CHAPTER

3

Roles for AXL and MERTK in Resistance to Cytotoxic and Targeted Therapies

Ryan J. Summers[*,**], *Katherine A. Minson*[*,**], *Deborah DeRyckere*[*,**,†], *Douglas K. Graham*[*,**,†]

***Emory University Department of Pediatrics, Atlanta, GA, United States**
****Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Atlanta, GA, United States**
[†]Winship Cancer Institute, Emory University, Atlanta, GA, United States

Abstract

AXL and MERTK are members of the TAM family (TYRO3, AXL, and MERTK) of receptor tyrosine kinases (RTKs). Ligand binding and subsequent activation of AXL and MERTK in human cancers lead to downstream signaling via pathways that mediate tumor cell survival and proliferation. TAM RTK signaling mediates resistance to conventional cytotoxic chemotherapies across a wide variety of both hematopoietic and nonhematopoietic malignancies. Similarly, TAM RTKs are upregulated and mediate bypass signaling in tumor cells with acquired resistance to a variety of different targeted kinase inhibitors. In addition, TAM RTKs can mediate therapeutic resistance through their physiological anti-inflammatory role in the immune system. These roles for AXL and MERTK in therapeutic resistance implicate them as attractive therapeutic targets, both in combination with other agents and in the context of acquired resistance. Accordingly, a number of TAM RTK-targeted agents are currently in development and several have progressed to clinical application, although challenges such as identification of appropriate biomarkers of TAM RTK inhibition remain.

ABBREVIATIONS

ADC	Antibody-drug conjugate
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
BAD	BCL-2-associated agonist of cell death
BAK	BCL-2 homologous killer
BAX	BCL-2 associated X

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BCL-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma-extra large
CLL	Chronic myeloid leukemia
CML	ezrin, radixin, moesin protein family
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-related kinase
FLT3-ITD	fms-like tyrosine kinase 3-internal tandem duplication
GAS6	Growth arrest-specific protein 6
GBM	Glioblastoma multiforme
IFN	Interferon; IGF-1R, Insulin-like growth factor 1 receptor
JAK	Janus kinase
LXR	Liver X receptor
MCL1	Myeloid cell leukemia 1
mTOR	mechanistic target of rapamycin
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	Non-small cell lung cancer; PD1, Programmed cell death protein 1
PKC	Protein kinase C
PI3K	Phosphoinositide-3-kinase
PUMA	p53 upregulated modulator of apoptosis
RTK	Receptor tyrosine kinase
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
VEGFR	Vascular endothelial growth factor receptor

3.1 INTRODUCTION

Receptor tyrosine kinases (RTKs) encompass a wide range of proteins involved in cellular signaling. Many RTKs have been implicated in human malignancies and some have been identified as oncogenic drivers. The TAM family of RTKs is composed of three transmembrane receptors—TYRO3, AXL, and MERTK [1–3]. These receptors have a similar overall structure consisting of an extracellular domain comprised of two immunoglobulin-like domains and two fibronectin type III domains, a transmembrane domain, and an intracellular kinase domain with a conserved KWIAIES sequence, which is unique to the TAM family [4]. The TAM receptors are ectopically or aberrantly expressed and contribute to oncogenesis in a wide range of human malignancies [3,5–7]. Among the TAM receptors, TYRO3 has been less studied compared to AXL and MERTK and therefore this chapter will focus on the latter two members of the TAM family. Although AXL and MERTK activate pathways that can mediate cell proliferation, such as the protein kinase B (AKT), extracellular signal-related kinase (ERK), and members of the signal transducer and activator of transcription (STAT) family pathways, their activation preferentially promotes tumor cell survival, rather than proliferation [8–11]. As such, the TAM RTKs have been the focus of intense study as potential therapeutic targets in a variety of malignancies and the subject of numerous recent reviews [4,5,12–18].

Activation of the TAM receptors in both physiologic and pathologic states is triggered by ligand binding followed by dimerization and auto-phosphorylation. Maximal TAM

stimulation requires binding to phosphatidylserine (PtdSer) in a complex with a protein ligand [4]. The TAM ligands are y-carboxylated proteins that bind to the receptors via their carboxy-terminal domain and to PtdSer via their amino terminus [19–22]. The first identified TAM ligand was the vitamin K-dependent growth arrest-specific protein 6 (GAS6), which binds to all three TAM RTKs [22], although AXL has the highest affinity for GAS6. There is evidence to suggest that AXL binds GAS6 constitutively *in vivo* but is not activated until PtdSer-mediated dimerization occurs [23]. In contrast, vitamin K-dependent protein S (PROS1) only binds MERTK and TYRO3 [23,24]. In addition, recent data demonstrate downregulation of AXL mRNA and protein in oral squamous cell carcinoma in response to PROS1 knockdown [25]. More recently, Tubby, tubby-like protein 1, and galectin-3 have been identified as MERTK ligands that regulate phagocytosis [26–28]. Although PtdSer is ubiquitously expressed, it is only available to activate the TAM receptors when externalized on apoptotic cell membranes, activated platelets, exosomes, and invading virus envelopes [29–34]. Ligand-independent activation of TAM kinases may also occur in pathologic states as there is evidence to suggest GAS6-independent activation of AXL in the setting of AXL overexpression in tumor cells. In this context, aggregation of AXL extracellular domains on adjacent cells [35] or ligand-independent homodimerization [36] can activate downstream signaling. Furthermore, AXL heterodimerizes with the non-TAM RTKs epidermal growth factor receptor (EGFR) and HER3 to initiate AXL-dependent signaling in breast cancer and glioblastoma cells, even in the absence of the dimerization partner ligand [37–39].

The best studied physiologic TAM RTK function is the role of MERTK in efferocytosis—the clearance of apoptotic material by monocyte- [29] and epithelial-derived cells [40]. TAM RTKs are also involved in regulation of the immune system with a net effect of protecting against a pro-inflammatory state. Their role in this context will be discussed later in the chapter. TAM RTKs also play a role in platelet aggregation by stimulating outside-in signaling via αIIbβ3 integrin [41]. These physiologic roles do not necessarily implicate TAM RTKs as oncogenic drivers. Nonetheless, TAM RTK signaling does promote survival, therapeutic resistance, motility, and invasion in neoplastic cells.

3.2 ROLES FOR TAM RTKs IN TUMORIGENESIS

3.2.1 TAM Receptor Expression and Signaling in Cancer

TAM receptors and their ligands are overexpressed in both hematologic and nonhematologic malignancies and their expression has been correlated with poor prognosis in a number of tumor types including glioblastoma multiforme (GBM), non-small cell lung cancer (NSCLC), oral squamous cell carcinoma, renal cell carcinoma, colon cancer, mesothelioma, and acute myeloid leukemia (AML) [4,42–49]. Interestingly, expression of AXL or GAS6 protein are both markers of poor prognosis in GBM and NSCLC [42,43], but decreased GAS6 mRNA expression has been correlated with poorer prognosis in NSCLC [43]. In one study, GAS6 mRNA levels had no prognostic significance in AML [45], but a larger study demonstrated GAS6 expression as an independent predictor of remission failure and shorter disease-free and overall survival [46]. Given that these two studies defined patients with GAS6 expressing AML differently (i.e., 90% [45] vs 26% [46]), further study is needed to draw firm conclusions on the prognostic role of GAS6 mRNA expression in AML.

Ectopic expression of MERTK was first noted in acute lymphoblastic leukemia (ALL) [1] and is present in 30–50% of pediatric B-cell [50] and T-cell [50,51] ALL patient samples. MERTK is also aberrantly expressed in 70%–90% of pediatric and adult AMLs [52]. Additionally, patients with multiple myeloma have an increased fraction of MERTK-expressing bone marrow plasma cells compared to healthy controls [53]. AXL expression has not been described in ALL, but overexpression is common in AML [45,54–56] and has also been demonstrated in chronic myeloid leukemia (CML) [54] and chronic lymphocytic leukemia (CLL) [57]. A complete list of solid tumors with TAM receptor overexpression can be found in the review by Graham et al. [4]. Most notably, MERTK overexpression has been demonstrated in lung cancer [58], glioma [59,60], and melanoma [61–64], and AXL overexpression has been demonstrated in lung cancer [58,65,66], glioblastoma [42,59,67], breast carcinoma [68–70], ovarian carcinoma [71–73], and melanoma [61,62], among others. It is unknown that what mechanisms underlie aberrant expression of TAM receptors across such a diverse range of tumor types, but it is possible that they reflect the physiologic mechanisms that regulate TAM expression in normal tissues. For example, *MERTK* transcription in macrophages is stimulated by liver X receptor (LXR) and LXR ligands such as 27-hydroxycholesterol, which is produced by both tumor cells and cells in the tumor microenvironment [74,75].

The prevalence of TAM receptor overexpression across such a wide range of malignancies implicates a critical oncogenic role for TAM receptor signaling. While expression of activated oncogenes and loss of tumor suppressor genes are critical to promote unregulated growth of some tumor cells, neoplastic cells can also be dependent on the function of endogenous, nonmutated genes for survival under stress (i.e., nononcogene addiction). For example, MERTK inhibition using short hairpin RNA (shRNA) promotes apoptosis in tumor cell cultures following serum starvation [52,58]. In an *in vivo* setting, limited oxygen and nutrients in the tumor microenvironment may also promote signaling through TAM-dependent survival pathways. As an example, GAS6 and PtdSer on exosomes from osteoblasts and bone marrow stromal cells provide ligands for TAM kinase activation in cancer cells in the bone marrow niche [76,77]. Together these data implicate MERTK signaling as a critical mediator of tumor cell survival.

Activation of the TAM receptors stimulates signaling through numerous downstream survival pathways including mitogen-activated protein kinase kinase (MEK)/ERK, phosphoinositide-3-kinase (PI3K)/AKT, p38, RHO family proteins, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Janus kinase (JAK)-STAT, and SRC family kinases (Fig. 3.1 and reviewed in [5]). Stimulation with GAS6 activates PI3K–AKT [50–52,58,64,78–80] and RAS–RAF–MEK [7,9,50,51,58,59,78–80] signaling in multiple cancers, and TAM receptor inhibition through both pharmacologic and genetic means can abrogate these signals. Signaling through MERTK also regulates downstream phosphorylation and transcriptional activity of STAT3, STAT5, and STAT6, and AXL similarly activates STAT3 in multiple tumor types [48,51,52,64,81]. In the case of TAM-mediated NF-κB signaling, there is a clear distinction between the effects in tumor cells vs normal tissues. In tumor cells, MERTK activates the NF-κB pathway [82–87] and potentially its apoptotic mechanisms. In other contexts, as part of their role in suppressing the innate immune inflammatory response, AXL and MERTK can inhibit NF-κB signaling, thereby decreasing inflammatory cytokine production [88–90]. This may represent a mechanism by which TAM signaling is co-opted in a context-dependent manner to enhance tumor cell survival. Inhibition of

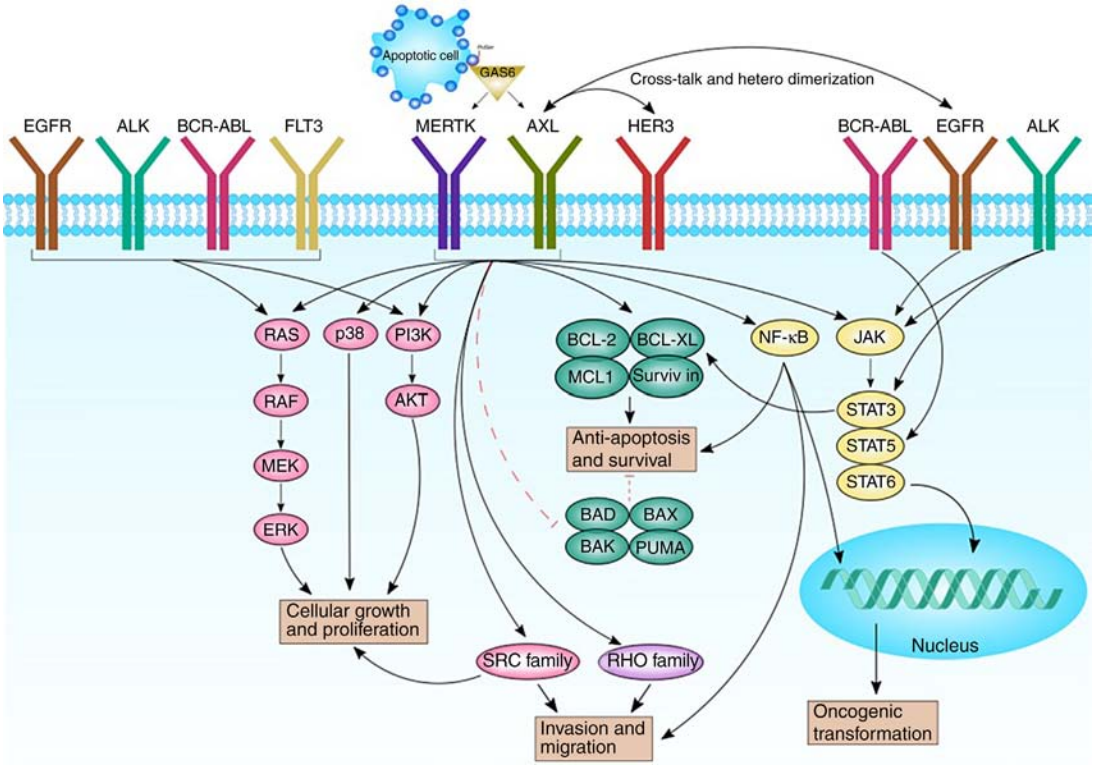

FIG. 3.1 AXL and MERTK activate oncogenic signaling to mediate therapeutic resistance. Activation of TAM RTKs by GAS6 complexed with PtdSer mediates downstream signal transduction through oncogenic pathways to promote tumor cell survival, proliferation, migration, and invasion. These effects are context dependent and vary based on the TAM RTK, ligand, and cell type involved. Oncogenic driver RTKs such as EGFR, ALK, FLT3, and BCR-ABL utilize many of the same downstream signaling pathways and inhibition of these RTKs by targeted therapies can be overcome by compensatory TAM signaling, leading to therapeutic resistance.

AXL or MERTK in tumor cells also regulates expression of numerous apoptotic proteins, including the antiapoptotic proteins B-cell lymphoma 2 (BCL-2), B-cell lymphoma-extra large (BCL-XL), myeloid cell leukemia 1 (MCL1) and survivin [45,57,58] and the pro-apoptotic proteins BCL-2-associated agonist of cell death (BAD), BCL-2 associated X (BAX), BCL-2 homologous killer (BAK), and p53 upregulated modulator of apoptosis (PUMA) [45,50,91]. TAM RTKs modulate these signaling pathways and apoptotic regulators to promote tumor cell survival in response to apoptotic stimuli.

3.2.2 Role of TAM RTKs in Mediating the Epithelial-to-Mesenchymal Transition

The epithelial-to-mesenchymal transition (EMT) is a reversible conversion of epithelial cells to a mesenchymal phenotype and plays an important physiologic role in embryonic

development and wound healing. In the context of cancer, EMT is associated with increased cell migration and invasion and ultimately with development of metastatic disease [92]. In cells undergoing EMT, typical epithelial markers such as E-cadherin are suppressed, whereas mesenchymal markers such as N-cadherin and fibronectin are induced. The transcription factors SNAIL1/2, TWIST1/2, and ZEB1/2 are well described as inducers of EMT [16]. Cells that have undergone EMT often exhibit properties characteristic of stem cells, such as decreased proliferation, and, as a result, are more resistant to antiproliferative therapy [93]. As such, intrinsically mesenchymal cells or cells that have undergone EMT are more resistant to both chemotherapy and targeted therapies [94].

AXL, in particular, has been implicated in EMT in a number of studies [94–96] and is one of the primary genes overexpressed in EMT-specific gene expression signatures [97–100]. It remains unclear as to whether AXL expression induces EMT or EMT induces AXL expression. The role of AXL in EMT likely has some relationship to therapeutic resistance, as AXL is upregulated in mesenchymal NSCLC cell lines, which are highly resistant to erlotinib compared to epithelial NSCLC cell lines [98]. Additionally, inhibition of AXL can reverse both the EMT phenotype in cancer stem cells [86] and EMT-associated resistance to EGFR inhibitors [98]. It is likely that the relationship between AXL and EMT is context-dependent, but the available data support a direct role for AXL in maintaining drug resistance and not simply as a biomarker of EMT.

3.2.3 Role of TAM RTKs in Angiogenesis

The TAM receptors are normally expressed in epithelial cells and in this context they function to mediate vascular integrity and promote angiogenesis. In the setting of vascular injury, TAM receptor signaling promotes platelet aggregation and restoration of the endothelial barrier [15,101–103]. In addition, AXL knockdown via shRNA impaired blood vessel formation and function in a mouse model of human angiogenesis [104]. Given this role for TAM RTK signaling, activation of the TAM pathways has been proposed as a mechanism for resistance to therapies targeting the vascular endothelial growth factor receptor (VEGFR) [102,103,105]. Indeed, a recent phase III clinical trial of cabozantinib, an MET, VEGFR, and AXL inhibitor, demonstrated superior outcomes for patients with renal cell carcinoma previously treated with VEGFR inhibitors, supporting a role for AXL in mediating resistance to anti-angiogenic therapies [106].

3.3 RESISTANCE TO THERAPY MEDIATED BY TAM RTKs

Signaling via TAM receptors has been linked to chemotherapy resistance via numerous mechanisms which are discussed in more detail below. TAM RTKs modulate downstream signaling through pathways important in the regulation of cell survival, proliferation, migration, and invasion. It is through these mechanisms that they are thought to promote resistance to traditional cytotoxic chemotherapy. Additionally, TAM RTKs mediate resistance to targeted therapies by allowing tumor cells to bypass the signaling blockade introduced by the targeted therapy (Fig. 3.1).

3.3.1 Role of TAM RTKs in Mediating Resistance to Traditional Chemotherapy

As has been noted, aberrant expression of TAM RTKs occurs in a wide range of both hematologic and nonhematologic malignancies. In many tumor types, higher TAM expression is associated with resistance to cytotoxic chemotherapies. For example, AXL mRNA is increased in cisplatin-resistant ovarian cancer cell lines [107] and AXL protein levels are increased in doxorubicin-resistant breast cancer and CML cell lines compared to nonresistant cell lines [108]. Similarly, AXL expression was upregulated in doxorubicin- and etoposide-resistant NSCLC cell-line derivatives, and ectopic expression of AXL in the parental cell line increased resistance to doxorubicin [83]. Similarly, introduction of a plasmid conferring AXL overexpression doubled the IC_{50} in an esophageal adenocarcinoma cell line treated with cisplatin relative to parental cell lines [109]. In this context, resistance to cisplatin was associated with decreased phosphorylation of c-ABL and p73β, a member of the p53 family. AXL and c-ABL also coprecipitated, suggesting a physical interaction that prevents shuttling of c-ABL to the nucleus where it usually binds and stabilizes p73 protein. Across a panel of cell lines derived from multiple tumor types (pancreatic, breast, prostate, colon, nasopharyngeal, renal cell carcinoma, melanoma, neural cancer, and leukemia), increased AXL protein correlated with increased resistance to doxorubicin, although interpretation of these data is complicated by inclusion of both liquid and solid tumor cell lines in the analysis [83]. AXL was upregulated in AML patient samples collected from patients who failed treatment with doxorubicin and cytarabine relative to diagnostic samples from the same patients, and AXL expression was induced in AML cell lines in the presence of chemotherapy agents in a manner dependent on methylation status of the AXL promoter [79]. Similarly, lymphocytes with ectopic MERTK expression isolated from MERTK transgenic mice were more resistant to dexamethasone than wild-type lymphocytes [7] and stimulation of a B-ALL cell line with GAS6 increased resistance to cytarabine-induced apoptosis by inducing cell-cycle arrest [77]. In an AML cell line, GAS6 stimulation conferred resistance to doxorubicin, etoposide, and cisplatin, and this effect was reversed by addition of a ligand trap to deplete GAS6. In this case, resistance correlated with increased AXL signaling as indicated by increased levels of phosphorylated AXL (pAXL) and concomitant increases in pAKT and pERK. MERTK activation was not examined. Additionally, the pro-survival proteins BCL-2, BCL-XL, and Twist were upregulated in this model [79].

Conversely, inhibition of TAM RTK signaling increases the sensitivity of tumor cells to a variety of chemotherapy agents. A large number of studies have demonstrated increased sensitivity to DNA damaging therapeutic agents in tumor cell lines in response to TAM RTK inhibition. Knockdown of AXL using small interfering RNA (siRNA) increased sensitivity to doxorubicin, vincristine, and paclitaxel in doxorubicin-resistant breast cancer and CML cell lines and abrogated cell migration and invasion induced by treatment with 5-fluorouracil in colon cancer cell lines [48,108]. MERTK knockdown with shRNA also sensitized B-ALL cell lines to dexamethasone, methotrexate, vincristine, and L-asparaginase, resulting in synergistic induction of cell death and increased apoptosis [50]. In T-ALL cell lines, shRNA-mediated MERTK knockdown sensitized cells to cytarabine, etoposide, 6-mercaptopurine, and methotrexate and increased apoptosis in response to treatment with cytarabine, 6-mercaptopurine, and methotrexate [51]. Inhibition of MERTK and AXL increased the sensitivity of astrocytoma cell lines to temozolomide, carboplatin, and vincristine [59] and

MERTK knockdown sensitized GBM cells to etoposide-induced apoptosis [60]. In addition, expression of MERTK was induced in GBM cell lines upon exposure to lomustine, cisplatin, temozolomide, etoposide, or irradiation [60,110]. In NSCLC, shRNA-mediated knockdown of AXL or MERTK increased sensitivity to carboplatin and cisplatin (AXL and MERTK) and etoposide and doxorubicin (AXL only) [58]. In hepatocellular carcinoma and breast cancer models, microRNA-mediated AXL inhibition increased sensitivity to cisplatin and paclitaxel, respectively [111,112]. Finally, AXL inhibition in pancreatic ductal adenocarcinoma enhanced radiation-induced apoptosis [113]. An additional study demonstrated increased sensitivity to methotrexate in an orthotopic B-cell ALL model in response to treatment with a MERTK tyrosine kinase inhibitor (TKI), leading to decreased tumor burden and increased survival in mice treated with combination therapy compared to mice treated with either single agent [114]. Decreased tumor growth was also observed in response to siRNA-mediated AXL inhibition in subcutaneous breast cancer and CML xenograft models generated from doxorubicin-resistant derivative cell lines, although in this case the decrease was independent of treatment with doxorubicin [108].

One major mechanism by which TAM RTK signaling mediates resistance to traditional cytotoxic chemotherapy is by downstream activation of pro-survival pathways. As described above, inhibition of MERTK or AXL signaling sensitizes cancer cells to treatment with chemotherapy in a variety of tumor types, both *in vitro* and *in vivo* (Table 3.1). These data implicate TAM RTKs as attractive targets for therapeutic inhibition in conjunction with cytotoxic agents that are currently the standard of care in many cancers.

3.3.2 Role of TAM RTKs in Mediating Resistance to Targeted Therapies

In addition to their roles in resistance to cytotoxic chemotherapy, TAM RTKs are important in mediating resistance to targeted kinase inhibitor therapies in a number of different tumor types (Table 3.2). Information regarding this aspect of TAM RTK biology is primarily related to AXL and was first demonstrated in response to treatment with BCR-ABL inhibitors. Several studies demonstrated AXL upregulation in CML and gastrointestinal stromal tumor (GIST) cell-line derivatives selected for resistance to imatinib [115], nilotinib [116], or PD-166326 [115] and in tumor samples collected from patients with imatinib-resistant GISTs [117] or CML [116]. In GISTs, both AXL and GAS6 overexpression have been associated with imatinib resistance. Knockdown of AXL with siRNA restored imatinib sensitivity, and transfection of AXL into an imatinib-sensitive CML cell line conferred protection against the antileukemic effects of imatinib, such as alteration of cell metabolism and induction of apoptosis [115]. Molecular modeling indicated that imatinib does not bind to AXL, suggesting that resistance is not mediated by off-target competition for inhibitor binding [117]. Further supporting this hypothesis, several studies have directly demonstrated weak or no binding of AXL and imatinib [118–120]. The mechanism of AXL upregulation in response to BCR-ABL inhibition in CML may be mediated through CBL, an E3 ubiquitin ligase that stabilizes AXL protein and mRNA [116]. Depletion of CBL in nilotinib-sensitive cells increased AXL mRNA and protein; conversely, forced expression of CBL in nilotinib-resistant cells decreased AXL expression and resensitized the cells to nilotinib. Other studies examining AXL in CML have implicated PKCα and β as regulators of AXL expression based on the observation that inhibition of PKC in imatinib-resistant cells via a small-molecule inhibitor or siRNA silencing leads

TABLE 3.1 TAM Receptor-Mediated Resistance to Conventional Therapies

TAM receptor	Cancer type	Therapy	References
AXL	AML	Cisplatin	[79]
		Cytarabine	[79]
		Doxorubicin	[79]
		Etoposide	[79]
	Astrocytoma	Carboplatin	[59]
		Temozolomide	[59]
		Vincristine	[59]
	Breast	Doxorubicin	[83,108]
		Paclitaxel	[108,112]
		Vincristine	[108]
	CML	Doxorubicin	[108]
		Paclitaxel	[108]
		Vincristine	[108]
	Colon	5-FU	[48]
		Doxorubicin	[83]
	Esophageal	Cisplatin	[109]
	Hepatocellular carcinoma	Cisplatin	[111]
	Melanoma	Doxorubicin	[83]
	Neural	Doxorubicin	[83]
	Nasopharyngeal	Doxorubicin	[83]
	NSCLC	Carboplatin	[152]
		Cisplatin	[58]
		Doxorubicin	[58,83]
		Etoposide	[58,83]
		Paclitaxel	[152]
	Ovarian	Cisplatin	[107]
	Pancreatic	Doxorubicin	[83]
		Irradiation	[113]
	Prostate	Doxorubicin	[83]
	Renal cell carcinoma	Doxorubicin	[83]

(*Continued*)

TABLE 3.1 TAM Receptor-Mediated Resistance to Conventional Therapies (*cont.*)

TAM receptor	Cancer type	Therapy	References
MERTK	ALL	6-MP	[51]
		Cytarabine	[51,77]
		Dexamethasone	[7,50]
		Etoposide	[51]
		L-asparaginase	[50]
		Methotrexate	[50,51,114]
		Vincristine	[50]
	Astrocytoma	Carboplatin	[59]
		Temozolomide	[59]
		Vincristine	[59]
	GBM	Cisplatin	[110]
		Etoposide	[60]
		Irradiation	[60,110]
		Lomustine	[110]
		Temozolomide	[110]
	NSCLC	Carboplatin	[58]
		Cisplatin	[58]

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CML, chronic myeloid leukemia; NSCLC, non-small cell lung cancer; GBM, glioblastoma multiforme.

to decreased AXL protein levels [115]. Together, these data suggest that PKCα/β mediates increased transcription of AXL, and CBL stabilizes AXL mRNA and protein, leading to overall increased AXL expression and consequent increased downstream survival signaling as a mechanism of resistance to BCR-ABL targeted therapies in CML [12].

AXL also plays a role in resistance to EGFR-family inhibitors. AXL expression was upregulated in EGFR-mutated NSCLC cell-line derivatives and xenografts with acquired resistance to erlotinib, an EGFR inhibitor, and AXL overexpression was both necessary and sufficient to confer resistance [121]. AXL inhibition with shRNA restored erlotinib sensitivity *in vivo*, and treatment with AXL inhibitors restored sensitivity to erlotinib and mediated synergistic antitumor activity in combination with erlotinib treatment in cell-line models. Moreover, upregulation of AXL was also observed in 20% of tumor samples collected from patients with NSCLC after development of resistance to erlotinib compared to matched pretreatment samples, demonstrating the clinical relevance of AXL upregulation as a resistance mechanism. In erlotinib-resistant NSCLC cell lines, activation of downstream signaling through ERK, AKT, and RELA (a subunit of NF-κB) was EGFR independent, suggesting AXL-mediated bypass signaling as a mechanism of resistance [121]. In addition, AXL and EGFR co-immunoprecipitate, suggesting the potential for heterodimerization [37,122]. Overexpression of AXL was also demonstrated in NSCLC cell lines and patient-derived xenografts resistant to cetuximab, and in this context EGFR directly regulates *AXL* mRNA expression via mitogen-activated

TABLE 3.2 TAM Receptor-Mediated Resistance to Targeted Therapies

TAM receptor	Cancer type	Therapy	References
AXL	AML	Midostaurin (FLT3)	[126]
		Quizartinib (FLT3)	[126]
	Breast	Erlotinib (EGFR)	[37]
		Lapatanib (HER2)	[39]
		PD1 inhibition	[143,144]
	CML	Imatinib (BCR-ABL)	[115,116]
		Nilotinib (BCR-ABL)	[116]
		PD-166326 (BCR-ABL)	[115]
	Colon	PD1 inhibition	[145]
	GIST	Imatinib (BCR-ABL)	[115]
		Nilotinib (BCR-ABL)	[116]
		PD-166326 (BCR-ABL)	[115]
	HNSCC	PI3K/AKT inhibition	[132]
	Melanoma	BRAF/MEK inhibition	[97]
		MEK inhibition	[127,128]
		NRAS inhibition	[99]
		PD1 inhibition	[140]
	Neuroblastoma	ALK inhibition	[133,134]
	NSCLC	ALK inhibition	[131]
		Cetuximab (EGFR)	[123]
		Erlotinib (EGFR)	[121,152]
		FGFR inhibition	[130]
		VEGF inhibition	[152]
	Renal cell carcinoma	Sunitinib	[47]
	Rhabdomyosarcoma	IGF-1R inhibition	[129]
MERTK	Breast	PD1 inhibition	[143,144]
	Colon	PD1 inhibition	[145]
	NSCLC	Erlotinib (EGFR)	[124]

Abbreviations: AML, acute myeloid leukemia; CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung cancer.

protein kinase (MAPK) signaling and the transcription factor c-JUN [123]. Furthermore, cetuximab-sensitive parental cells were rendered resistant by overexpression of AXL. AXL expression has also been associated with relative resistance to EGFR inhibitors in triple-negative breast cancer cell lines [37,122] and is overexpressed in estrogen receptor-positive breast cancer cell lines that express HER-2, an EGFR-family member, but are resistant to lapatinib,

an HER-2 inhibitor [39]. In this context, AXL and HER3 heterodimerized to bypass HER-2 signaling inhibition mediated by lapatinib and treatment with foretinib (a multikinase inhibitor targeting AXL, MET, and VEGFR) or AXL siRNA restored lapatinib sensitivity. Although roles for MERTK in resistance to EGFR-targeted therapy have not been thoroughly explored, overexpression of MERTK in an NSCLC cell line was sufficient to confer erlotinib resistance, and treatment with an MERTK kinase inhibitor reversed this phenotype [124]. Further, it was recently demonstrated that MERTK and EGFR co-immunoprecipitate in NSCLC cells, suggesting heterodimerization [125]. Additionally, the combination of small-molecule inhibitors targeting MERTK and EGFR synergized to inhibit cell expansion in NSCLC cell lines.

In AML with an fms-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) mutation, AXL expression was necessary for constitutive activation of FLT3 [56], and both total AXL and pAXL were increased in FLT3-ITD AML cell lines and patient samples resistant to the FLT3-targeted TKI midostaurin [126]. In addition, treatment with the FLT3-targeted TKIs quizartinib and midostaurin increased pAXL, consistent with a role for AXL in TKI resistance in this model. Further validating this hypothesis, inhibition of AXL with a small-molecule inhibitor, shRNA, or GAS6 ligand-trap resensitized resistant AML cell lines to FLT3-targeted TKIs.

Overexpression of AXL mRNA has also been described in BRAF- and NRAS-mutated melanoma cell lines and patient samples that are resistant to BRAF/MEK inhibitors [97,99]. In this context, two distinct populations of melanoma cells expressing high levels of either AXL or MITF were observed at baseline, but upon relapse after BRAF ± MEK inhibition tumor cells predominantly expressed high levels of AXL [97]. This observation suggests that AXL-expressing cells present from the outset may harbor intrinsic resistance leading to inevitable relapse. Additional studies revealed decreased extracellular shedding of numerous kinase receptors, including AXL, in response to treatment with an MEK inhibitor, leading to increased receptor expression on the surface of tumor cells and consequent increased downstream signaling [127]. This alternative mechanism to increase TAM RTK signaling may be particularly important in patients with melanoma, where progression-free survival following treatment with BRAF/MEK inhibitors is correlated with high serum levels of soluble TAM RTKs before treatment and reduced shedding of TYRO-3, AXL, and MERTK post-treatment [127,128].

Roles for AXL in resistance to other kinase inhibitors have also been described, but have been less well studied. In a derivative of the Rh41 rhabdomyosarcoma cell line selected for resistance to treatment with an anti-insulin-like growth factor 1 receptor (IGF-1R) monoclonal antibody, AXL mRNA and protein were overexpressed compared to parental cells [129]. Interestingly, AXL upregulation was not observed in Rh41 rhabdomyosarcoma cells selected for resistance to a TKI targeting IGF-1R, demonstrating differential mechanisms of resistance to therapeutic agents with different modes of action in the same cell line. Upregulation of AXL has also been observed in NSCLC cell lines with acquired resistance to fibroblast growth factor receptor (FGFR) [130] and anaplastic lymphoma kinase (ALK) [131] inhibitors and in head and neck and esophageal squamous cell carcinomas, where AXL forms a heterodimer with EGFR and induces PKC/mTOR pathway signaling to mediate resistance to PI3K/AKT pathway inhibition [132]. Ligand-dependent activation of AXL decreased sensitivity of ALK-mutated neuroblastoma cells to crizotinib, an ALK TKI, and AXL inhibition via siRNA restored the activity of crizotinib [133]. Additionally, AXL inhibition with a small-molecule inhibitor increased apoptosis in response to treatment with crizotinib in ALK-mutated neuroblastoma

cell lines and enhanced the efficacy of ALK inhibition *in vivo*. In ALK-mutated neuroblastoma cells resistant to crizotinib, ligand-dependent AXL activation conferred resistance to the ALK inhibitor ceritinib through upregulation of ERK signaling [134]. Chronic treatment of renal cell carcinoma cell lines with the anti-angiogenic agent sunitinib activated AXL and increased cell invasion, and AXL inhibition with cabozantinib impaired metastatic behavior *in vitro* and rescued acquired sunitinib resistance *in vivo* [47].

3.3.3 Role of TAM RTKs in Immunosuppression

Another mechanism by which TAM RTKs promote therapeutic resistance in cancer is through modulation of the immune system. Tumors arise from "self" tissue and in order to mount an effective antitumor immune response tolerance in the adaptive immune system must be overcome. Immune checkpoint therapeutics represent one approach that has been used successfully, with a goal of reactivating $CD8^+$ cytotoxic T-cells to mediate tumor cell killing. The innate immune system, however, has a built-in "braking" system mediated by TAM RTKs that functions to reduce inflammation and resulting tissue damage in the context of physiologic tissue remodeling and wound healing [15]. AXL and MERTK bind interferon (IFN) α receptors to promote downstream signaling via STAT1 and activate transcription of suppressor of cytokine signaling (SOCS) proteins, which inhibit JAK [15], thereby mediating a negative feedback loop to dampen inflammation [16]. Furthermore, activation of TAM RTKs in macrophages leads to a switch from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype [135]. M2 macrophages are unable to activate $CD8^+$ cytotoxic T-cells, and increased incidence of M2 macrophages in the tumor microenvironment correlates with poor prognosis in some tumor types [136]. In addition, MERTK signaling in macrophages promotes biosynthesis of immunosuppressive long-chain fatty acid-derived lipids called specialized pro-resolving mediators [137]. Thus, physiologic TAM RTK signaling can lead to blunt innate and adaptive immune responses to cancer. On the contrary, inhibition of TAM RTKs promotes an M1 phenotype in macrophages, which then secrete pro-inflammatory cytokines leading to increased recruitment and/or activation of T-cells in the tumor microenvironment and reduced tumor incidence and growth in syngeneic mouse models [135]. These data validate TAM RTKs as potential therapeutic targets in the tumor microenvironment.

In an effort to overcome adaptive immune tolerance in the tumor microenvironment, immune checkpoint therapeutics targeting programmed cell death protein 1 (PD1) or its ligand (PD-L1) have recently been developed and have shown promise in clinical trials (reviewed in [138]). The PD1 immune checkpoint pathway mediates suppression of cytotoxic T-cells, and PD-L1 and PD-L2 ligands can be expressed on tumor cells to inhibit antitumor immunity [139]. Interestingly, intrinsic resistance to PD1-targeted therapy is associated with increased AXL expression in metastatic melanoma [140]. In addition, MERTK expression in an epithelial cell line was sufficient to induce expression of the checkpoint pathway ligands PD-L1 and PD-L2, and shRNA-mediated inhibition of MERTK suppressed PD-L1 expression in a breast cancer cell line [141]. Similarly, in an NSCLC cell line, PtdSer binding to TAM RTKs mediated upregulation of PD-L1 expression and AKT-dependent therapeutic resistance [142]. Together, these data suggest that therapeutic strategies combining TAM RTK inhibition with immune checkpoint inhibitors may be particularly effective for induction of antitumor immunity. Indeed, several recently

reported studies demonstrated synergistic inhibition of tumor growth in mouse models of colon and breast cancer upon dual inhibition of AXL and the PD1 or CTLA4 checkpoint pathways [143–145], lending support to this hypothesis.

3.4 TARGETING AXL AND MERTK

3.4.1 Agents and Approaches to Inhibit TAM RTKs

As the TAM RTKs represent attractive therapeutic targets across a wide range of malignancies, there has been significant effort to identify targeting methods with the potential for clinical translation. Several strategies have been used to target the TAM RTKs. These include disruption of ligand-receptor binding using antibodies or ligand traps, inhibition of kinase activity with ATP-competitive small-molecule inhibitors, and antibody-mediated downregulation of cell-surface TAM RTK expression. One group has used an aptamer approach to target AXL [146] and others have developed ligand traps [21,56,147–149] consisting of the TAM RTK extracellular domain fused to the Fc domain of human IgG, including an AXL decoy receptor that binds GAS6 with higher affinity than AXL itself and abrogates AXL signaling [150,151]. Monoclonal antibodies targeting the extracellular domains of TYRO3, AXL, and MERTK have all been developed. Antibody binding inhibits TAM RTK activity either through disruption of ligand binding [105,152] or through increased receptor internalization and subsequent degradation [153,154]. Several groups have used anti-AXL antibodies in preclinical studies in Kaposi sarcoma, NSCLC, and pancreatic cancer models [80,152,154]. RU-301 and RU-302 bind the extracellular Ig-1 ectodomain of AXL and inhibit binding of GAS6, and modeling suggests they will bind MERTK and TYRO3 as well [155]. Two anti-AXL antibodies [151,156] have been used to generate antibody–drug conjugates (ADCs) and have shown promising results in preclinical studies [156,157]. An antibody targeting MERTK cell-surface expression has also been described and mediates decreased migration in glioblastoma cells [158] and decreased colony-forming potential and therapeutic resistance in NSCLC cells [153]. The most common approach to TAM RTK inhibition, and the tool most likely to translate to clinical application, is the use of ATP-competitive small-molecule TKIs. The first TKIs with TAM RTK-inhibitory activity were multikinase inhibitors. Due to similar ATP binding sites between TAM RTKs and MET, many TKIs target both. Both TAM RTKs and MET play roles in therapeutic resistance, especially in NSCLC, so there may be some advantage to targeting both receptors [16]. The multikinase inhibitor sitravatinib has a low IC_{50} for both AXL and MERTK and was superior to imatinib and crizotinib in preclinical sarcoma models [159]. Clinical trials are ongoing to test the multikinase inhibitors cabozantinib [160], sitravatinib [161], and glesatinib [161] in NSCLC patients, including those with high expression of or genetic changes in AXL. Sitravatinib and glesatinib are being used in combination with the PD-1 inhibitor nivolumab with the goal of bypassing adaptive immune tolerance, as described above [161]. Sitravatinib is also being tested as a single agent in patients with advanced solid tumors demonstrating genetic changes in a number of RTKs, including AXL [162].

There are also several AXL or MERTK selective inhibitors in preclinical and clinical development (Table 3.3 and reviewed in [4,12,16]). The AXL inhibitor TP-0903 has been used in preclinical studies in CLL [163,164], AML [126], and SLC-0211, another AXL

TABLE 3.3 TAM-Selective Targeted Therapies Currently in Development

			Inhibitory activity (IC_{50})						
			AXL		MERTK				
Compound	**Mechanism**	**Tumor types**	**Enzymatic**	**Cell-based**	**Enzymatic**	**Cell-based**	**Phase**	**Other kinase targets**	**References**
CAB-AXL-ADC	ADC	Solid tumors	*	*	*	*	Preclinical	N/A	[157]
HuMax-AXL-ADC	ADC	Solid tumors	*	*	*	*	I	N/A	[156]
Aravive-S6 (MYD1–72)	Ligand trap	AML	*	*	*	*	Preclinical	N/A	[151]
BGB324	TKI	AML, NSCLC, melanoma	14 nM	14 nM	220 nM	700 nM	I/II	RET, VEGFR, FLT3, ABL, TIE2	[78,175]
Gilteritinib (ASP2215)	TKI	NSCLC, AML	0.7 nM	*	2.9 nM	*	II	FLT3, ALK, LKT	[176,177]
MRX-2843	TKI	AML	15 nM	*	1.3 nM	*	Preclinical	FLT3	[169]
TP-0903	TKI	Solid tumors	27 nM	222 nM	<200 nM	*	I	AURKA, AURKB, JAK2, ALK, ABL1	[178]
UNC2025	TKI	ALL, AML	14 nM	122 nM	0.74 nM	2.7 nM	Preclinical	FLT3, TYRO3	[114,179]

Abbreviations: ADC, antibody–drug conjugate; ALK, anaplastic lymphoma kinase; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AURK, aurora kinase; FLT3, fms-like tyrosine kinase 3; IC_{50}, half maximum inhibitory concentration; JAK, Janus kinase; NSCLC, non-small cell lung cancer; RET, rearranged during transfection; TKI, tyrosine kinase inhibitor; TAM, TYRO3, AXL, and MERTK; VEGFR, vascular endothelial growth factor receptor.
* *Information not published.*

inhibitor, has been effective in NSCLC and CML animal models [16,165]. The AXL inhibitor BGB324 is currently being tested in phase I/II trials in adults with NSCLC and metastatic melanoma and a phase I trial in adults with AML. Preliminary data indicate that the compound was well tolerated and demonstrated an antileukemic effect. Importantly, biologic correlates demonstrated decreased activation of AXL and downstream signaling through ERK and AKT in leukemic blasts after 21 days of treatment [166]. Additional preclinical studies demonstrated therapeutic activity in CML, irrespective of sensitivity to BCR-ABL inhibition [167]. Gilteritinib, a dual FLT3 and AXL inhibitor, is currently being studied in AML clinical trials [160]. At the time of this writing, there are no clinical trials of MERTK-selective TKIs ongoing; however, there are robust preclinical data to support further development of several compounds. UNC2025 and MRX-2843 are dual MERTK and FLT3 inhibitors that were effective as monotherapy in preclinical models of leukemia [168,169], NSCLC [153], and GBM [110] and mediated synergistic antileukemia activity in combination with methotrexate [168].

3.4.2 TAM RTKs as Dual Targets

The roles for TAMs in promoting tumor cell survival and resistance to both targeted and cytotoxic chemotherapies coupled with their roles in immunosuppression in the tumor microenvironment suggest that they could be effective therapeutic targets in many human cancers. Given the context-dependent expression of TAM RTKs, optimum targeting could be modified based on the tumor type. For example, dual targeting of AXL and MERTK could be considered in NSCLC where both are expressed and have independent roles in tumor progression [58], whereas in pediatric ALL, an MERTK-selective approach might be better as AXL is not expressed in this setting [50,51]. Similarly, subtypes of colon cancer and triple-negative breast cancer with a mesenchymal phenotype overexpress AXL, providing a rationale for targeting AXL in combination with chemotherapy in patients with these tumors as a tool to overcome innate resistance associated with the mesenchymal phenotype [170–172].

3.4.3 TAM RTK Biomarkers

One of the challenges in the use of TAM-targeted therapeutics is the lack of identified robust biomarkers. The signaling pathways downstream of TAM kinases can be regulated by a number of other RTKs, making them less valuable as biomarkers. In addition, the signaling pathways downstream of TAM kinases are tissue dependent, further complicating the identification of biomarkers of TAM inhibition that can be applied across multiple tumor types. Because TAM RTK activity is regulated by ligands, their activity also varies in different tumor contexts, e.g., GAS6 can be secreted by bone marrow stromal cells to activate TAM RTK signaling in leukemia [77] or can be affected by the tumor organizational structure in solid tumors [173]. In current clinical trials, AXL expression and activation are measured by assessing AXL protein levels and AXL phosphorylation at Tyr702 [16]. Interestingly, some inhibitors decrease phosphorylation of AXL at the Tyr 702 site [47] and others such as BGB324 [78] and S49076 [174] do not, a finding which remains of undetermined significance. Development of robust biomarkers of TAM RTK inhibition will be critical for effective clinical application of TAM RTK inhibitors and is an active area of research in the field.

3.5 SUMMARY

Resistance to both conventional and targeted therapy represents a significant challenge in the treatment of cancer. Further studies to explore mechanisms of resistance and identification of drivers of resistance will allow for the development of additional agents targeting drug-resistant tumor cell populations. The TAM receptors have been identified as key mediators of resistance to both cytotoxic chemotherapy and targeted agents across a wide range of malignancies. TAM RTKs provide bypass signaling to facilitate resistance to targeted therapies and function to attenuate the immune response to cancer. As such, they represent attractive therapeutic targets. A number of TAM-selective TKIs have been developed. Clinical trials of AXL-selective agents are ongoing and MERTK-selective TKIs have demonstrated promising activity in preclinical studies. Development of adequate biomarkers of TAM RTK inhibition will be critical to facilitate optimal clinical application of these inhibitors. The current data suggest that treatment with TAM RTK inhibitors in combination with conventional chemotherapy, targeted agents, or immune checkpoint inhibitors will prove useful to overcome or prevent therapeutic resistance in a variety of tumor types.

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