The Brk protein tyrosine kinase as a therapeutic target in cancer: opportunities and challenges

Amanda J. Harvey[a] and Mark R. Crompton[a]

Brk is an intracellular protein tyrosine kinase that is significantly overexpressed in a majority of breast tumors, while being detected at appreciable levels in only a limited range of adult tissues that does not include the mammary gland. It has recently been demonstrated to have a role in promoting the proliferation of carcinoma cells, one that it is unlikely to perform in normal adult cells, and it therefore represents an exciting target for the development of novel cancer therapies based on specifically or selectively interfering with its functions. The strategy of pharmaceutical kinase inhibition is clinically proven and widely pursued in oncology programmes directed at a variety of tumor types. However, a potentially kinase-independent role for Brk in regulating proliferation suggests that alternative approaches, such as inhibiting protein–protein interactions, may prove more successful. Further research into Brk's signaling functions will underpin progress towards turning the potential suggested by these observations into rational drug discovery, from which a large number of patients stand to benefit. ***Anti-Cancer Drugs*** **15:107–111 © 2004 Lippincott Williams & Wilkins.**

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[a]School of Biological Sciences, Royal Holloway University of London, Egham, Surrey, UK.

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Correspondence to M. R. Crompton, School of Biological Sciences, Royal Holloway University of London, Egham, Surrey TW20 0EX, UK.
Tel: +44 1784 443554; fax: +44 1784 434326;
e-mail: m.crompton@rhul.ac.uk

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Introduction

Brk (PTK6) is a human intracellular protein tyrosine kinase. An analysis of its predicted amino acid sequence reveals that it comprises a Src homology 2 (SH2) and an SH3 domain in addition to the catalytic domain, an overall architecture reminiscent of the well-studied Src family of kinases [1]. There are several features of the amino acid sequence and gene structure that clearly distinguish it from Src family kinases, and Brk has been classified as a member of a small distinct family of kinases whose functions are generally not yet well understood (reviewed in [2]).

Based initially on its expression patterns, notably its frequent *de novo* expression in breast tumor development, and subsequently on assays of its proliferation promoting functions, we have proposed that Brk could be targeted for the development of novel tumor therapeutic agents. In this review, we discuss the extents to which Brk meets the criteria ideally required of a therapeutic target, how targeting might be achieved and where the priorities for future research may lie.

Brk's tissue expression patterns

In normal adult human tissues, Brk has a relatively restricted expression pattern. Thus far, appreciable expression has been detected in terminally differentiating cells of the gastrointestinal tract (specifically esophagus, stomach, duodenum and colon) [3] and luminal epithelial cells of the prostate [4]. The murine Sik protein shares only 80% amino acid sequence identity with Brk; however, the genes encoding these proteins are orthologous based on Southern blotting and gene mapping [3]. Sik, like Brk, has been found to be expressed in the gastrointestinal tract and also in the skin [5]. While Brk expression has been detected in a variety of cultured cells, the studies that have been performed on primary human tumor samples are the most informative with respect to Brk's potential role in pathology. Brk expression is retained in prostate tumor progression [4] and modestly increased in colon tumorigenesis [3]. Strikingly, Brk is expressed at significant levels in around 60% of human breast tumors, including lymph node metastases, but not in normal mammary tissue or fibroadenomas [1,6]. In support of the hypothesis that *de novo* Brk expression is specifically associated with tumor development in the breast, Sik expression is absent at various stages of development in the normal murine mammary gland [3]. Studies with cultured cells have also suggested that a small proportion of metastatic melanomas may acquire Brk expression during their development (7).

Based on these expression studies alone, there are compelling reasons to study Brk further in the context of breast cancer, in order to dissect the mechanisms by

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which a majority of tumors acquire Brk expression. These changes have not been reported to be associated with increased Brk gene number in breast tumors and it is therefore likely that studies of Brk gene regulation will pinpoint regulatory pathways that are subverted in breast tumorigenesis. To date there have been no published studies on the basis for the breast tumor-specific expression of Brk and this remains a fruitful avenue for future research.

Brk's cellular functions

The provocative findings on Brk's tumor- and tissue-specific expression have stimulated studies into its functions in cell regulation. In order to determine whether pharmacologically targeting Brk's function could be of therapeutic value we will need to understand how it contributes to the phenotypes of normal and tumor cells, and progress has been made in both directions. In early studies, the role of Brk in breast tumor development was modeled using an immortalized human cell line (HB4a) [8] which retains many of the features of primary luminal epithelial cells, these being the likely precursors of the majority of human breast tumors. HB4a cells express little or no endogenous Brk and, although immortalized, retain a dependence on exogenous growth factors for their proliferation. Experimental expression of Brk in HB4a cells, to levels comparable with those detected in breast carcinoma cell lines (i.e. pathologically relevant levels), results in them being sensitized to the mitogenic effect of epidermal growth factor (EGF) [9]. Since there is no concomitant change in the number or affinity of cell-surface EGF receptors, the most likely interpretation is that Brk in some way potentiates the coupling of EGF receptor signaling with proliferative responses. EGF receptor family signaling has an important role in promoting mammary tumorigenesis (reviewed in [10]) and Brk is therefore functionally implicated in signaling pathways with well-characterized pathological relevance. Brk expression is also found to increase both the proportion of HB4a cells that can proliferate anchorage independently, and the size of the resulting clones, pointing to the possibility that Brk expression may influence anchorage-independent survival and/or proliferation. The results of these studies are therefore suggestive of an important functional role for Brk in the breast tumor phenotype, e.g. by imparting on mammary cells a proliferative advantage in the presence of limiting concentrations of growth factors.

Recent advances in the use of RNA interference (RNAi) to specifically suppress gene function in mammalian cells have the potential to revolutionize gene function analysis (reviewed in [11]). We have recently employed the transfection of small interfering RNAs (siRNAs) targeting the Brk mRNA to specifically suppress Brk expression in the T-47D breast carcinoma cell line by RNAi [12]. This has allowed us to dissect Brk's function in the context of cells that are derived directly from a human breast tumor, and have therefore acquired co-operative genetic and epigenetic changes representative of tumor immortalization and progression *in vivo*. In particular, since Brk overexpression in breast tumors has not been attributed to alterations to its gene, e.g. amplification, the selectively advantageous change(s) that results in Brk expression *in vivo* is presumably accompanied by alterations to the expression levels of multiple other molecules; thus cells that have acquired Brk expression through pathological evolution are the best context in which to study it. Transient reductions in Brk expression of approximately 70% or more result in a significant (around 50%) reduction in cell number accumulation over a period of 4 days post-treatment. Since we have failed to detect any induction of cell death in the cultures, the most likely explanation for this is that cells with reduced Brk expression proliferate more slowly, an interpretation supported by the observation that Brk targeting results in a reduced rate of DNA synthesis as assessed by bromodeoxyuridine incorporation. These studies strongly corroborate the general conclusions arrived at from the expression of Brk in non-transformed mammary cells, i.e. that Brk expression in breast tumor progression may confer on cells a proliferative advantage. The ever-improving strategies for siRNA delivery into mammalian cells, e.g. the use of various viral expression vectors, will allow studies to be performed on further breast carcinoma cell line models and potentially in short-term cultures of primary tumors.

In contrast to breast carcinoma cells, it is unlikely that Brk serves a proliferation promoting function in most normal adult cells, since in the adult skin and gastrointestinal tract Brk/Sik expression is localized to cells that have exited the proliferative zones of the tissue [5,3]. In the only other adult tissue type in which appreciable Brk expression has been detected and analyzed, the prostate, it is not yet clear how Brk expression correlates with proliferative status [4]; however, this is also a tissue with a highly differentiated non-proliferative compartment. The coincidence of Brk/Sik expression with terminal differentiation is suggestive of a functional connection and there is evidence to support this. Overexpression of Sik in a murine keratinocyte cell line results in the increased accumulation of the differentiation marker filaggrin after induction of differentiation in response to calcium ions [13].

Brk as a therapeutic target

We have proposed that Brk is a potentially exciting target for the future development of novel treatments for breast cancer, a disease that is now reported to be the commonest cancer in the UK (Cancer Research UK data). When considering the proportion of patients who

may benefit from a putative Brk-targeted therapy, it is worthwhile considering that *de novo* Brk expression in approximately 60% of breast tumor cases is significantly higher than the incidences of other common molecular alterations: p53 mutation (around 20%) or c-*erbB-2* overexpression (25–30%) [14,15]. In addition to its status as a 'marker' of breast tumorigenesis in many patients, there is now a body of evidence that points to Brk having a direct role in supporting carcinoma cell proliferation (see above). Thus, pharmaceutically targeting Brk might be anticipated to have the prospect of slowing or arresting tumor growth and molecular profiling of individual tumors will allow the identification of those patients whose disease is likely to respond. By incompletely inhibiting Brk expression, we have achieved a suppression of proliferation in the order of 50%. It is likely that a more complete growth arrest will be achieved by more effective targeting and the application of novel vector systems to deliver stable siRNA expression will allow this issue to be addressed. Taking the extremely pessimistic view that we may already have achieved close to the maximal phenotypic response, growth retardation on this scale could nonetheless be clinically valuable. For example, delaying the growth of tumor recurrences at secondary sites after primary treatment, which can manifest after many years of apparently disease-free remission, would potentially provide a significant improvement in prognosis.

In considering the feasibility of therapeutically targeting Brk it will be important to address the issue of potential drug side-effects and Brk is an exciting prospect in this respect. In the major sites of physiological expression, which are encouragingly relatively restricted, Brk is very unlikely to have a role in proliferation (see above), thus providing the possibility of fine-tuning a pharmacological intervention to interfere with a subset of Brk's functions and thus enhance therapeutic specificity. From the point of view of predicting where possible unwanted side-effects of therapies directed at normal Brk may arise, it will be extremely valuable to assess the phenotype of experimental animals completely lacking the Brk ortholog. Tyner and colleagues [2] have reported, as unpublished data, that Brk/Sik-deficient mice are viable, fertile and show no obvious physical deformity. Tempered with the caveats that important deficits may become apparent on further investigation, that developmental plasticity may have allowed the enactment of compensatory signaling that would not occur in the context of acute drug action and that the function of Brk in human tissues may be distinct from mice (bearing in mind the relatively diverged amino acid sequences), this is clearly an encouraging finding. The results of detailed investigations into the phenotypes of these mice are eagerly awaited.

In addition to proliferation there are several other important facets to the carcinoma cell phenotype, in which a participating role for Brk has not yet been investigated. Brk expression can support the anchorage-independent expansion of mammary luminal epithelial cell clones [9] and it is therefore possible that Brk expression could contribute to insensitivity to apoptosis in the absence of epithelial cell attachment (anoikis). Such a survival capacity is likely to enhance the progression and metastatic potential of tumor cells, as are other evolved traits such as migration/chemotaxis, invasion through extracellular matrices and the support of neoangiogenesis. Our current knowledge of Brk's signaling partners supports the view that research in these directions will be fruitful. Brk associates with two members of the EGF receptor/erbB family, EGF receptor and erbB3 [9,16], and these interactions may be necessary for Brk's potentiation of EGF-mediated mitogenic signaling. EGF receptor activation has been shown to upregulate matrix metalloproteinase and vascular endothelial growth factor production (e.g. [17,18]), and Brk expression may therefore influence invasion and neoangiogenesis through a role in downstream EGF receptor signaling. Brk expression in HB4a mammary epithelial cells enhances EGF-induced phosphoinositide 3-kinase (PI3 K) recruitment to erbB3 and subsequent phosphorylation of Akt [16], and Brk expression may therefore potentiate anti-apoptotic signaling [19] in the context of carcinoma cell survival during dissemination or therapy. Future studies on Brk's roles in tumor progression will clarify whether therapeutic targeting will have important clinical benefits beyond the inhibition of cell proliferation.

Potential strategies for Brk targeting

An important consideration in assessing Brk as a potential therapeutic target will be to determine the best way in which to inhibit its function. There is currently no suggestion that the amino acid sequence of Brk is altered by mutation in tumors, since a full-length cDNA clone derived from the breast carcinoma cell line T-47D [1] encodes a protein of identical sequence to that encoded within normal human genomic DNA [20,21]. Additionally, since expression from this T-47D-derived cDNA clone enhances the proliferation of mammary cells [9] and targeting the mRNA in T-47D cells by RNAi results in reduced proliferation [12], it is the normal protein that mediates proliferative regulation and whose function will need to be targeted in breast carcinomas. There is therefore little prospect of sequence specificity-based 'molecular biological' approaches such as site-directed RNAi to target tumor cells alone, leaving aside the likely problems that would be associated with the efficient delivery of such therapies.

The hopes of those who have lead research into developing small molecule kinase inhibitors as tumor therapies are now beginning to be realized, with the

clinical application of STI571 leading the way [22]. This is a promising and well-tolerated tyrosine kinase targeted drug, in spite of the fact that it inhibits several widely expressed enzymes (Abl, Arg, Kit, PDGFr) in addition to its pathological target Bcr–Abl. Thus, the prospect of designing further drugs on a similar premise, without having to achieve absolute target specificity, is an entirely realistic one. Since the activities of many different protein kinases have been implicated in the development of a variety of human tumors, including breast cancer, the development of kinase inhibitors is an extremely active area of current pharmaceutical research. In the case of Brk we will have to tread with caution before fully committing to this strategy. While Brk's kinase activity is required to partially transform NIH3T3 murine embryonic fibroblasts to anchorage independent proliferation [9], expression of a kinase-inactivated Brk mutant in T-47D breast carcinoma cells promotes proliferation [12]. The generality of the latter finding will need to be addressed in further experimental models, since it suggests that kinase inhibition may not be the optimal strategy for blocking Brk's promotion of carcinoma cell proliferation. In the event that kinase inhibition were deemed a promising approach, it would have to be borne in mind that the catalytic activity has been implicated in the regulation of keratinocyte differentiation [13].

It can be confidently predicted that regulated protein–protein interactions will be found to be pivotal in Brk's activities. Brk associates with the EGF receptor [9], the adapter-like protein BKS/STAP-2 [23], GAP-associated p65 protein [13], erbB3/HER3 [16] and the Sam68 RNA-binding protein [24]. The latter two interactions form in the absence of Brk's kinase activity and are therefore candidates for an involvement in any kinase-independent activities. In general, the functional outcomes of Brk's known intermolecular interactions are still poorly understood; while the Brk–erbB3 interaction may be necessary for enhancing PI3 K recruitment and activation, studies of the Brk–Sam68 interaction have provided the most complete picture of a Brk signaling function to date. In phosphorylating it, Brk inhibits Sam68's RNA binding function and thereby interferes with its ability to increase expression from a reporter gene containing a specific (rev responsive) regulatory element [24]. Brk also inhibits Sam68-mediated enhanced expression from mRNAs containing constitutive transport elements and introns [25], and it therefore appears that Brk functions in regulating gene expression through modulating the utilization of mRNAs. Brk relocalization from the nuclear to cytosolic compartment correlates with advanced stage prostate tumorigenesis, and nuclear exclusion prevents association with Sam68 and is possibly associated with a reduced catalytic activity [4]. This raises the possibility that there may be tumor-specific modifications to Brk's signaling functions beyond 'simple' changes in expression levels and that these could be exploited therapeutically if better understood.

Brk-targeting strategies based on interfering with specific protein complex formation might focus on its SH3 and SH2 domains. These modules typically participate in intra- and inter-molecular interactions whose general structural principles are now relatively well understood; SH3 domains bind a family of target motifs containing proline residues and SH2 domains bind phosphorylated tyrosine residues in restricted local sequence contexts (reviewed in [26]). In Brk's case, both the SH3 and SH2 domains have been implicated in regulating its activity through intramolecular associations [27] and in mediating binding of the Sam68 RNA-binding protein [24]. Additionally, the SH2 domain binds tyrosine phosphorylated GAP-associated p65 protein [13]. No doubt these domains in Brk are important for a variety of functionally important protein–protein interactions that remain to be fully characterized and dissecting these details will be an important goal for the future. While the exploitation of SH2 and SH3 mediated protein–protein interactions for therapeutic intervention is not currently as advanced as enzymatic inhibition, significant progress is being made (reviewed in [26]). Structural studies on Brk, which have been initiated [28,29], coupled with imaginative chemistry, may allow the design of pharmacological agents that target Brk-containing complexes in breast tumor cells.

Conclusions

The expression of Brk in mammary tissues is striking; it is expressed at significant levels in around 60% of tumors, but not in normal tissue or benign lesions. By comparison with other molecular alterations in breast cancers, this is a very frequent aberration. Brk expression is likely to confer a proliferative advantage on the carcinoma cells and interfering with its functions would therefore be of great therapeutic benefit. Since Brk's role in normal tissues is unlikely to be in the promotion of proliferation, there is the prospect of designing anti-proliferative breast cancer drugs with few side-effects in normal tissues, once the specific mechanisms underlying this aspect of carcinoma cell regulation by Brk are understood.

Brk's kinase activity, and SH2 and SH3 domains, represent molecular targets for drug design. Whether the 'proven' kinase inhibition strategy will be the most appropriate is currently in doubt; however, approaches to exploiting SH2 and SH3 interactions therapeutically are becoming increasingly sophisticated. Detailed investigations into Brk's signaling mechanisms in normal and tumor cells will point to the best route forward.

The basis of Brk's *de novo* expression in breast tumors is not understood, but it most likely reflects the subversion of signaling pathways regulating gene expression, rather

than a locally restricted alteration to the Brk gene itself. Future research in this area could therefore uncover further exciting therapeutic opportunities.

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