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## Src inhibitors in early breast cancer: a methodology, feasibility and variability study

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**Abstract** Early clinical trials of anticancer agents may be enriched by robust biomarkers of activity. Surrogate measures used in trials of cytotoxic agents, such as tumor size regression, may not be informative when investigating targeted agents that act principally to inhibit invasion or proliferation. This study aimed to determine the validity of invasion-related biomarkers of activity for AZD0530, a potent Src inhibitor currently in clinical development. Focal adhesion kinase (FAK) and paxillin are downstream phosphorylation substrates of Src and mediate tumor cell adhesion and invasiveness. These were therefore selected as biologically relevant markers of Src inhibition. Early breast cancer was chosen as a model as multiple samples can be collected during standard treatment and there is an intervening period in which experimental intervention can be applied. Tumor tissue was collected from diagnostic core biopsies and subsequent surgical tumor excision samples in 29 women with early breast cancer attending a single center. Protein levels were assessed quantitatively by Luminex<sup>®</sup> and qualitatively by immunohistochemistry. AZD0530 inhibited tumor growth in a manner independent of dose and inhibited phosphorylation of FAK and paxillin

in a dose-dependent manner in a Calu-6 xenograft model. In the clinical study, agreement of within-visit and also of between-visit measurements was high and the estimated number of patients required to detect a drug effect would be low enough to allow use of these markers as endpoints in future dose selection studies.

**Keywords** Src kinase · AZD0530 · Early breast cancer · Focal adhesion kinase · Paxillin · Immunohistochemistry · Phosphorylation

### Background

Rationale for a Src inhibitor in breast cancer

Src kinase and its potential role in cancer development has been reviewed extensively in the literature [1, 2]. As with many other common solid tumors, Src expression and activity is elevated in human breast cancers [3–5] with high activity being associated with poor outcome [6]. Preclinical data support a role for Src in the regulation of breast cancer cell motility, invasion and metastasis [7–9]. Evidence also exists linking Src signaling to estrogen receptor signaling [9]. It is therefore possible that inhibition of Src could have an effect in tumors that fail to respond to hormone or growth factor therapies [10, 11], a group where there remains a high level of unmet clinical need.

Methodological rationale in early breast cancer

Early breast cancer is an attractive disease model for pharmacodynamic studies using tumor tissue biomarkers. In most centers, the routine diagnosis and management of breast cancer is a two-stage process. Patients undergo

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tumor biopsy—usually a core biopsy—to confirm the diagnosis of invasive cancer at which point samples can be taken for experimental investigation. This is followed by surgical excision of the tumor permitting the use of tissue, which is excess to diagnostic requirements, for further biomarker assessment. Clinical logistics usually dictate that there will be a window of time between the biopsy and definitive surgery during which an experimental intervention (such as drug administration) can take place without compromising the patient's routine cancer care. Hence, this approach provides an ideal model for measuring the effects of novel drugs on tissue biomarkers in patients with early, treatment-naïve cancer (Fig. 1).

### AZD0530

AZD0530 is a potent Src inhibitor that modulates multiple key signalling pathways in cancer. In keeping with its mechanism of action, AZD0530 has little effect on tumor growth in preclinical models, but does inhibit cancer cell invasion and metastasis *in vitro* and *in vivo* [12].

### Rationale for choice of biomarkers

Multiple substrates have been described for Src. It is likely that the main mode of action of Src during cancer cell invasion is via regulation of focal adhesion complexes. Focal adhesion complexes contain transmembrane integrins that interact with the extracellular matrix (ECM) and a multi-component, intracellular complex which mediates and regulates interactions between the ECM and the intracellular signaling machinery and cytoskeleton. Src

activation leads to phosphorylation of focal adhesion kinase (FAK) and paxillin, both of which are components of the focal adhesion complex [13, 14].

The Luminex<sup>®</sup> platform (Luminex Corporation, Austin, TX, USA) used in this study, presents a reproducible system for quantifying relative concentrations of analytes in tumor lysates. Immunohistochemistry (IHC) allows qualitative assessment of these analytes (and hence the ability to control for intra-biopsy heterogeneity).

## Methods

### Preclinical xenograft model

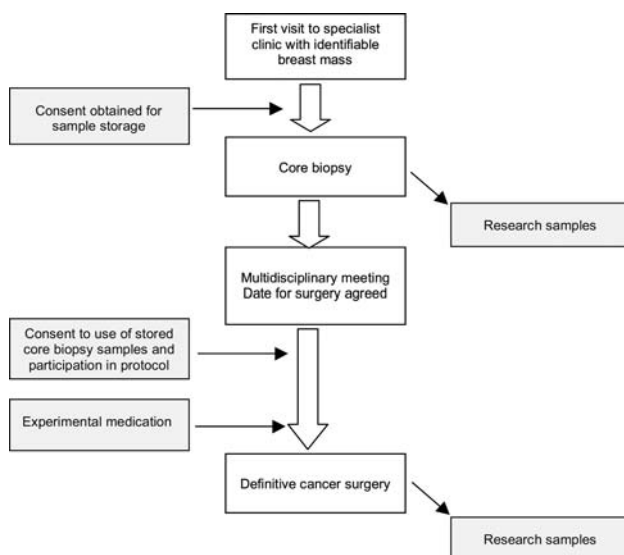
The preclinical study was conducted at Oncodesign (Dijon, France). Nude female rats (RH-rnu/rnu)<sup>1</sup> were sublethally irradiated prior to implantation using a cobalt (Co<sup>60</sup>) radiation source. Treatment began 1 h prior to implantation and the four groups were given once-daily oral doses of AZD0530 5, 10 or 20 mg/kg or vehicle alone. Each animal was inoculated subcutaneously with  $2 \times 10^7$  Calu-6 (human pulmonary carcinoma) cells. Tumor volume was measured twice-weekly using callipers and tumor growth was recorded. Dosing continued for 28 days. The animals were sacrificed 6 h after receiving the final dose and tumor tissues were collected.

### Patients

Female patients with early breast cancer undergoing core biopsy and subsequent surgical excision of their breast tumors presenting to a single center were enrolled. The planned recruitment was 30 patients as this was a number considered likely to provide appropriate information about the distribution of the endpoints being investigated. Patients who had received investigational therapies within 4 weeks of study entry were excluded. All women gave signed, informed consent to participate in the study and the study was approved by the local area ethics committee.

### Tissue sampling procedure

At the time of diagnostic core biopsy, following informed consent, six extra samples were taken using a 14-gauge core biopsy needle. Five further samples from each cancer were collected using a 14-gauge core biopsy needle from the excised tumor immediately after surgical removal. Two samples from the diagnostic and surgical cores were prepared for Luminex analysis, and three samples from each



**Fig. 1** Methodological feasibility of biomarker studies in early breast cancer

<sup>1</sup> Calu-6 nude female rats were generated by Oncodesign (Dijon, France).

time point for frozen-section IHC. The sixth sample, taken at the diagnostic core biopsy, was prepared for paraffin-embedded sectioning for IHC. All samples were immersed in the respective fixative within 5 min of removal from the patient.

#### Tissue fixation and storage

Samples for Luminex analysis were snap frozen in liquid nitrogen. Samples for paraffin-embedded IHC were immediately immersed in 10% neutral buffered formalin for 24 h prior to paraffin-block preparation. Samples for frozen IHC were immediately immersed in liquid nitrogen-precooled isopentane. All frozen specimens were stored at  $-80^{\circ}\text{C}$ , and paraffin blocks were stored at room temperature.

#### Lysate preparation

Samples were transported to a central laboratory on dry ice, mechanically disrupted together with a 15-fold (wt/vol) quantity of frozen lysis buffer, pH 7.4 (10 mM Tris, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 2 mM  $\text{Na}_3\text{VO}_4$ , 1% Triton-X100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF and 5% Sigma P2714 Protease Inhibitor Cocktail (Sigma Aldrich Co Ltd., Poole, Dorset, UK)) using a SPEX 6850 liquid nitrogen freezer mill (SPEX CertiPrep, Metuchen, NJ, USA) to produce a mixed powder of tissue and lysate solution at  $-80^{\circ}\text{C}$ . Samples were thawed, mixed and centrifuged. Total protein in the supernatant was quantified using the Pierce BCA optical protein assay (Pierce and Warriner, Chester, UK). A 1.5 mg/ml lysate was prepared from cultured mouse NIH 3T3 cells overexpressing active Src in the same lysis buffer for use as an assay standard.

Rabbit polyclonal capture antibodies were coupled to Luminex beads using the Bio-Rad Amine coupling kit as described in the manufacturer's instructions (bead sets and kit: Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK; other reagents: Sigma Aldrich Co Ltd.).

Tyr861 on FAK and Tyr31 on paxillin were chosen as specific substrates, although other tyrosine residues are also phosphorylated in a Src-dependent manner on both proteins. As the amount of any phosphorylated protein is dependent on the total amount of that protein, total paxillin was also measured. Reliable reagents for quantification of total FAK could not be identified. The primary measure of Src activity at focal adhesions was therefore expressed as the ratio of phosphorylated paxillin to total paxillin. Antibodies were supplied without bovine serum albumin or azide. Antibody (5  $\mu\text{g}$  per reaction) was used for both anti-p-FAK Y861, and anti-paxillin pY31 (BioSource International, Camarillo, CA, USA) and 10  $\mu\text{g}$  total paxillin

antibody per reaction (Cell Signaling Technology Inc., Beverly, MA, USA).

#### Luminex assay

The assay technique, reagents and controls have been described in detail [15]. In brief, 25  $\mu\text{l}$  of diluted bead antibody conjugate (at 100 beads/ $\mu\text{l}$ ) was added to each well. Lysate (100  $\mu\text{l}$ ) was then added to each well. A c-Src3T3 lysate standard dilution range was prepared starting at 100  $\mu\text{g}/\text{ml}$  and the test lysates were diluted to 175  $\mu\text{g}/\text{ml}$ . All samples and standards were run in triplicate. Phycolink anti-phosphotyrosine (type 1) R-phycoerythrin conjugate (50  $\mu\text{l}/\text{well}$ ) (Prozyme via Europa Bioproducts Ltd., Ely, Cambridgeshire, UK) was added prior to being read on the Bio-Rad Bio-Plex using Bio-Plex Manager v3 for data collection and quantitation of the unknowns against the c-Src3T3 standard curves. In the total paxillin assay, the c-Src3T3 lysate concentration was 300  $\mu\text{g}/\text{ml}$  and the test lysate concentration was reduced to 100  $\mu\text{g}/\text{ml}$ . Detection for these plates was by incubation with 50  $\mu\text{l}/\text{well}$  anti-paxillin (BD Transduction Laboratories, Franklin Lakes, NJ, USA) (diluted 1 in 83) followed by further incubation with 50  $\mu\text{l}/\text{well}$  diluted (1 in 100) anti-mouse IgG/biotin (Upstate Biotechnology (UBI), Lake Placid, NY, USA) and a further incubation with 50  $\mu\text{l}/\text{well}$  (diluted at 1 in 100) streptavidin-phycoerythrin conjugate (UBI). The beads used for the total phosphorylation assay were Qiagen LiquiChip Avidin Beads (Qiagen GmbH, Hilden, Germany), which were allowed to bind to Cell Signaling Technology pY100-Biotin (75  $\mu\text{l}/\text{well}$  of a 1:1600 dilution).

#### Immunohistochemistry

For the preclinical studies immunohistochemical (IHC) analyses were conducted as described in Jones et al. [15]. The following commercial antibodies were used in the range of 5–10  $\mu\text{g}/\text{ml}$ :

- Total paxillin (Santa Cruz, SC-5574)
- Phospho (p)-paxillin (BioSource, pY31 #44-720)
- Total FAK (Santa Cruz, SC-932)
- p-FAK (BioSource, pY861 #44-626G)

For the clinical studies, sections (7 and 4  $\mu\text{m}$ ) were prepared from frozen and paraffin-embedded samples, respectively, and processed using the Dako EnVision Detection Kit.

The following primary antibodies (1–10  $\mu\text{g}/\text{ml}$ ) were used in analyses of frozen sections:

- Activated Src—mouse monoclonal clone 28 (supplied by Dr. Hisaaki Kawakatsu, Dean Sheppard Laboratory,

San Francisco, CA, USA), stock concentration 1.9 mg/ml, assay concentration 1 µg/ml. This antibody targets the C-terminal tail of Src, which is only detectable when Src is in its active conformation [16]

Frozen sections were scored in a blinded fashion according to the intensity of staining of carcinoma cells on a scale of –, +/–, +, ++ or +++ by one observer (VJ) and confirmed by an independent reviewer (CW).

For paraffin sections, staining in nuclear, cytoplasmic and membrane subcompartments was scored on a scale of – (0), + (1), ++ (2) or +++ (3), and then multiplied by the percentage of cells stained in each subcompartment to give a H score out of 300. The H scores for each section were then summed to give a total score out of 900 (final H score).

## Statistics

Previous experience indicated that the Luminex data conformed to a log-normal distribution; therefore, data were logarithmically transformed prior to analysis. Two separate analysis of variance (ANOVA) models were fitted to the Luminex data for each parameter. The first used individual results from the two separate samples available from each patient at each visit. This model included visit as a fixed effect and random effects for patient and the visit by patient interaction. This model gives an estimate of the variability between individual patients, between samples from the same patient at the same visit, and between samples from the same patient at different visits. Data were also analyzed in terms of mean value for each patient at each visit, again using an ANOVA model this time with visit fitted as a fixed effect and patient fitted as a random effect. This model gives an estimate of variability between individual patients and between visits for individual patients.

## Results

### In vivo tumor growth and biomarker expression

Growth of tumors in Calu-6 xenograft bearing rats was shown to be significantly decreased following treatment with AZD0530 relative to vehicle (AZD0530 5 and 20 mg/kg,  $P < 0.0001$ ; AZD0530 10 mg/kg,  $P = 0.0002$ ) and was similar with all AZD0530 doses (Fig. 2a). Expression of phosphorylated Src substrates, FAK and paxillin, was also markedly reduced in these tumors following treatment with AZD0530 for 28 days as shown by IHC (Fig. 2b) and Luminex assay (Fig. 2c). These effects were dose-dependent.

### Patients

Thirty patients entered the study. One patient was found to have metastatic disease prior to surgery and so was excluded from the analysis.

### Quantitative analysis using Luminex

Luminex results from diagnosis and surgery samples were obtained for 29, 28, 28 and 26 patients for p-paxillin, total paxillin, total p-tyrosine and p-FAK, respectively. The p-paxillin:total paxillin ratio was chosen as the primary endpoint of this study as this was considered the best reflection of Src activity. In addition, by correcting for total paxillin, this reduces inter-sample variability due to tissue heterogeneity. Twenty-eight p-paxillin:paxillin values were obtained in this study.

### Intrapatient variability

For all analytes there was a wide range of values among different patients. Agreement between samples taken simultaneously from one patient was generally good (Fig. 3).

Furthermore, average readings from core biopsies had a high level of agreement with average readings at definitive surgery (Fig. 4).

The estimated components of variation for each of these endpoints are illustrated for individual values at each visit in Table 1 and for the mean values at each visit in Table 2.

The total between-patient and within-patient variability is reduced for all variables compared to the model using the individual values.

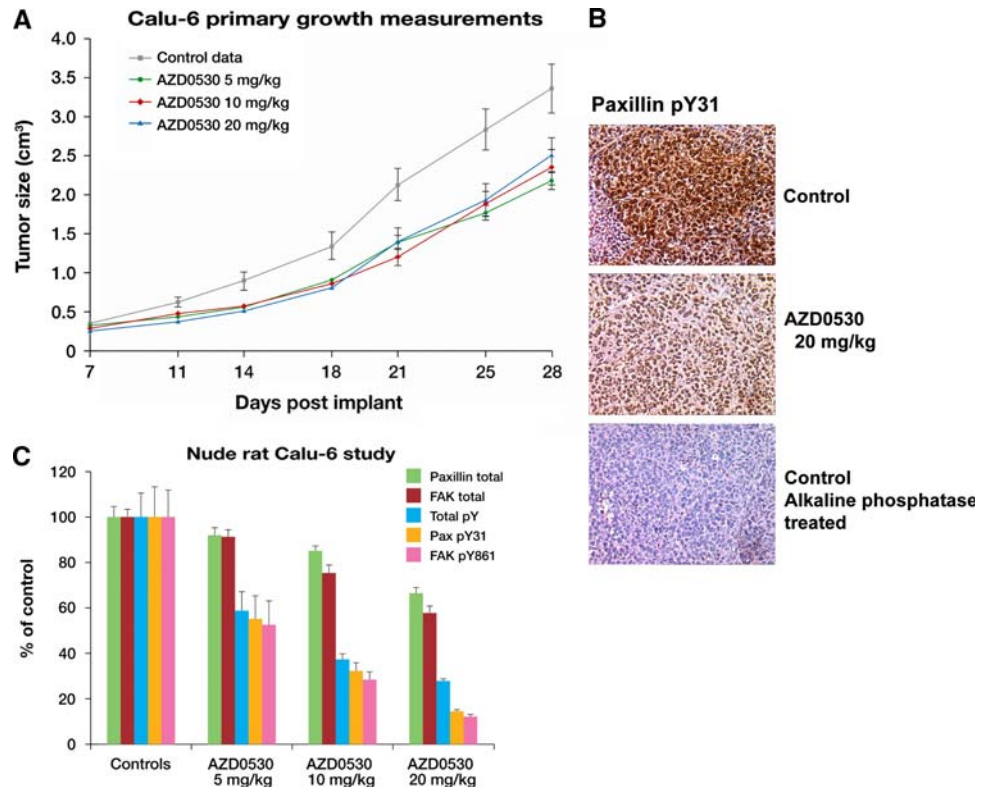
### Semi-quantitative analysis by immunohistochemistry

#### *Frozen samples*

Of samples from 29 patients: 19, 24, 14, 25 and 20 provided evaluable IHC images using antibodies against activated Src, total paxillin, p-paxillin, total FAK and p-FAK, respectively. Discrimination was low between different levels of IHC staining in the frozen tissue sections, particularly for activated Src. All samples analyzed expressed active Src, with one classified as +, five as ++, and 13 as +++ at diagnosis. Representative sections are shown in Fig. 5. Agreement between visits was high: 15 assessments remained the same, and four changed by one category. The generally poor morphology of the frozen tissue sections, despite the use of a rigorous preparation protocol, meant that it was not possible to discriminate subcellular localization of antibody staining.



**Fig. 2** (a) Calu-6 tumor growth, (b) Immunohistochemistry detection of p-paxillin (pY31) and (c) Luminex detection of total paxillin and FAK, total phosphotyrosine levels and p-paxillin (Y31) and p-FAK (Y861) in rats bearing Calu-6 xenografts following treatment with AZD0530 5, 10 or 20 mg/kg for 28 days



### Paraffin-embedded samples

In contrast to the generally poor quality of the frozen tissue sections, the excellent morphological preservation observed in the paraffin-embedded tissues meant that subcellular distribution of p-paxillin and p-FAK could be visualized in sections from paraffin-embedded tissue (Figs. 6 and 7).

H-score analyses provided information on the subcellular distribution of p-paxillin and p-FAK (Tables 3 and 4).

## Discussion

### Rational drug development and pharmacodynamic biomarkers

Most oncology drugs entering Phase I trials never attain marketing approval. One common reason for failure is lack of efficacy in Phase II or III clinical trials [17, 18]. This has wide-ranging consequences. For example, patients and volunteers are exposed to toxic, sometimes life-threatening, side effects from drugs, which subsequently prove no clinical benefit. Furthermore, valuable resources (patients, skilled staff and funds) are consumed at the expense of other research needs. It is also probable that some drugs, which are tolerable and effective, are failing clinical development because efficacy testing is conducted using the incorrect dose, schedule or patient population. The

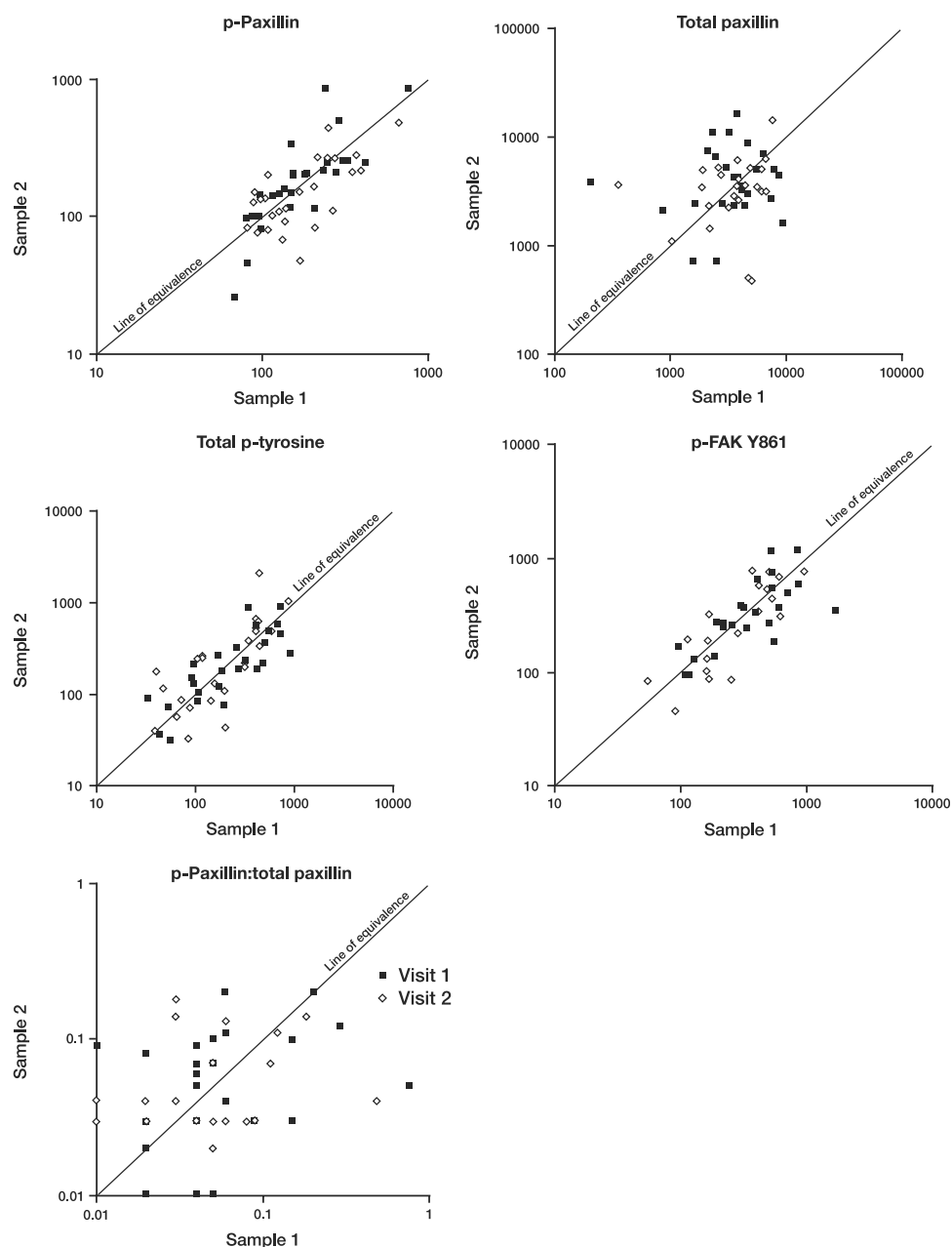
consequence is that effective new treatments may be discarded, often late in clinical development.

The challenges facing clinical drug development have become more intense in recent years for several reasons:

- i. The focus has shifted from cytotoxic drugs to biological agents targeting specific oncogenic pathways; thus, therapeutic hypotheses are dependent upon specific molecular interventions. This leads to the reasonable assertion that if a drug can be robustly demonstrated to have failed to inhibit its target, then the therapeutic hypothesis will also fail.
- ii. New drugs often target oncogenic processes other than tumor growth. Because of this, tumor shrinkage (response rate) is a less valid surrogate for early clinical proof-of-concept testing than it is for cytotoxic agents. More relevant endpoints (such as disease stabilization, time to progression or overall survival) demand that larger numbers of patients be tested before concluding that an inactive drug is, indeed, inactive.
- iii. As the size of the predicted incremental benefits from additional new treatments declines, the number of patients required to demonstrate these benefits increases. This demands more resource and increases the cost of failure.

One response to these challenges is to find new ways to discover more information about a drug, in particular about its pharmacodynamic effects, at the very earliest stages of

**Fig. 3** Correspondence between Luminex values for separate core samples taken at same visit. Values are expressed in c-Src3T3 lysate equivalent  $\mu\text{g/ml}$

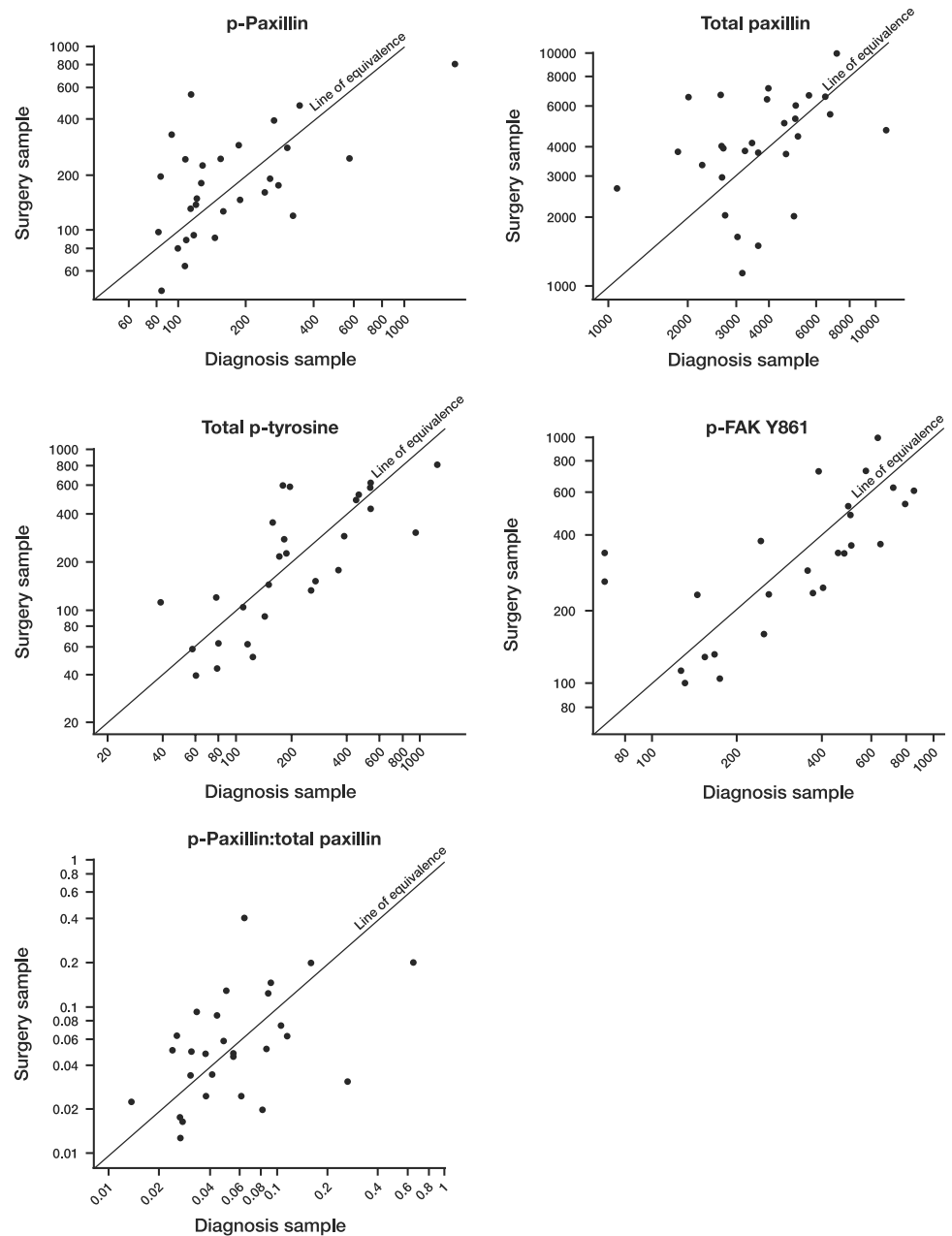


clinical development. This information can then be used for efficient decision making (for example, to terminate development prior to Phase II) and to guide later development (for example to optimize dose and schedule).

We have described part of the pre-Phase I programme for AZD0530, with a view to its potential application in early breast cancer. We have demonstrated that AZD0530 can reduce tumor growth, and modulate tumor FAK and paxillin phosphorylation in a rat xenograft model. In the clinical setting, we have shown that, in the majority of cases, it is feasible to obtain adequate amounts of tumor tissue from women undergoing routine care of early breast cancer on two separate occasions. Furthermore we have demonstrated that samples taken simultaneously and at a

relevant time interval show good levels of agreement for the biomarkers in question. These data should enable a clinical trial in which these markers can be measured to assess the pharmacodynamic response to AZD0530. This approach could be used to explore a range of safe and tolerable doses so that the optimum biological dose is chosen for further development in early breast cancer. Furthermore, by estimating the variability of the proposed primary pharmacodynamic endpoint, we can predict the likely number of patients that would be required to demonstrate a true effect of a Src inhibitor such as AZD0530 in early breast cancer (Table 5). In this case, the number of patients required is sufficiently small to permit exploration of more than one dose in such a study.

**Fig. 4** Correspondence between diagnosis and surgery visit Luminex values. Footnote for total p-tyrosine: one patient is not plotted as the baseline value was zero. Value at visit 2 was 179.92



**Table 1** Components of variability of individual values for Luminex analysis

Endpoint	Between patient	Within patient, between visit	Within patient, within visit
p-Paxillin/total paxillin	0.3049	0.0000	0.5064
p-Paxillin	0.2114	0.1094	0.3545
p-FAK Y861	0.3365	0.0984	0.1255
Total p-tyrosine	0.6306	0.5373	0.3798
Total paxillin	0.0343	0.0437	0.4549

Tyrosine phosphorylation levels (such as those used in the present study) are, by their nature, labile, being dependent on the balance of activity of both phosphatases and kinases, themselves dependent upon upstream regulators

and the availability of ATP. As ATP production is itself dependent upon oxidative metabolism, it was considered important to limit the delay between biopsy and tissue fixation. In a previous study, we have demonstrated that



**Table 2** Components of variability of mean values for Luminex analysis

Endpoint	Between patient	Within patient
p-Paxillin/total paxillin	0.2910	0.3329
p-Paxillin	0.2493	0.1845
p-FAK Y861	0.3625	0.1518
Total p-tyrosine	0.6011	0.6432
Total paxillin	0.0881	0.1588

minor variation in this short delay is unlikely to have a significant impact on the results of Luminex analysis [15]. One limitation of the Luminex assay described here is that it is not possible to control for inter-biopsy tissue heterogeneity. However, we have demonstrated that by averaging data from two biopsies at each patient assessment, the overall variability is sufficiently small to permit the use of these endpoints in pharmacodynamic studies.

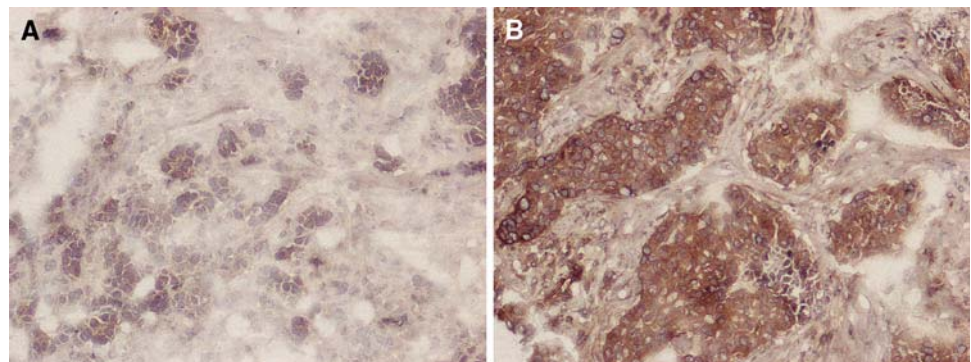
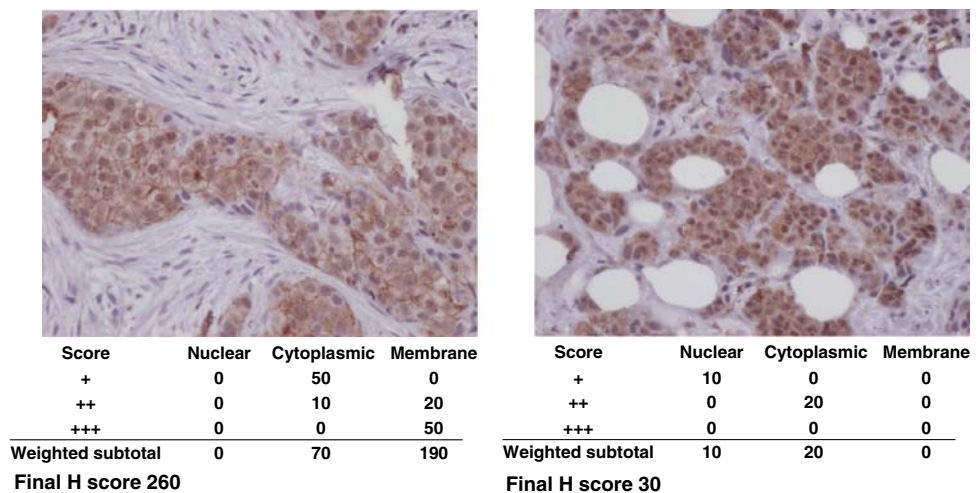
There is currently no known clinical benefit in administering AZD0530 to patients with early breast cancer. Clinical trials in which the primary endpoint is not known to relate directly to clinical benefit are relatively commonplace, although they are usually conducted in healthy volunteers who are financially compensated for their

inconvenience. In addition to the risks of administering drugs with very little prior exposure in man, a clinical trial of a novel agent in women with early breast cancer carries the additional risk that unforeseen toxicity could prevent or delay the implementation of conventional curative treatment. In particular, experience of the novel agent in association with general anesthesia and surgical wounding in man is absent. Ethically, it may be reasonable to conduct such studies in patients provided that fully informed consent is given, including information about the predicted absence of clinical benefit, and provided that toxicity is low and the risks of compromising the outcome of conventional cancer treatment are minimized.

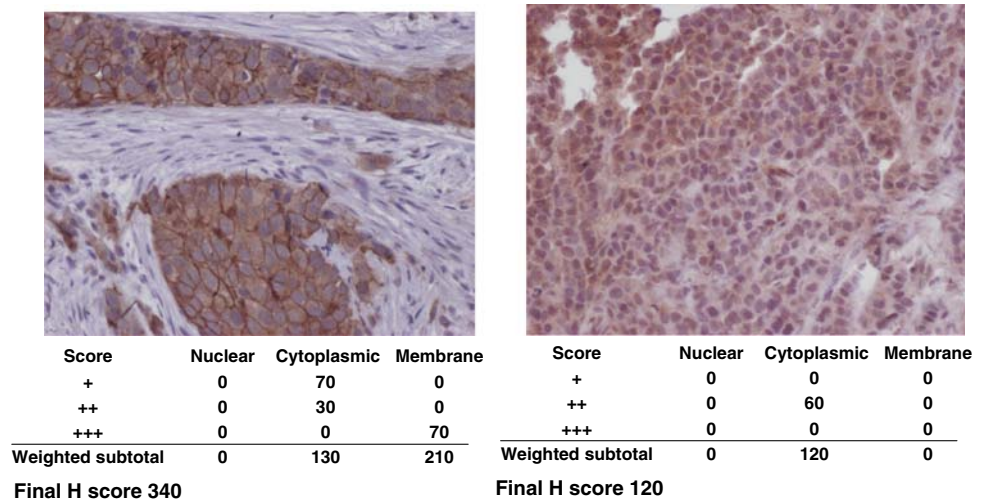
From an ethical perspective, a trial such as that proposed in this model could only be justified if there were a reasonable degree of confidence that the result would be statistically valid. Therefore methodology studies, such as this one, are vital before such a trial could proceed in order that the trial could be sized with confidence.

#### Rationale for a Src inhibitor in early disease

Src family members have been implicated in the regulation of multiple oncogenic processes, but the weight of

**Fig. 5** Selected frozen tumor biopsy sections stained for active Src. (a) Patient 24, graded '+' intensity. (b) Patient 4, graded '+++' intensity**Fig. 6** Selected paraffin-embedded tumor biopsy sections stained for p-paxillin. (a) Patient 11. (b) Patient 6

**Fig. 7** Selected paraffin-embedded tumor biopsy sections stained for p-FAK. (a) Patient 11. (b) Patient 3



**Table 3** Composite H scores for p-paxillin staining of paraffin-embedded tissue

Patient number	Nuclear			Sub total	Cytoplasmic			Sub total	Membrane			Sub total	Final H score
	+	++	+++		+	++	+++		+	++	+++		
1	10			10	10			10	40	40	120	140	
2				0		80		240			0	240	
3				0				0			0	0	
4				0	10			10			0	10	
5				0	50	20		90	70		140	230	
6	10			10	20			20			0	30	
7				0		90		180			0	180	
8				0	40	40		120	40		80	200	
9	5			5	50			50	50		100	155	
10 <sup>a</sup>													
11				0	50	10		70	20	50	190	260	
12				0	20	20		60	10		20	80	
13				0	40			40	30		30	70	
14	30			30	30	30	10	120		30	90	240	
15	10			10	70			70	10		10	90	
16	10			10	30	30		90			0	100	
17				0	20			20	10	50	110	130	
18	10			10	10			10	10		10	30	
19	10			10	70			70	40		40	120	
20	10			10		50		100		50	100	210	
21	10			10	50	20		90		50	100	200	
22	10			10	10	40	10	120	30		30	160	
23	10			10	10		40	130		20	40	180	
24	10			10	80			80	40	40	120	210	
25	20			20	70	20		110	50	30	110	240	
26	10			10	10			10	10		10	30	
27	10			10	10			10			0	20	
28				0				0			0	0	
29	10			10	20			20			0	30	

<sup>a</sup> Insufficient evaluable material

**Table 4** Composite H scores for p-FAK staining of paraffin-embedded tissue

Patient number	Nuclear			Sub total	Cytoplasmic			Sub total	Membrane			Sub total	Final H score
	+	++	+++		+	++	+++		+	++	+++		
1				0		30		60		70		140	200
2				0	50	50		150		50		100	250
3				0		60		120				0	120
4				0		60		120				0	120
5				0		80		160		50		100	260
6				0		70	5	155		5		10	165
7				0		70		140		5		10	150
8				0		90		180		70		140	320
9				0		90		180		40	40	200	380
10				0		100		200			50	150	350
11				0	70	30		130			70	210	340
12				0		40	60	260			50	150	410
13				0	100			100	50	50		150	250
14				0		100		200	30	30	30	180	380
15				0	50	50		150		50		100	250
16				0	30	20	10	100		10		20	120
17				0				0		50		100	100
18				0	10	40		90		50		100	190
19				0		50	50	250		60	20	180	430
20				0		40	40	200			50	150	350
21				0		100		200		50		100	300
22				0		100		200	30	30		90	290
23				0	80	20		120		50		100	220
24				0	50	50		150	50	10		70	220
25				0	30	50	20	190	10	80		170	360
26				0	90		10	120		10		20	140
27				0	90		10	120			10	30	150
28				0	100			100				0	100
29				0	90	10		110				0	110

**Table 5** Sample size estimates<sup>a</sup>

Endpoint	Estimate of SD	Number of patients per group
p-Paxillin/total paxillin	0.790	8
p-Paxillin	0.659	6
p-FAK Y861	0.717	7
Total p-tyrosine	1.115	15

<sup>a</sup> Sample size estimates are based on a 50% treatment difference, 1 sided 20% significance level and 80% power

preclinical evidence suggests that their predominant role may be in the regulation of cancer cell invasion [19]. The clinical development of drugs that target invasion presents a particular challenge, as we predict that these drugs may be of little benefit to patients with established metastatic

disease. Rather they may be effective in preventing progression from early invasive disease to advanced metastatic disease. If this prediction is correct, then efficacy testing would be necessary in patients with early disease stages, using stage progression/disease-free survival as the primary endpoints. Prior to such studies it will be necessary to identify the dose which inhibits Src in vivo and to demonstrate that such a dose is safe for long-term use.

AZD0530 is currently in clinical trials in patients with advanced solid malignancy.

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