZnSe crystals to obtain two arrays of sample replicates, respectively. A third array will be prepared with fresh, unfixed cells stained with Annexin-V in order to identify changes occurring at the cell surface and plasma membrane at the early stages of apoptosis (exposition of phosphatidylserine at the external surface of the cell). Single isolated cells will be selected and IR signals will be acquired in transmission mode from spot areas fitting the dimensions of a cell (diameter ~10-15μm); 36X condenser-objective will be used. We plan to process 30 samples (each K562 clone = 5 spot samples, 1 CTRL + 4 treatments; 3 K562 clones deposited on a single ZnSe crystal, 2 replicates) maintaining the IR sampling interval of wavenumbers from 800 to 4000 cm^{-1} (spectral resolution $4cm^{-1}$). The FT-IR spectra of one hundred individual cells will be acquired in each spot sample co-adding 256 scans on the single-channel MCT detector. With a scanner velocity of 20KHz and an average acquisition time per cell of 2 minutes, roughly 200 spectra of single cell will be obtained per shift, taking into account also the time needed to select cells in the sample and to acquire spectra

from corresponding backgrounds. By visible microscopy some cells with particular shapes, for instance giant cells and shrinking cells, will be mapped at sub-cellular level fully exploiting the high brightness of SR in order to gain major details on intracellular events. Statistical analysis and a detailed comparison among K562 cell clones and treatments will be performed in order to establish, if existing, a correlation between early spectral changes induced by STI571 in cells and the evolution of morphological and biochemical features leading to programmed cell death and revealed by complementary biochemical investigation in samples. For instance, to measure DNA strand breaks associating to early nuclear changes during apoptosis, the TUNEL assay will be applied to formalin-fixed samples and apoptotic cells will be counted by fluorescence microscopy. Fluorescence data and IR data will be compared in the same spot area. Unsupervised HCA will be applied to selected intervals of frequencies in the spectrum and utilized to classify sensitive and resistant cancer cells based on the differences of their IR spectral profiles that will be validated by the results of TUNEL assay.

4. Explain why this work calls for access to Elettra

The complexity of the planned experiment requires a FTIR microscope with fast acquisition capabilities and high signal-to-noise ratio (SNR) values at high spatial resolution. Therefore, SR IR source is more indicated than Globar source to obtain the FT-IR absorbance spectra of single cells with reasonable SNR values and within a reasonable acquisition time of IR signals from spot areas of reduced sizes (<20x20 μm squared) on the single element MCT detector. We also expect that technical improvements at SISSI@Elettra will allow to analyze the spectra of thousands single cells and to compare spectral region of frequencies below 1100 cm^{-1}.

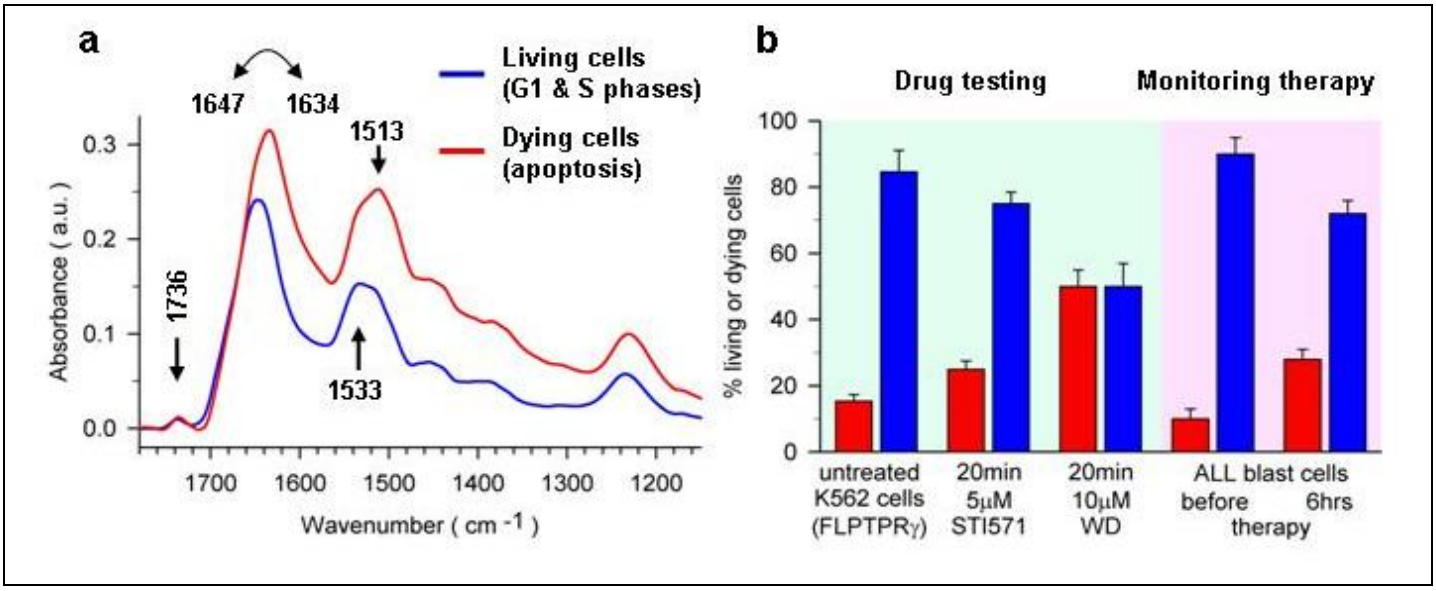

Figure 1: FT-IR spectroscopic approach for the evaluation of drug-resistance/-sensitivity in leukaemic cells (proposal 2008232).
a: representative FT-IR spectra of living and apoptotic FLPTPRγ K562 cells identified by HCA on hundred spectra of single cells. Major differences are observed in the region of amides I and II (1700-1480 cm^{-1}) sensitive to the environment of peptide linkage and reflecting the overall secondary structure in proteins and in the relative intensity of CO stretching at ~1736 cm^{-1} attributed to plasma membrane phospholipids.
b: the relative variations (mean±SD) in the number of apoptotic (drug-sensitive, red) and living (drug-resistant, blue) K562 cells after a short (20 min) exposition to STI571 or WD, inhibiting BCR-ABL tyrosine kinase posphorylation and targeting protein tyrosine phosphatase PTPRγ, respectively. Significant differences are observed in treated with respect to untreated K562 cells and between the two different treatments. The percentages of living and apoptotic blasts identified by FT-IR absorbance spectroscopy and HCA on the spectra of single leukaemic cells isolated from the peripheral blood of a patient with Acute Lymphobastic Leukaemia (ALL) are also compared before and after the administration of a standard therapeutic protocol. STI571: Imatinib mesylate (or Gleevec from Novartis Pharmaceuticals, Basel, Swiss); WD: penetratin-conjugated Wedge domain peptide targeting PTPRγ.

5) References

[1] H-Y. N. Holman, et al., *Biopolymers (Biospectroscopy)* **57**, 329-335, (2000).

[2] K.Z. Liu, et al., *Apoptosis* **6**, 267–276 (2001).

[3] Jamin N., et al., *Biopolymers* **72**, 366–373 (2003).

[4] Deininger M., Druker B.J. *Pharmacol Rev* **55**, 401-423 (2003).