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Heterogeneous Sensitivity of Hepatocellular Carcinoma to Sorafenib Revealed by the Short-term Culture of Tumor Fragments

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Abstract. *Background/Aim: Sorafenib is currently the only medical treatment with proven efficacy against hepatocellular carcinoma (HCC). HCC cell lines display heterogeneous sensitivity to sorafenib, but little is known about the sensitivity of clinical tumors. We aimed to examine this aspect. Materials and Methods: Using experimental tumors generated in nude mice, we set up a technique for short-term culture of HCC fragments. We applied this technique to six human HCC samples obtained from surgical resection. Results: HCC fragments in culture retain their morphology and viability for at least 48 h, permitting an in vitro analysis of the effect of sorafenib on the Extracellular signal-regulated kinase (ERK) cascade. HCC exhibit heterogeneous individual responses, ranging from potent inhibition to paradoxical activation of this oncogenic cascade. Conclusion: Our observations highlight the heterogeneous sensitivity of HCC to sorafenib, and point to the potential interest of short-term culture of tumor fragments for personalizing the medical treatment of HCC.*

Hepatocellular carcinoma (HCC) is the most frequent form of primary liver cancer (1). Sorafenib, a kinase inhibitor that is active against RAF kinases, is currently the only medical treatment proved to extend the survival of patients with advanced HCC (2). Sorafenib essentially acts as a stabilizer of tumor growth, a fact that complicates the individual evaluation of its efficacy (2, 3). Consequently, little is known about the heterogeneity of individual responses of patients to sorafenib. We recently found great differences among a panel of HCC cell lines, some being more resistant to sorafenib than others (4). We examined the rational basis for this heterogeneity, and observed that the antiproliferative efficacy of sorafenib correlates with its ability to control the ERK kinase cascade in each cell line. In resistant HCC cells, sorafenib lost its ability to inhibit, and sometimes even paradoxically activated the ERK kinase cascade (4). We hypothesized that Epidermal growth factor receptor (EGFR) activation could be a possible source of resistance of HCC cells to sorafenib. In some HCC cells resistant to sorafenib, EGFR inhibition by erlotinib prevented the paradoxical activation of the ERK cascade and augmented the anti-oncogenic efficacy of sorafenib (4). These observations point to the importance of the ERK kinase cascade as a therapeutic target of sorafenib in HCC, and indicate that HCC cells may not all be equally sensitive to sorafenib (4). In the present work, our aim was to translate these observations to a more clinically-relevant setting. Using experimental tumors grown as xenografts and clinical samples obtained from surgical resections, we validated a technique for the short-term culture of tumour fragments and used it to examine the sensitivity of individual HCC to sorafenib.

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Materials and Methods

Cell lines and cultures. Hep3B cells were obtained from Dr. Wychowski (Institut de Biologie de Lille, France) and were authenticated using profiling of short tandem repeats at 16 loci (LGC Standards, Strasbourg, France) (data not shown). Hepatocytic

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Table I. *Baseline characteristics of the patients with hepatocellular carcinoma.*

				Non-tumoral liver		
Patient no.	Age	M/F (years)	Tumour size	Fibrosis * (cm)	Child-Pugh	Aetiology# class
1	84	M	4	A1F2-F3	A	NASH, T2D
2	71	M	4.5	A1F3	A	NASH
3	76	F	3.6	A1F1	A	Hemochromatosis
4	63	M	16	A2F3-F4	A	-
5	48	M	50	A1F4	B	Alcohol
6	78	M	30	A1F2-F3	A	Alcohol, T2D

*Histological findings are quantified using the Metavir algorithm (8). Fibrosis is scored between 0 and 4 (F_0=non fibrosis, F_1=portal fibrosis without septa, F_2=portal fibrosis with rare septa, F_3=numerous septa and bridges without cirrhosis, F_4=cirrhosis) and histological activity between 0 and 3 (A_0=no activity, A_1=mild, A_2=moderate, A_3=severe). #Alcohol consumption defined as >20 g/day for females and >30 g/day for males. NASH: Non-alcoholic steatohepatitis. T2D: Type-2 diabetes.

differentiation was verified during the course of the experiments by measuring alpha-fetoprotein secretion in the culture medium (data not shown). Cells were cultured in DMEM (Dulbecco's modified Eagle's medium) purchased from Sigma (Saint Quentin Falavier, France) and supplemented with 10% fetal calf serum (Jacques Boy, Reims, France), 2 mM glutamine, penicillin and streptomycin. Cells were maintained at 37˚C in a humidified atmosphere with 5% CO_2.

Animals. Research carried out on animals at our University complies with French standards of ethics, and was performed after approval by the local Ethics Committee (CREMEA, approval number: 1107-02). Tumours of about 8 mm in diameter were recovered 14 to 21 days after the subcutaneous injection of 5×10^6 Hep3B cells in female BALB/c nude mice (Charles River, Wilmington, MA, USA). Mice were killed by cervical dislocation prior to tumor recovery.

HCC samples. Clinical HCC samples were obtained from patients undergoing surgical resection with curative intent (n=6) performed between November 2011 and May 2012 in the Amiens University Hospital. The corresponding protocol was approved by the Comité de Protection des Personnes Nord-Ouest (CPP NO). Patients' consent was obtained for the use of resected tumors. A summary of the pathological findings and baseline characteristics of each patient is given in Table I.

Antibodies and chemical reagents. Sorafenib was a kind gift from Bayer Healthcare (Wayne, NJ, USA). Primary antibodies directed against ERK and phosphorylated ERK were from Cell Signaling Technology (Danvers, MA, USA), those against actin from Sigma, and secondary antibodies coupled to peroxydase from GE Healthcare (Velizy Villacoublay, France).

Histology. Tissue specimens were formalin-fixed and paraffin-embedded; 4-μm sections were cut and stained with hematoxylin-eosin-saffron.

Immunoblot analysis. Following homogenization in sample buffer, protein concentrations were measured with a BCA kit (Pierce, Rockford, IL, USA). Fifty micrograms of protein were loaded on denaturing polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. Immunoblots were performed with relevant antibodies and the Enhanced chemiluminescence reaction was used for revelation. Western blots were scanned and quantified using the ImageJ software (National Institute of Health, NIH, USA).

Lactate dehydrogenase (LDH) release assay. Tumor cell viability was determined using the CytoTox 96 assay (Promega, Madison, WI, USA), measuring the release of LDH in the culture medium. All analyses were performed on the culture medium collected at different time points. The total LDH content, taken as 100%, was obtained after tumor homogenization in 1% Triton ×100.

Statistical analyses. The Student's *t*-test was used and a value of $p<0.05$ was considered as the threshold for significance.

Results

In order to explore the individual sensitivity of HCC to sorafenib, we set up experimental culture conditions that would preserve the architecture and the viability of the tumour tissue. We initially used Hep3B tumors grown as xenografts in nude mice. Tumors obtained by subcutaneous injection of Hep3B cells in female BALB/c nude mice are histologically similar to human HCC, presenting a morphology with high cell density, few or no areas of necrosis or inflammation, and a rich vascular supply (data not shown). The tumors were recovered after reaching an approximate diameter of 8 mm. Mice were killed by cervical dislocation and the tumours were surgically recovered under sterile conditions. Immediately after tumor resection, tumors were cut into homogeneous cubic fragments (each 4 to 6 mm^3). The tumor fragments were rinsed once in DMEM and incubated in 24-well tissue culture plates in DMEM supplemented with 10% fetal calf serum, glutamine and antibiotics. At different time intervals, the fragments were fixed and processed for histological analysis (Figure 1A). We found that the fragments retained the initial tumour organization for up to 48 h. After 72 h, areas with reduced cellular density evocative of cellular necrosis were apparent (Figure 1A). To further assess tumor cell viability, we measured the release of the intracellular enzyme LDH in the culture medium (Figure 1B). We found that the levels of tumor cell lysis remained low (<20% of total LDH) during the first 48 h of culture (Figure 1B), confirming that tumor cell viability was preserved for at least 48 h. Sorafenib was applied under these conditions, in order to evaluate its impact on oncogenic kinases in the tumor fragments. After 48 h of treatment with increasing concentrations of sorafenib, protein extracts prepared from tumor fragments were analyzed by immunoblotting in order to measure the phosphorylation levels of ERK. As shown in Figure 1C, increasing

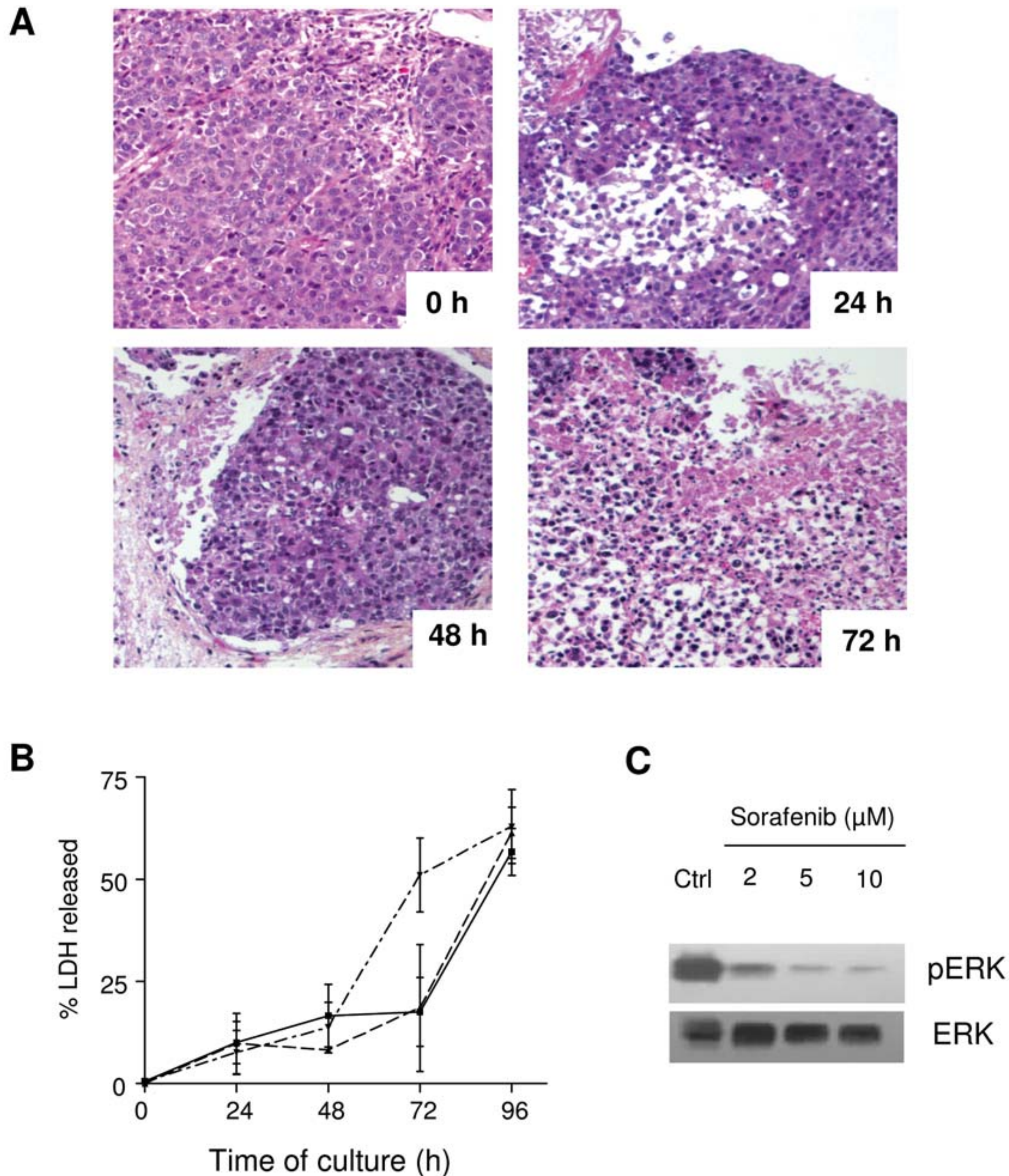

Figure 1. *Short-term culture of fragments of hepatocellular carcinoma. A: Histological examination of fragments prepared from Hep3B tumours grown as xenografts in nude mice and maintained in culture for 0 to 72 h (magnification, ×20). Note that the architecture and the heterogeneous composition of the tumours are preserved for up to 72 h. B: Analysis of tumor viability by Lactate dehydrogenase (LDH) release assay. Results are expressed as a percentage of total LDH for three independent Hep3B tumors. For each tumor, average values were obtained from four fragments analyzed at the indicated time points. C: Experimental verification of the kinase-blocking activity of sorafenib in Hep3B tumour fragments. Tumor fragments were exposed to increasing concentrations of sorafenib (0 to 10 μM) for 48 h, and protein extracts were analyzed by immunoblotting to detect Extracellular signal-regulated kinase (ERK) phosphorylation.*

concentrations of sorafenib reduced the activation level of the ERK pathway in a concentration-dependent manner. We concluded that our experimental conditions permitted the functional analysis of the impact of sorafenib on its target oncogenic kinases.

We further proceeded with the analysis of six HCC samples obtained from surgical resections (Table I). Active, non-necrotic parts of the tumours were morphologically identified and small tumour fragments were prepared as previously described. The fragments were exposed to

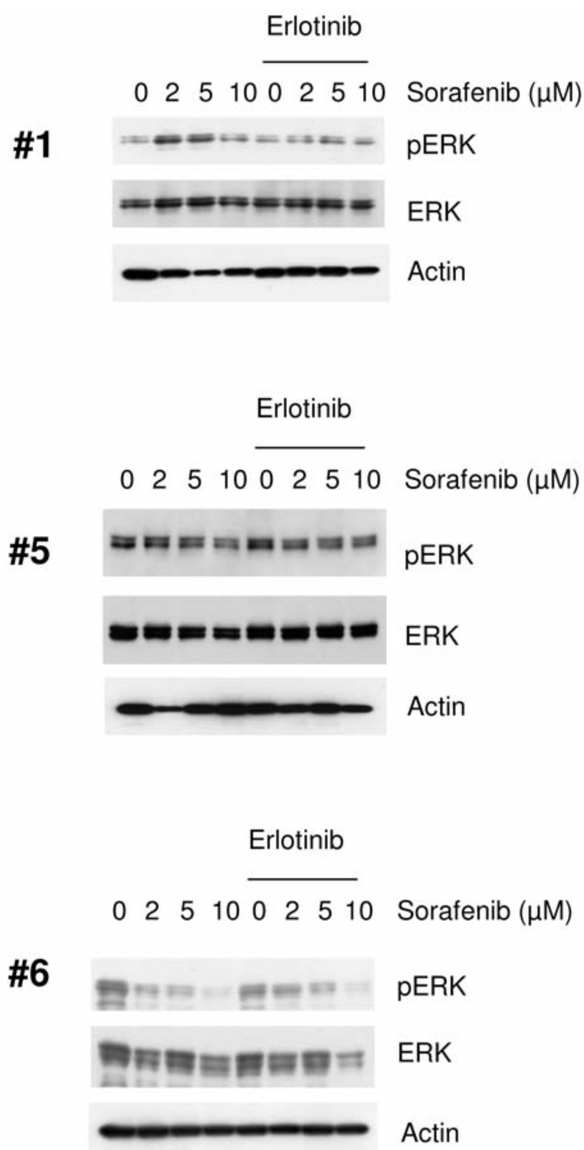

Figure 2. *Heterogeneous impact of sorafenib on the ERK kinase in human hepatocellular carcinoma fragments. Tumour fragments were incubated for 48 h with increasing concentrations of sorafenib (0 to 10 μM), either alone or with erlotinib (1 μM) and processed for the analysis of ERK phosphorylation by immunoblotting. Representative immunoblots are shown for the three types of responses that were observed, i.e. tumors that are either sensitive to the inhibitory action of sorafenib (patient #6), poorly-sensitive to sorafenib (patient #5), or present a paradoxy activation of ERK phosphorylation (patient #1).*

increasing concentrations of sorafenib (0, 2, 5, 10 μM) for 48 h, alone, and in combination with erlotinib (1 μM). We noticed that ERK phosphorylation in tumour fragments followed three patterns (Figures 2 and 3): i) concentration-dependent inhibition of ERK phosphorylation, leading to a potent inhibition of the pERK signal at 5 μM, *i.e.* at clinically relevant concentrations of sorafenib (3) (n=1; patient #6); ii) little or no effect of sorafenib on pERK, even when applied at high, supra-clinical concentrations (n=3; patients #2, #4 and #5); iii) paradoxical activation of ERK phosphorylation by sorafenib (n=2; patients #1 and #3). Interestingly, this paradoxical activation was in both cases somewhat abrogated by erlotinib (Figures 2 and 3). We conclude that tumour tissues prepared from resected human HCC present important differences in their responses to sorafenib.

Discussion

Short-term culture of tumour samples is a promising approach for predicting tumour sensitivity to drugs in a patient-specific manner (5). To our knowledge, the present report constitutes the first application of this technique to HCC and a targeted therapy. Tumor tissue culture preserves the architecture as well as the heterogeneous composition of the tumour, including both the stromal and non-tumoral cells. This is an essential point because the tumor microenvironment is a potent determinant of tumor cell sensitivity to targeted therapies directed against RAF kinases (6). Another important advantage of this technique is that it takes into account the variety of HCC aetiologies. Indeed, most HCC cell lines were established in a context of viral carcinogenesis mediated by the hepatitis-B virus (4). Short-term culture of tumor fragments is potentially interesting in order to explore *in vitro* the response of HCC associated with chronic alcohol intake or metabolic alterations, for which there are no cell line models.

Because of previous findings reported by our group and others, indicating that the ERK kinase cascade is an important target of sorafenib in HCC (3, 4), the present report was centred on the effects of sorafenib on this pathway. We found that HCCs are strikingly heterogeneous regarding their sensitivity to sorafenib, with individual responses ranging from a potent inhibition of the ERK cascade to its paradoxical activation. Interestingly, a chemical blocker of EGFR improved the control exerted by sorafenib over the RAF kinase cascade in two tumors, in accordance with our recent description of EGFR activation being a potential source of resistance to sorafenib in HCC cell lines (4). The finding that RAF kinases can be paradoxically activated by sorafenib in HCC is potentially important. Such a paradoxical activation was, for example, recently reported to promote skin carcinogenesis induced by vemurafenib, another inhibitor of RAF kinases (7). There are currently no validated genomic or biological analyses able to predict the sensitivity of HCC to sorafenib at the individual patient level. Although more investigations are clearly required to expand the characterization of individual responses of HCC to sorafenib, short-term culture of tumour fragments holds potential for predicting the individual efficacy of this treatment.

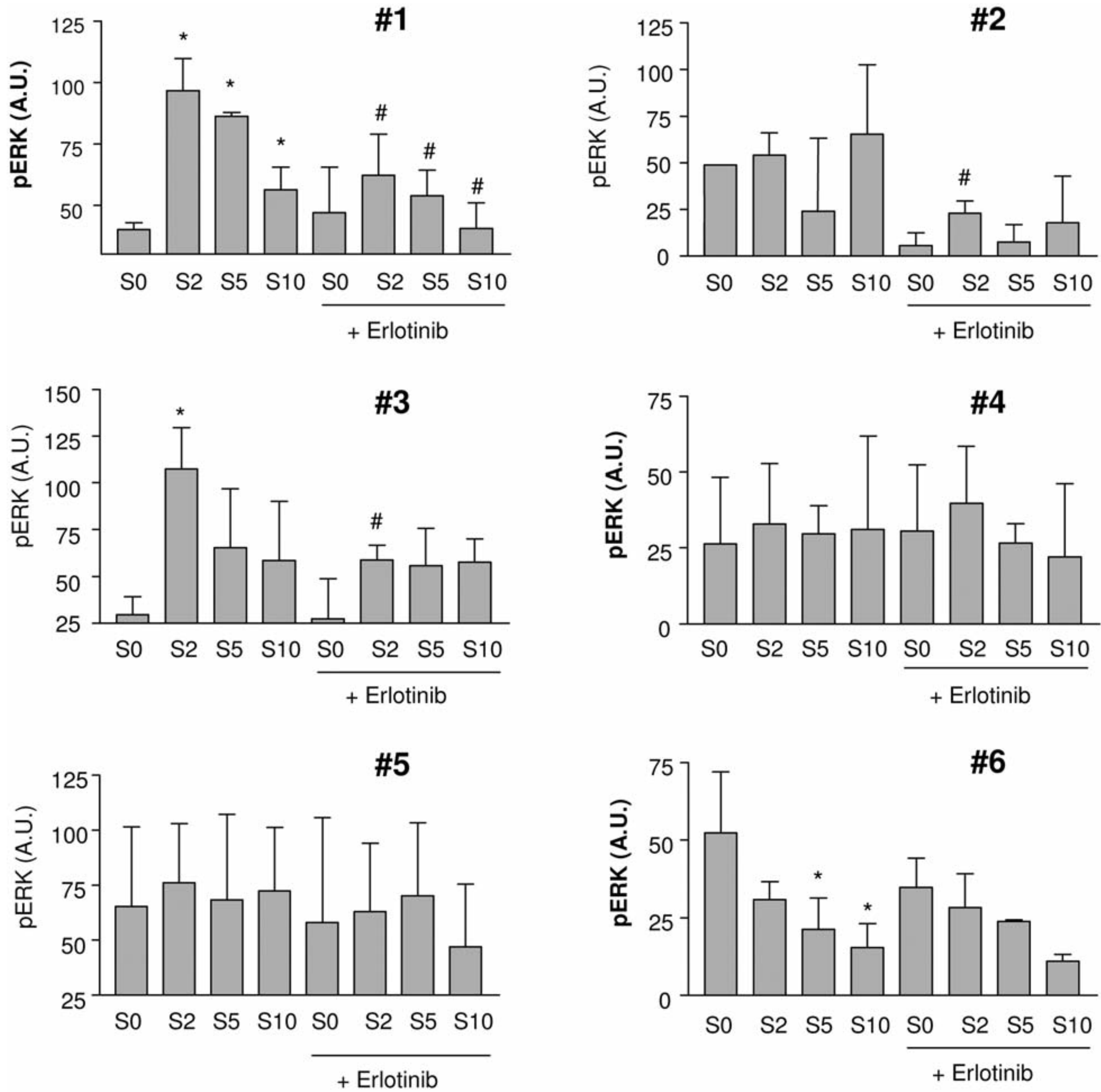

Figure 3. *Quantification of the impact of sorafenib on ERK phosphorylation in human hepatocellular carcinoma fragments. Quantification of the ERK phosphorylation level is presented for each patient under the same experimental conditions as in Figure 2. The results are average measurements calculated from three fragments of the same tumour exposed to the indicated concentrations of sorafenib (0–10 μM), alone, and in association with erlotinib (1 μM). *p<0.05 compared to the condition without sorafenib (S0); #p<0.05 compared to equivalent conditions without erlotinib.*

Conflicts of Interest

None declared.

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