Clinical Development of Src Family Kinase Inhibitors in Malignant Melanoma

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Abstract: Currently available systemic therapies for malignant melanoma are unsatisfactory and there is an urgent need for effective and well tolerated drugs for use in both early and advanced disease. The Src family of cytoplasmic tyrosine kinases (SFKs) have been implicated in the regulation of many of the hallmarks of malignancy making them attractive targets in solid tumours including melanoma. The first generation of selective SFK inhibitors to enter the clinic (AZD0530, dasatinib, bosutinib) have demonstrated safety, tolerability and target modulation in phase I trials. Phase II trials in patients with advanced melanoma are now planned in the USA and Europe. Here we discuss the rationale for, and challenges facing, the successful development of SFK inhibitors in melanoma. Furthermore, as dasatinib is also a potent inhibitor of the receptor tyrosine kinase (RTK), c-Kit, we reconsider the utility of targeting this kinase in the light of recent molecular epidemiological data.

INTRODUCTION

Malignant melanoma remains the deadliest of skin cancers. Its incidence has steadily risen with approximately 60 000 new cases diagnosed in Europe in 2000 [1]. Melanoma detected whilst localised to its site of origin is amenable to cure by complete surgical excision. In such cases the risk of occult micrometastatic disease and subsequent relapse is best predicted by pathological factors such as the Breslow thickness (depth of invasion) and the presence of epidermal ulceration. Disease involving regional lymph nodes confers a significantly worse prognosis although cure remains possible with radical lymph node dissection. The presence of distant metastases confers a bleak prognosis with a median survival of six to nine months [2]. Current systemic therapies for melanoma are poorly tolerated and / or of limited efficacy, both in the peri-operative and advanced disease setting [3, 4]. Effective and well tolerated therapies for use in both early and advanced disease are therefore urgently required.

TYROSINE PHOSPHORYLATION IS INCREASED IN MELANOMA

The modification of proteins by the addition of phosphate groups is a major activating event in signal transduction and is involved in the regulation of nearly every aspect of cell biology. Abnormally high phosphorylation of proteins on tyrosine residues is a common finding in malignant cells and results from the increased activity of receptor or non-receptor tyrosine kinases (RTKs) or from the decreased activity of tyrosine phosphatases [5].

Immunohistochemical analysis of melanoma surgical specimens with a pan-phosphotyrosine antibody revealed a significant increase in phosphotyrosine staining in melanomas in comparison to adjacent normal melanocytes or melanocytic nevi [6]. Strikingly, the frequency and intensity of phosphotyrosine staining increased in a stepwise fashion with the greatest phosphorylation occurring in later (vertical growth phase and metastatic melanoma) rather than earlier (nevi, radial growth phase melanoma) lesions. This elevation in phosphotyrosine content may be explained by increased activity of receptor (e.g. c-Met, EGFR, FGFR, Ephrins) and non-receptor (e.g. SFKs, FAK) tyrosine kinases and by the reduced activity of tyrosine phosphatases [6-8]. However, the relative contribution of individual kinases or phosphatases in melanoma is poorly understood. Here we focus specifically on the potential role of SFKs but also consider, where relevant, c-Kit and other tyrosine kinases susceptible to inhibition by dasatinib, AZD0530 and / or bosutinib.

SRC-FAMILY KINASES (SFKS) IN CANCER

Spanning the 20th century, the venerable position of the viral oncogene *v-Src* and its cellular homologue *c-Src* (hereafter referred to as *Src*) in the developing fields of cell and cancer biology has been reviewed elsewhere [9]. Src (Fig. **1**) is the prototypic member of a family of nine cytoplasmic tyrosine kinases and displays significant functional redundancy with the two other ubiquitously expressed SFKs, Yes and Fyn [10]. Expression of the remaining SFKs (Lyn, Lck, Hck, Blk, Fgr and Yrk) is restricted primarily to haematopoietic cells. SFKs are basic components of the cell signalling machinery and are involved in diverse signalling pathways regulating growth, survival, motility and adhesion which originate from receptor tyrosine kinases, integrins, cadherins, cytokine receptors and G-protein coupled receptors [10].

The kinase activity of Src is regulated by several mechanisms which have in common the ability to influence the stability of inhibitory intra-molecular interactions [11]. When phosphorylated, Src tyrosine-530 forms an intra-

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molecular interaction with the SH2 domain whilst a further intra-molecular interaction occurs between the SH3 domain and a short stretch of amino acids between the SH2 domain and kinase domain. In this conformation not only is the Src kinase domain inaccessible but access of potential binding partners to the protein-protein interacting SH2 and SH3 domains is sterically hindered. v-Src, the oncoprotein isolated from Rous Sarcoma Virus, lacks the C-terminal regulatory sequences present in Src and so is constitutively active [12].

Src expression and / or activity is upregulated in a variety of human tumours [13]. The largest body of evidence exists for colorectal cancer in which the activity of Src is elevated in tumour specimens when compared to adjacent normal mucosa and the degree of activity increases in parallel with advancing stage of disease [14-16]. However, elevated Src kinase activity has also been reported in pathological specimens from human breast [17, 18], gastric [19], pancreatic [20] and oesophageal cancers [21].

SFK EXPRESSION AND ACTIVITY IS INCREASED IN MELANOMA

Importantly, Daud and colleagues have determined the in-situ activity of SFKs in melanoma by immunohistochemical analysis of tumour biopsies with an anti-Src phosphotyrosine-419 antibody [23]. It should be noted that, due to homology within the activation loop, phospho-specific antibodies raised against Src phospho-tyrosine-419 do not discriminate between Src and other SFKs. Utilising this antibody, SFK activity was detected in 48% of melanomas with the strongest staining occurring in metastatic lesions.

Complementary *in vitro* data suggest that Yes rather than Src may be the most significant contributor to the elevated SFK activity observed in melanoma. Specifically, Loganzo reported 5-10 fold elevation of Yes activity, but not Src activity, in 18 of 20 melanoma cell lines in comparison to normal melanocytes [24]. Similarly, Gallick and colleagues have described elevated Yes but not Src activity in metastatic versus poorly metastatic melanoma cell lines [25]. In addition, O'Conner reported no increase in Src activity in 6 melanoma cell lines in comparison to normal melanocytes [26]. However, the activity of Src itself also varies considerably among melanoma cell lines [27, 28] and among clinical specimens, including variability of Src activity among metastatic deposits within a single patient [29]. Finally, the elevated SFK activity seen in the *Xiphophorus* model of melanoma appears to be attributable to Fyn [30]. Taken together these data suggest that activation of SFKs may be a common occurrence in melanoma, although the precise contribution of individual family members may vary and remains ill-defined.

MECHANISMS OF SFK ACTIVATION IN MELANOMA

Data obtained in melanoma cell lines, viewed in concert with data obtained in other tumour types, support the existence of several non-mutually exclusive events leading to SFK activation.

1. Increased SFK Protein Levels

A distinction may be made between increased SFK kinase activity which is directly proportional to an increase in protein levels versus an increase in specific activity i.e. an increased kinase activity which occurs in the absence of, or disproportionately to, a change in protein levels and which reflects post-translational regulation. For example, the increased Yes activity observed in 18 of 20 melanoma cell lines was attributed to a parallel increase in protein levels [24]. This was associated with an increase in mRNA transcripts without evidence of genomic amplification. However, other workers have described alterations in specific SFK activity occurring independently of alterations in protein levels, supporting the existence of additional levels of regulation in melanoma [25, 31].

2. Mutational Activation

A C-terminal truncating and activating *Src* mutation has been reported in a subset of colorectal cancers [32]. However, this finding has not been widely replicated [33-35] suggesting that mutational activation of Src is an infrequent event in colorectal cancer. This mutation has either not been detected in other solid tumours, including transitional cell carcinoma [36] or ovarian carcinoma [37], or has been detected at very low frequency, as in endometrial cancer [37]. Loganzo *et al* detected no mutations in the C-terminal regulatory region or, indeed, any coding region, of *Yes* in 20 melanoma cell lines tested [24]. Activating mutations, at least for *Yes*, would therefore appear unlikely to be a common mechanism of SFK activation in melanoma.

3. Downstream of Activated RTK Signalling

Typically, on encountering its cognate ligand, a RTK undergoes dimerisation and transphosphorylation of tyrosine residues within the cytoplasmic domain. These residues form high-affinity binding sites which the SFK SH2 domain favours in preference to the lower affinity intra-molecular tyrosine-530 interaction hence resulting in simultaneous re-

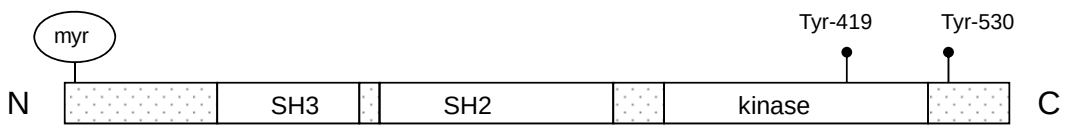

Fig. (1). Structure of Src: Src, the prototypic SFK, has a modular structure comprising an N-terminal unique region containing a myristylation site (myr) which is essential for membrane localisation, an SH3 domain, an SH2 domain, a kinase domain and a C-terminal regulatory sequence [11]. The SH2 and SH3 domains mediate protein-protein interaction; the SH3 domain binds proline rich sequences whilst the SH2 domain binds regions containing phosphorylated tyrosines. Within the activation loop of the kinase domain tyrosine-419 undergoes autophosphorylation and is therefore often used as a readout of Src kinase activity. Tyrosine-530, lies in the C-terminal regulatory region and, in contrast to tyrosine-419, its phosphorylation is associated with inactivation of Src [22].

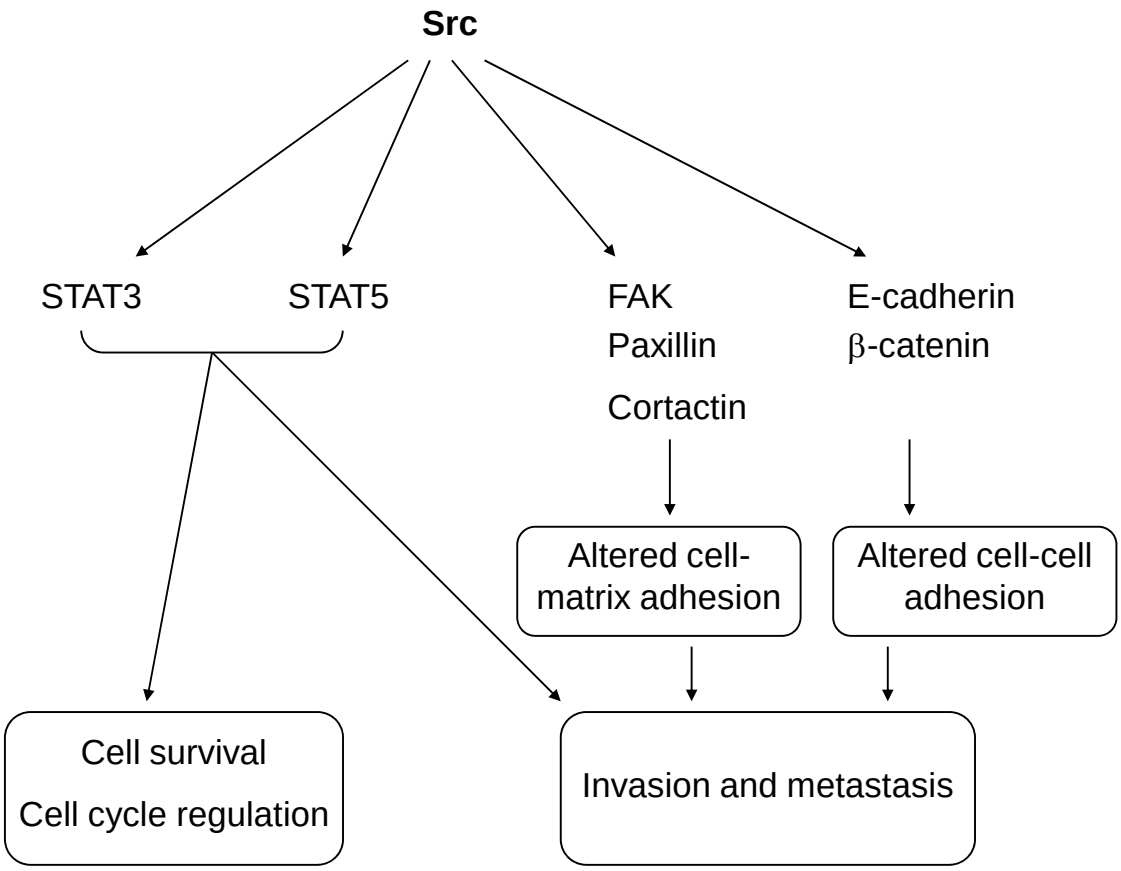

Fig. (2). Schematic diagram of key SFK-dependent pathways in melanoma.

cruitment and activation of the SFK [38]. Therefore, elevated SFK activity may be secondary to activation of an upstream RTK to which it is coupled [39, 40].

To date several RTKs have been implicated in the development or progression of melanoma, including c-Kit (discussed below), c-Met [41], EGFR [42, 43], and FGFR [7]. A causal role for RTK signalling in melanomagenesis is supported by the frequent development of melanomas in transgenic mice overexpressing hepatocyte growth factor (the ligand for c-Met) [44]. Furthermore, the Xiphophorus melanoma receptor kinase (Xmrk) is responsible for a hereditary melanoma syndrome in the fish *Xiphophorus* (recently reviewed in [45]). *Xmrk* is a mutated form of the fish orthologue of *EGFR* which has acquired 2 mutations in the extracellular domain leading to constitutive dimerisation and ligand-independent activation [46]. In both of these models the melanomas are highly invasive, reminiscent of the observation that phosphotyrosine content is generally highest in vertical growth phase and metastatic lesions in human melanoma.

Several observations directly support RTK signalling as one mechanism of SFK activation in melanoma. First, the constitutive activation of a SFK in 586 mel melanoma cells was abrogated by expression of a dominant negative FGF receptor [31]. Protein levels were unchanged indicating that the reduction was in specific activity. Second, Yes (but not Src) has been implicated downstream of NGF signalling in melanoma cell lines and its activity correlated with their invasive and metastatic potential [25]. Third, Xmrk recruits and activates Fyn (although not Yes or Src) in fish [30] or mammalian melanocytes [47]. Finally, Src is activated and forms a complex with EGFR upon stimulation of A375 human melanoma cells with EGF [48].

4. Displacement of Inhibitory Intramolecular Interactions

Analogous to the action of activated RTKs, cytoplasmic proteins may similarly displace inhibitory SFK intramolecular interactions if they contain sequences which will bind the SFK SH2 or SH3 domains with greater affinity. For example, focal adhesion kinase (FAK), a cytoplasmic TK implicated in the regulation of cell adhesion, migration and survival, is localised to integrin-rich cell-matrix contacts and becomes phosphorylated on integrin ligation to create a high affinity SH2 domain binding motif thus recruiting and activating Src [49]. A consensus SH3 domain binding motif also contributes to FAK's ability to activate Src [50]. As described below, FAK expression and / or phosphorylation is often upregulated in melanoma cell lines suggesting that this may be an important mechanism of SFK activation [51, 52].

5. Reduced Phosphorylation of Regulatory Tyrosine-530

The phosphorylation state of Src tyrosine-530 acts as a molecular switch with phosphorylation favouring inactivity and de-phosphorylation favouring activation. Reduced phosphorylation of tyrosine-530 may occur via a reduction in the expression of C-terminal Src Kinase (Csk) as has been reported in some hepatocellular cancers [53] or by the increased expression of phosphatases. For example, PTP-1B dephosphorylates tyrosine-530 and activates Src in several

breast cancer cell lines [54]. Interestingly, this phosphatase is also overexpressed in several melanoma cell lines [8] whilst the increased specific activity of Src in melanocytes in comparison to fibroblasts was associated with a reduction in phosphorylation of tyrosine-530 [26].

Other mechanisms of Src activation in cancer have also been proposed including elevated N-myristoyltransferase activity, altered intracellular targeting, altered proteasomal degradation and altered serine/threonine phosphorylation [55, 56]. However, no direct data exist to link these mechanisms to melanoma.

CONSEQUENCES OF SFK SIGNALLING IN MELANOMA

A growing body of evidence indicates that SFKs can regulate many processes relevant to neoplasia including cell cycle progression, survival, motility and invasion (Fig. **2**) [55, 56].

1. Invasion and Metastasis

One of the major contributions of SFK signalling in cancer may be to confer an invasive or metastatic phenotype. Indeed, many SFK substrates including FAK, cortactin, $p130^{cas}$ and p120-catenin are proteins directly implicated in the regulation of cell adhesion and migration [57]. In particular, a significant body of evidence has been generated to support a cooperative and critical role for Src and FAK in cancer cell migration [57]. Consequently, it is not surprising that several lines of evidence support a central role for the Src-FAK axis in the migration and invasion of melanoma cells. For example, SFK activity correlated with the metastatic capacity of melanoma cell lines [25, 58]. Similarly, both the level of FAK expression and the degree of phosphorylation of FAK at tyrosine-379 and -576 correlated with invasiveness and / or motility of melanoma cell lines [51, 52]. Consistent with these observations was the demonstration that the ability of exogenous Xmrk to render poorly motile murine melanocytes highly motile required the coordinated action of Fyn and FAK [59]. Furthermore, the migration and invasion of C8161 melanoma cells was abrogated by expression of a dominant negative FAK protein [52]. The requirement for SFK-FAK signalling in melanoma is further highlighted by the finding that treatment of melanoma cell lines with dasatinib blocked phosphorylation of FAK and completely inhibited the motility and invasiveness of A2058 melanoma cells [60].

2. Increased Proliferation

Studies in both fibroblasts and epithelial cells have identified a role for SFKs in cell cycle progression, particularly in the context of growth factor signalling [10]. In keeping with these observations the proliferation of both normal melanocytes and melanoma cells in response to FGFR signalling is associated with SFK activity [31]. Furthermore, the transformation and proliferation of mouse melanocytes in response to expression of Xmrk is mediated by Fyn [47]. Despite this, in most tumour types studied only a subset of cell lines appear to depend on SFK activity for proliferation. For example, concentrations of dasatinib which fully blocked Src activity inhibited proliferation in only 2 of 12 colon cancer cell lines tested [61]. Similarly, pharmacologically relevant concentrations of dasatinib did not inhibit proliferation in 13 of 24 breast cancer cell lines and 4 of 15 lung cancer cell lines [62]. In-keeping with these observations, anti-proliferative effects of AD0530 were also observed in only a minority of cell lines treated [63]. The extent to which SFK inhibition might inhibit the proliferation of melanoma cells is therefore uncertain. Existing data obtained in melanoma cell lines suggest that, in common with other solid tumours, the anti-proliferative effects of SFK inhibitors may be quite variable [23, 60, 64]. Smalley and Herlyn have recently suggested that vertical growth phase and metastatic melanoma cells may be relatively insensitive to the anti-proliferative effects of SFK inhibition in comparison to radial growth phase cells. This intriguing but preliminary observation would clearly have implications for development of SFK inhibitors and requires further investigation [64].

3. Enhanced Survival

Accumulating evidence supports an important pro-survival role for Src-dependent activation of the signal transducer and activator of transcription (STAT) transcription factors in melanoma. First, expression of a dominant negative Stat3 protein caused apoptosis of B16 melanoma cells *in vitro* and *in vivo* [28]. Stat3 was subsequently shown to be constitutively activated in 16 of 18 melanoma cell lines [28] and in the majority of tumour specimens examined [23, 28]. Second, in cell lines and tissue specimens there was a correlation between Src activity and phosphorylation / activation of Stat3 whilst pharmacological inhibition of SFK activity caused a reduction in Stat3 activity in cell lines [23, 28]. Third, either dominant negative Stat3 expression or pharmacological SFK inhibition induced apoptosis in JW and A2058 melanoma cell lines and this was associated with inhibition of expression of the anti-apoptotic genes *Bcl-*X_L and *Mcl-1* [28]. Finally, Stat5 was hyperphosphorylated in 62% of melanoma metastases tested [48]. Stat5, like Stat3 was also activated by Src and inhibition of Stat5 expression caused enhanced apoptosis in A375 and BLM melanoma cells which, in this case, was associated with reduced expression of Bcl-2 [48].

4. Enhancement of Chemosensitivity

SFK inhibitors have been reported to enhance cell kill or to overcome drug resistance in a variety of models [65-67]. In this context, dasatinib has been reported to enhance the sensitivity of melanoma cell lines to platinum and topoisomerase I inhibitors [23].

5. SFK Signalling in Stromal Cells

Regardless of the consequences of inhibiting SFK signalling in melanoma cells, SFK inhibitors may independently exert effects by interfering with SFK-dependent functions in stromal cells. For example, angiogenesis, a promising target for melanoma therapeutics, may be susceptible to SFK inhibition [68-71]. In addition, lytic bone metastases which are a common feature of advanced melanoma might be ameliorated by inhibiting SFK signalling in osteoclasts [72, 73].

SFK INHIBITORS IN CLINICAL DEVELOPMENT

Dasatinib

Dasatinib (BMS-354825; Bristol-Myers Squibb, Princeton, NJ) is a potent, orally active, ATP-competitive inhibitor of 5 kinases / kinase families: SFKs, c-Kit, Abl, PDGFR and

EphA2 [74]. Dasatinib inhibits both Abl and c-Kit more potently than imatinib although whether this might translate into greater clinical efficacy in CML and GIST is the subject of ongoing clinical investigation [75]. Crucially, however, dasatinib retains activity against 21 of 22 tested imatinib-resistant Bcr-Abl mutants [76-78] as well as imatinib-resistant c-Kit mutants [79, 80]. Molecular insights provided by the crystal structure of the dasatinib-Abl complex demonstrate less stringent conformational requirements in the activation loop for dasatinib-binding in comparison to imatinib. In addition, dasatinib does not form critical interactions with most of the P-loop residues whose mutation interferes with imatinib binding [81]. Similarly, less stringent conformational requirements may also explain the activity of dasatinib against imatinib-resistant forms of c-Kit [80].

Preclinical studies demonstrated efficacy of dasatinib against Bcr-Abl positive leukemias [76] and, on the basis of efficacy in phase I and II trials [82-86], dasatinib was granted approval by the FDA in 2006 for use in patients with CML or Philadelphia chromosome positive acute lymphoblastic leukaemia with resistance to or intolerance of imatinib. The development and use of dasatinib in haematological malignancy has been extensively reviewed elsewhere [87].

The development of dasatinib in solid tumours is less advanced. However, data obtained in preclinical models suggest dasatinib may interfere with several of the hallmarks of malignancy. Specifically, pharmacologically achievable concentrations of dasatinib have influenced proliferation [61, 62, 88, 89], survival [88-90], migration and invasion [61, 88-91], metastasis [92], and drug sensitivity [23] in a variety of solid tumour preclinical models. In melanoma Jove and colleagues have presented data showing treatment of cell lines with dasatinib inhibited phosphorylation of FAK, caused rounding and altered cell matrix adhesion and could completely abolish invasion and migration [60]. Dasatinib has also been reported to inhibit proliferation of melanoma cell lines and to synergise with platinum and topoisomerase I inhibitors [23].

Preliminary results of a phase I study of dasatinib in patients with GIST and other solid tumours including malignant melanoma have been presented [93]. In contrast to patients with Bcr-Abl positive haematological malignancies, no significant myelosuppression was seen in patients with solid tumours. Non-haematological toxicities were predominantly grade 1 and included anorexia, nausea, diarrhoea, rash and fluid retention. Grade 3 hypocalcaemia was reported in one patient, perhaps reflecting inhibition of Src-dependent osteoclast function. No objective responses were reported although disease stabilisation was seen in patients with GIST, sarcoma, biliary tract cancer, mesothelioma and melanoma. Phase II evaluation of dasatinib in patients with advanced melanoma is underway [94].

AZD0530

AZD0530 (AstraZeneca, Macclesfield, UK) is a potent, selective, orally available dual inhibitor of Abl and the SFKs currently in phase I and II trials in patients with common solid tumours. Unlike dasatinib, it is not a potent inhibitor of c-Kit or the PDGFR family [63]. It also differs from dasatinib by having a longer half life enabling continuous exposure with once daily dosing in man [95]. There are currently no registered trials examining the role of AZD0530 in CML or other haematological malignancies.

Clinical data demonstrate that AZD0530 inhibits the downstream effects of SFK activity (namely phosphorylation of FAK and Paxillin) in tumour tissue when delivered at tolerable doses to patients with a variety of advanced solid tumours in the phase I setting [96]. Likewise, in healthy volunteers, there is almost complete suppression of markers of bone resorption, supporting the notion that SFK inhibitors may have clinical application inhibiting normal bone turnover and malignant bone disease [97].

Pre-clinical data suggest that AZD0530 is a poor inhibitor of tumour growth, even in tumour models where potent inhibition of Src can be demonstrated [63]. However, at clinically achievable concentrations, AZD0530 inhibits a variety of *in vitro* models of tumour cell invasion, scattering and migration [63, 98]. In animal models of bladder and pancreatic cancer it inhibits metastasis formation with little effect on growth of the primary tumour [70, 99].

Although there are no specific data regarding AZD0530 in melanoma, its emerging good tolerability profile [95], apparent selectivity for SFKs, and its ability to inhibit its targets in a wide range of solid tumours would suggest that it may be an interesting investigational drug in this context.

Bosutinib

Bosutinib (SKI-606; Wyeth Pharmaceuticals Inc, Philadelphia, PA) is an orally available inhibitor of SFKs and Abl. Like AZD0530 it has no significant activity against PDGFR or c-Kit [100]. Preclinical data demonstrate that it can inhibit growth in models of colorectal [101-103] and breast [104] cancers. In addition, it also inhibits experimental models of invasion and cell migration in these cancers [102, 104]. It is also active in several (but not all) imatinib resistant CML cell lines [100].

Preliminary results of a phase I trial in patients with solid tumours were reported at ASCO 2007 [105]. Dose limiting toxicities were grade 3 diarrhoea and rash. Stable disease was demonstrated in patients with breast, colorectal and non-small cell lung cancer and one patient with pancreatic cancer achieved stable disease for greater than 52 weeks. There are currently no published data concerning the use of bosutinib in melanoma.

C-KIT IN MELANOMA

Gain of function mutations in c-Kit, a type III receptor tyrosine kinase potently inhibited by dasatinib, occur in several malignancies, most notably in the majority of GISTs, but also in some haematological malignancies and in testicular seminoma [106]. In GIST most mutations occur within exon 11 and result in in-frame deletions, insertions or point mutations in the juxtamembrane region [107]. By causing loss of function of the auto-inhibitory role of the juxtamembrane domain they facilitate ligand independent dimerisation and constitutive activation.

c-Kit is expressed by normal melanocytes and its signalling is important for melanocyte proliferation, differentiation, survival and migration [108]. However, the role, if any, of c-Kit in melanomagenesis has been controversial. Several immunohistochemical studies have reported downregulation

of c-Kit in melanoma tissue, particularly in vertical growth phase [109, 110] and also in metastatic lesions [111]. Furthermore, expression of c-Kit in c-Kit-negative metastatic melanoma cells inhibited their growth and metastatic potential in nude mice [112]. Taken together this suggested a potential tumour or metastases suppressor role for c-Kit, perhaps via the induction of differentiation or the inhibition of growth [7]. However, recent data have added complexity to this model and suggest that, in at least a subset of melanomas, aberrant c-Kit activity may contribute to oncogenesis.

Mutation or Increased Copy Number of *c-Kit* Occurs in a Subset of Melanoma

Curtin and colleagues have recently identified *c-Kit* mutations and / or copy number increases in 39% of mucosal and 36% of acral melanomas and in 28% of melanomas arising on skin showing changes of chronic skin damage [113]. Conversely, no amplification or mutation of *c-Kit* was found in melanomas arising in skin without chronic sun damage. Sequencing of *c-Kit* in adjacent normal tissue, where possible, confirmed that mutations were somatic. *BRAF* and *c-Kit* mutations were essentially mutually exclusive. Interestingly, c-Kit protein levels were elevated in the majority of tumours in which *c-Kit* was mutated or amplified. A second group independently identified one of these mutations, a L576P substitution, in 3 of 20 anal melanomas [114]. Again these tumours were all strongly immuno-positive for c-Kit. A third group have also reported the L576P mutation in 2 of 100 melanomas although their subtype as per Curtin's classification was not described [115].

Identical *c-Kit* mutations are implicated in the pathogenesis of GIST, haematological malignancy and seminoma [106]. The observation that mutant *c-Kit* appeared to be selected for further supports, albeit indirectly, a similar functional significance in melanoma. However, the effects of c-Kit signalling may be cell lineage-dependent. For example, expression of c-Kit with the D814Y activating mutation caused increased proliferation in mast cells whilst in melanocytes proliferation was reduced but migratory potential dramatically increased [116]. Therefore, proof-of-principle experiments to ascertain whether melanoma cell lines harbouring increased copy number or activating mutations in *c-Kit* demonstrate an enhanced sensitivity to c-Kit blockade are necessary.

c-Kit Inhibition in Melanoma: Experience with Imatinib

Imatinib is a potent, selective, small molecule inhibitor of Abl, c-Kit and PDGFR [117, 118]. *In vitro*, imatinib inhibited the proliferation of 8 of 19 melanoma cell lines at pharmacologically relevant concentrations [119]. Although no clear relationship between expression of c-Kit, PDGFR-α and PDGFR-β existed, the two cell lines which expressed the highest levels of c-Kit were also the most sensitive to growth inhibition by imatinib. Mutations were not detected in exon 11 in any cell line although mutations elsewhere were not excluded. *In vivo*, imatinib was able to inhibit the growth of xenografts of B16F10 melanoma cells [120] but not of A375SM or MeWo cell lines [121]. The level of expression of c-Kit did not predict growth inhibition, nor did inhibition of PDGFR phosphorylation correlate with growth inhibition [121]. These data may indicate the involvement of alternative imatinib-sensitive kinases in melanoma growth.

Three phase II trials of imatinib in patients with advanced melanoma have been reported, all with disappointing results

Table 1. Phase 2 Studies of Imatinib in Melanoma

	Ugurel *et al.* [122]	**Wyman *et al.* [123]**	**Eton *et al.* [124]**
No of Patients	18	26	21
Median Age (yrs)	58	54.2	59
ECOG Performance status	0 – 33% 1 – 50% 2 – 17%	0 – 42% 1 – 50% (8% undefined)	Median = 1 (range 0-2)
Site of primary tumour	Cutaneous – 11 (all non-CSD) Acral – 1 Mucosal -1 Ocular – 1 Unknown Primary - 4	Cutaneous – 23 Ocular – 2 Mucosal - 1	Not reported
Previous lines of treatment for Stage IV disease. (chemotherapy, biotherapy or combination)	0 – 0 1 – 44% 2 – 44% $\geq$3 – 12%	0 – 38% 1 – 31% 2 – 23% $\geq$3 – 8%	0 – 38% $\geq$1 – 62% (includes adjuvant)
Survival data (median)	OS 3.9m TTP 1.9m	OS 6.5m PFS 2.0m	OS 7.5m 20 of 21 pts progressed within 3m
Response data	CR – 0% PR – 0% SD - 6% (1pt, duration 2.6m)	CR – 0% PR – 0% SD – 8% (2pts, duration: 3.2m and 3.6m)	1 patient "near CR in in-transit, inguinal/ iliac and lung metastases"; duration 12.8m

OS = overall survival; PFS = progression free survival; TTP = time to progression; CR= complete response; PR = partial response; SD = stable disease.; CSD = chronic sun damage.

[122-124] (Table **1**). The frequency of *c-Kit* genetic aberrations in these phase II studies has not been described. However, in Curtin's survey, increased copy number and / or mutation of *c-Kit* was usually associated with increased c-Kit protein levels [113]. In this regard the absence of strong c-Kit expression in all but one tumour of the 27 for which material was available in the Wyman and Ugurel studies may indicate a lack of activation of the putative target pathway. In-keeping with this observation, the patient population in at least one of these trials primarily harboured melanomas arising in skin lacking signs of chronic sun damage which would not be expected to harbour *c-Kit* aberrations [125]. Tantalisingly, the one patient in whom imatinib has induced an objective response had an acral melanoma and expressed c-Kit at a high level [124]. No *c-Kit* mutations were detected in this patient; the reported deletion of serine-715 likely represents a normal splice variant [126].

Taken together, these data support a re-evaluation of the potential role of c-Kit inhibition as a therapeutic strategy in melanoma. Should proof-of-principle preclinical data confirm the functional significance of activating mutations or increased copy number of *c-Kit* in melanoma (which seems likely by analogy to other tumours) then there is sufficient rationale to justify a further study in which patients are selected on the basis of *c-Kit* amplification or mutational status. Such a trial has already been proposed for imatinib [125]. In the existing phase II studies significant toxicity was reported, perhaps reflecting the high dose of imatinib (800 mg / day) in a poor prognostic group of patients. Eton *et al* reported a reduction in phosphorylated relative to total c-Kit as determined by dual immunofluorescence in baseline and 2nd week tumour specimens in five of five patients tested indicating that target modulation was achieved at this dose level [124]. Therefore, an important question to be addressed, with appropriate pharmacodynamic monitoring, is whether a reduced dose of imatinib may be sufficient for target inhibition but better tolerated. It should also be noted that substitutions of aspartic acid at codon 816 (comprising 12% of the c-Kit mutations reported by Curtin *et al*. [113]) are associated with imatinib resistance [127]. Dasatinib retains activity against D816 mutant c-Kit, albeit with some loss of potency [79, 80] and therefore might offer a broader spectrum of activity than imatinib in this setting. In addition, phase II studies of other multi-targeted TKIs with c-Kit activity such as sunitinib (Pfizer Inc, New York, NY) and AZD2171 (AstraZeneca, Macclesfield, UK) are already in progress in unselected patients with advanced melanoma [128].

OTHER RELEVANT KINASES

Three other tyrosine kinases known to be potently inhibited by dasatinib (although not AZD0530 or bosutinib) may be relevant to the action of this agent in melanoma and are considered briefly below:

PDGFR

Platelet-derived growth factors (PDGFs) and their receptors (PDGFRs) have been implicated in the pathogenesis of a variety of cancers and play an important role in angiogenesis [129]. Both PDGF and PDGFR are highly expressed in primary and metastatic melanoma suggesting the existence of a possible autocrine loop [130]. Additionally, the level of expression of PDGFR correlated with the metastatic potential of melanoma cell lines [121]. Nevertheless, clinical experience with imatinib indicates that targeting PDGFR, at least in an unselected patient population, is unlikely to be efficacious.

EPH-A2

Although most studied for their role in development, increasing evidence links members of the ephrin family of RTKs to cancer [131]. EPH-A2, which is potently inhibited by dasatinib, has been shown to be overexpressed in many melanoma cell lines [132, 133] and in a subset of melanoma biopsies [134]. Ephrin-A1, a ligand for EPH-A2, was expressed in 43% of primary melanomas and 67% of metastatic melanomas [134]. *In vitro*, Ephrin-A1 stimulated proliferation, although not migration or invasion, of EPH-A2 expressing melanoma cell lines suggesting the potential existence of an autocrine loop *in vivo* [133].

Abl

Whilst the Bcr-Abl oncoprotein is responsible for the majority of cases of chronic myeloid leukaemia, a significant role for Abl signalling in solid tumours has yet to be demonstrated. Elevated levels of Abl and the related kinase Arg have been reported in breast cancer cell lines [135] and Abl signalling has been implicated in epidermal mesenchymal transition (EMT), a hallmark of invasion, in colon cancer cells [136]. Moreover, at least in some contexts Abl may be an effector of Src-induced transformation [137]. Whether and how Abl signalling may impact on melanomagenesis has not been defined.

CHALLENGES IN PHASE II / III DEVELOPMENT OF SFK INHIBITORS IN MELANOMA

Phase II trials of dasatinib in melanoma are underway or imminent in both USA and Europe. Although there are phase II programmes of AZD0530 and SKI-606 in a variety of solid tumours, there are currently no publicised studies in this indication. Whilst the commercial opportunity for new drugs in the treatment of melanoma is high, their effective and efficient development raises several challenges:

Which Stage of Disease?

As discussed above, the exact role of SFKs in melanomagenesis is not clear, but the weight of evidence in solid tumours is that their principle role is in the regulation of tumour cell invasion, rather than tumour growth. It is therefore unlikely that significant tumour shrinkage will be seen in advanced cancers. Although invasion is an ongoing process contributing to cancer morbidity and mortality at all stages of the disease, the opportunity for anti-invasive drugs, such as SFKs, may lie in the prevention of progression from early to disseminated disease. This raises the challenge of developing a drug in early disease (with the attendant cost and time implications) in the absence of a prior signal in advanced disease; whilst evidence of disease stabilisation or regression in advanced disease would present a straightforward 'go' decision in early disease, the absence of such an effect should not necessarily be seen as a 'no go' signal.

Proof of Concept: Appropriate Endpoints

Cytotoxic drug development pathways have been dependent on tumour response (shrinkage) for early decision

making in phase II. As we do not predict tumour shrinkage as a likely outcome from SFK inhibition in melanoma, response rate is probably not an appropriate endpoint in phase II design in this setting. Indeed, even with conventional cytotoxics, objective response rates in melanoma in early phase trials do not predict subsequent regulatory approval [138]. Cytostatic endpoints such as time to progression may be valid alternatives, but these present methodological challenges. In particular, historical controls can provide misleading assumptions about anticipated tumour behaviour in untreated patients.

Phase II Design Issues

For the reasons outlined above, single-arm phase II studies of SFK inhibitors in advanced melanoma using response rate as the primary measure of efficacy are probably not appropriate. Randomized phase II designs should be considered in order that more accurate control data can be obtained, and such designs should include time to progression among the efficacy endpoints. One interesting trial design which specifically addresses disease stabilisation is the randomized discontinuation study [139], although practical implementation of this design has proved challenging in oncology [140]. Randomized designs are inevitably larger, and usually longer and more costly than single arm phase II studies.

Patient Selection and Personalized Medicine

Biomarkers which truly differentiate between patients who will and will not benefit from a given therapy are few in oncology. Nonetheless, as the number of agents increases it is essential that we continue to search for rational ways of selecting the most appropriate therapies for individual patients. This seems a tempting opportunity for molecularly targeted agents such as the SFKs, but our current understanding of the targets does not indicate a clear molecular hypothesis for rational patient selection. In particular, no data exist to support the selection of trial subjects on the basis of SFK expression or activity. Preclinical studies in breast and lung cancer have suggested an mRNA expression profile based on a small set of transcripts which is predictive of growth inhibition in cell lines [62]. However, it is not intuitive that this signature would be predictive of clinical benefit in melanoma or, fundamentally, whether such a gene signature would have relevance should clinical activity be mediated via inhibition of invasion or via effects on stromal cells.

In contrast, for c-Kit, a case for patient selection can be made; specifically to test the utility of c-Kit inhibition in the subset of patients harbouring the molecular abnormalities in c-Kit described above.

Pharmacodynamic Endpoints

Any clinical development pathway for a novel anticancer drug presents significant risk, and, as the number of patients and costs rise, the value of alternative risk-minimisation strategies increases. Thus the ability to demonstrate the therapeutic principle (i.e. that the drug does inhibit the intended pathway within the tumour when administered at tolerable doses) may be important, because failure to do so could be a rational reason to terminate development in that indication. In addition, pharmacodynamic endpoints may enable rational decision making about optimal dose and schedule of a drug. The relative ease of access to cutaneous tumour deposits in melanoma should facilitate studies incorporating serial biopsies for pharmacodynamic studies. In this context potential readouts of SFK inhibition would include reduction of auto-phosphorylation of Src at tyrosine-419 as well as reduced phosphorylation of SFK substrates such as FAK and paxillin [61, 96].

Duration of Exposure and Safety Issues

For drugs which stabilise advanced cancer, or drugs which inhibit progression from early disease, the optimum duration of therapy may be many months or even years. All three drugs discussed are simple-to-take oral preparations but such therapy demands high levels of tolerability and safety; the exact safety and tolerability burden of these drugs in chronic administration remains largely unexplored.

Combination Strategies

A reasonably wide body of literature suggests that Src expression and activity is associated with acquired or innate anti-cancer drug resistance [141]. Furthermore, pharmacological inhibition of Src can overcome this resistance [67, 142, 143]. This hypothesis underpins some of the combinational drug development programmes in place for SFK inhibitors in other solid tumours, and may also be a productive approach in melanoma [23].

CONCLUSIONS

The recent discovery of SFK inhibitors and their successful progress through phase I trials offers an exciting opportunity to directly evaluate the clinical utility of SFK inhibition in melanoma. However, our understanding of the role of SFK signaling in melanoma biology remains incomplete and the successful development of SFK inhibitors in this context is by no means guaranteed. Considerable challenges lie in the design of trial strategies to reliably detect activity of an agent whose action may be primarily to inhibit invasion and metastasis rather than growth. Significantly, work to discover predictive biomarkers for SFK inhibitors is at an early stage and such information will most likely be gleaned retrospectively. Nevertheless, close cooperation between laboratory and clinic may maximize the chances of successful development of a new class of agents in this refractory disease.

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