THERAPEUTIC RESISTANCE — OPINION

Environment-mediated drug resistance: a major contributor to minimal residual disease

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Abstract | Environment-mediated drug resistance is a form of *de novo* drug resistance that protects tumour cells from the initial effects of diverse therapies. Surviving foci of residual disease can then develop complex and permanent acquired resistance in response to the selective pressure of therapy. Recent evidence indicates that environment-mediated drug resistance arises from an adaptive, reciprocal signalling dialogue between tumour cells and the surrounding microenvironment. We propose that new therapeutic strategies targeting this interaction should be applied during initial treatment to prevent the emergence of acquired resistance.

Resistance to tumour therapy can be subdivided into two broad categories: *de novo* and acquired. Acquired resistance develops over time as a result of sequential genetic changes that ultimately culminate in complex therapy-resistant phenotypes. Conversely, one form of *de novo* drug resistance is environment-mediated drug resistance (EMDR), in which tumour cells are transiently protected from apoptosis induced by either chemotherapy, radiotherapy or receptor-mediated cell death[1,2]. This form of drug resistance is rapidly induced by signalling events that are initiated by factors present in the tumour microenvironment and can be subdivided into two categories: soluble factor-mediated drug resistance (SFM-DR), which is induced by cytokines, chemokines and growth factors secreted by fibroblast-like tumour stroma; and cell adhesion-mediated drug resistance (CAM-DR), which is mediated by the adhesion of tumour cell integrins to stromal fibroblasts or to components of the extracellular matrix (ECM), such as fibronectin, laminin and collagen (FIG. 1). As a continuation of this theme, Cordes *et al.* have coined the analogous term CAM-RR to refer to cell adhesion-mediated resistance to radiotherapy[3].

Whereas SFM-DR is primarily mediated by the induction of gene transcription, CAM-DR is mediated largely, but not entirely, by non-transcriptional mechanisms. Non-transcriptional mechanisms include the degradation of activators of apoptosis[4] or subcellular redistribution[5] and increased stability of suppressors of apoptosis and cell cycle regulators[6].

The main obstacle to effective treatment is the failure of initial cancer therapy to eradicate a sufficient number of tumour cells to prevent disease recurrence, which significantly affects long-term survival. This population of surviving cells following therapy is called minimal residual disease (MRD), and these cells can go on to find refuge in protective microenvironments. For example, the presence of bone marrow micrometastasis in around 30% of patients with breast cancer at the time of diagnosis is a strong predictor of relapse, despite aggressive treatment[7,8], and 15–20% of patients still have disseminated tumour cells in the bone marrow following treatment[9,10]. The selective pressure of therapy eventually leads to the development of acquired resistance in these surviving cells and the outgrowth of MRD, causing disease relapse. EMDR contributes substantially to MRD[11] and to the development of acquired resistance by protecting tumour cells from therapy until they evolve acquired-resistance phenotypes (FIG. 2).

To understand the mechanistic differences between CAM-DR, which is a component of EMDR, and acquired resistance, Hazlehurst *et al.* used an *in vitro* cell culture model of myeloma drug resistance to compare their respective gene expression profiles. In this study, a myeloma cell line selected in the absence of ECM for acquired resistance to melphalan[12] was compared with a drug-sensitive parental cell line that was adhered to the ECM component fibronectin to induce CAM-DR[13]. Although the two types of resistance protected tumour cells from melphalan-induced apoptosis at equivalent levels, at the transcriptional level acquired resistance was more complex than CAM-DR when these two groups were compared with sensitive parental cells. Oligonucleotide microarray analysis showed that the acquired-resistance phenotype was associated with 1,479 gene expression changes, compared with only 69 for CAM-DR[13]. This example is consistent with the non-transcriptional mechanisms that have been described for CAM-DR and suggests that treatment strategies could more efficiently target the less complex CAM-DR phenotype at earlier stages of disease, before the development of acquired resistance.

In recognition of the important role of EMDR in treatment failure, research is increasingly focusing on therapeutic strategies that target its pathways in the tumour microenvironment[2]. Importantly, the EMDR phenotype is transient, appearing only while the tumour cells are in contact with the microenvironment, and they rapidly revert to drug sensitivity when removed from the microenvironment. Therefore, in an effort to limit MRD, compounds that block key EMDR pathways that are mediated by integrins and soluble factors are now entering clinical trials. Nevertheless, limited clinical studies have used these compounds as secondary treatments, with the goal of overcoming resistance after

primary therapy. Because EMDR precedes MRD and the development of acquired resistance, these new strategies would probably be more effective if used as an initial, preventive treatment in conjunction with traditional therapy. In this Perspective, we outline the preclinical evidence of EMDR and describe its molecular mechanisms, with emphasis on treatment strategies. In doing so, we highlight recent evidence that advocates focusing research and interventions on common pathways that mediate stroma–tumour communication and are the basis of EMDR.

Minimal residual disease

The presence of surviving tumour cells immediately after therapy suggests that they must be protected by some form of *de novo* drug resistance, because acquired drug resistance takes time to develop, as indicated by *in vitro* and *in vivo* models of acquired drug resistance[12,14]. There are two types of *de novo* resistance: intrinsic and extrinsic. Intrinsic *de novo* resistance is thought to be caused by pre-existing random genetic mutations that are selected for through selective pressures imposed by drugs when these mutations offer a survival advantage (as first shown by the Luria–Delbrück experiment)[15]. Because of the complexity of acquired resistance, further mutations may be needed in addition to these intrinsic factors to produce highly resistant phenotypes. By contrast, extrinsic factors such as the mediators of EMDR could protect tumour cells that contain intrinsic mutations while other mutations develop (FIG. 2). Therefore, a combination of both types of *de novo* resistance may be required to generate acquired and more complex resistant phenotypes (BOX 1).

In the past decade, sensitive techniques have allowed researchers to detect single disseminated tumour cells in the early phases of disease, enabling MRD levels in patients to be monitored with a high degree of sensitivity and reproducibility. The most sophisticated of these techniques, quantitative PCR amplification of specific genetic tumour markers and the detection of abnormal marker patterns by flow cytometry, have been developed to monitor the haematological malignancies known as acute lymphoblastic lymphoma (ALL) and acute myeloid leukaemia (AML)[16,17]. For this reason, MRD has been studied primarily in these malignancies. The presence of MRD above a certain threshold after primary therapy predicts relapse. For example, in ALL the presence of MRD above a threshold of one leukaemic cell per 1,000 bone marrow cells after primary therapy correlates with a high probability of relapse, and among patients who relapse the level of MRD is inversely proportional to the length of remission[18]. Similarly, MRD levels in the bone marrow also correlate with markedly decreased relapse-free survival in patients with AML after each of three rounds of chemotherapy[16]. Disseminated epithelial tumour cells can also be isolated from the bone marrow of patients with breast or gastric cancer after therapy and before the onset of clinical metastatic disease[7,9,19], and their presence correlates with poor survival. Because of the strong correlation between MRD level and relapse, treatment of patients with ALL and AML who have higher MRD levels is currently intensified to decrease the probability of relapse[20,21]. However, to date most strategies do not consider the microenvironmental factors that cause MRD, such as EMDR. A better understanding of how these factors contribute to therapeutic resistance and MRD would lead to more effective therapeutic interventions to avoid treatment failure.

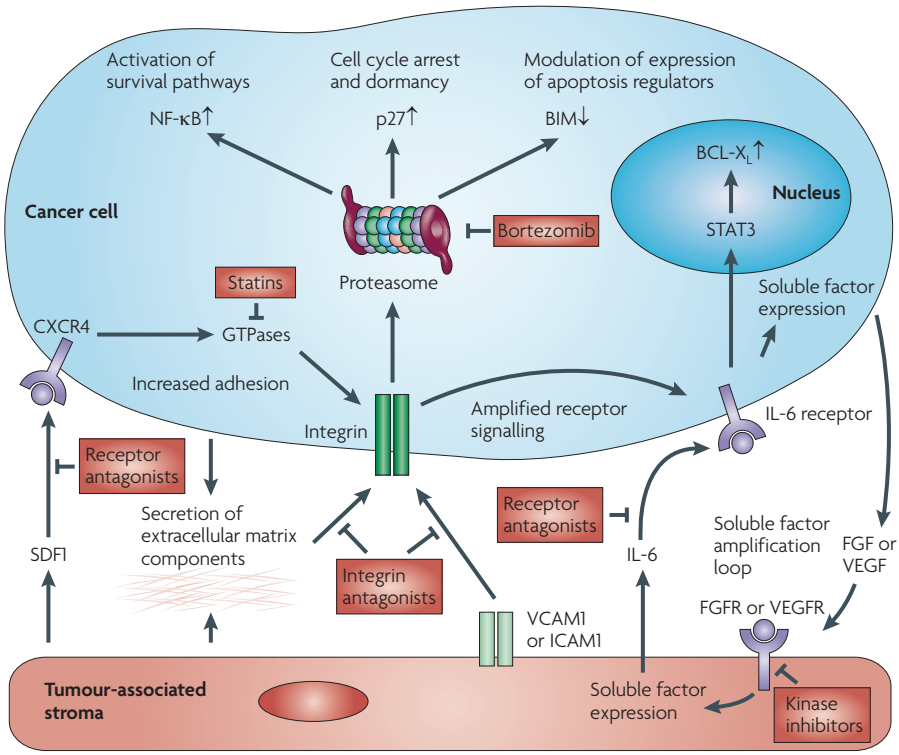

Figure 1 | **Tumour–stroma communication is the basis of EMDR.** Dynamic signalling interactions between tumour cells and mesenchymal stroma in the microenvironment induce a transient, resistant state that protects tumour cells from therapy by inducing the environment-mediated drug resistance (EMDR) phenotype. Integrins on tumour cells bind to fixed extracellular matrix components secreted by both tumour cells and stroma, and to receptors expressed on stroma, such as vascular cell adhesion protein 1 (VCAM1). This adhesion of haematopoietic and epithelial tumour cells induces quiescence and modulates the regulation of pro- and anti-apoptotic molecules, conferring cell adhesion-mediated drug resistance (CAM-DR) in microenvironments.Although CAM-DR is mediated largely by proteasome-dependent mechanisms[4,6,48,49,96], the mechanisms by which adhesion modulates proteasome activity are not understood. A paracrine amplification loop of soluble factors secreted by both tumours and stroma induce cell proliferation[65], the upregulation of anti-apoptotic molecules[55] and increased adhesion[50,63,92]. As a result of interactions between tumour cells and stroma, the expression patterns of soluble factors, integrins and extracellular matrix proteins change with tumour progression to increase EMDR[32,62,79]. Therapeutic strategies designed to disrupt EMDR and limit minimal residual disease include integrin and soluble factor antagonists that function extracellularly, as well as inhibitors of downstream intracellular resistance pathways in both tumour cells and their associated stroma (indicated in red). BCL-X_L, BCL2-like protein 1; BIM, BCL2-interacting mediator of cell death; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; ICAM1, intercellular adhesion molecule 1; IL-6, interleukin 6; NF-κB, nuclear factor-κB; SDF1, stromal cell-derived factor 1; STAT3, signal transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Preclinical studies. Preclinical experiments in mouse models have been used to study the mechanisms of MRD. Results from these studies indicate that specific EMDR mechanisms contribute to MRD and can be directly targeted. The concept of EMDR is exemplified by the work of Teicher *et al.*, who used a mouse model of chemotherapy resistance to show that tumours develop complex resistance mechanisms *in vivo* that are crucially dependent on their interaction with host factors[14]. In this system, mouse mammary tumour cells that developed resistance by passage through drug-treated mice lost their resistant phenotype when cultured *in vitro*.

One specific mechanism of EMDR is CAM-DR, which relies on the integrin-mediated adhesion of tumour cells with microenvironmental factors such as the ECM and ligands expressed on stromal cells. In studies that compared integrin expression in sensitive and drug-resistant myeloma cell lines, Damiano[1] showed that α4β1 expression was increased in myeloma cells with acquired resistance. Similarly, α4β1 integrin was shown to be important for the development of MRD by correlating results from a mouse model of AML MRD with the clinical outcome of 25 patients with AML[11]. Blocking this interaction *in vivo* led to a 100% survival rate in mice treated with cytosine arabinoside, whereas treatment with the drug alone only modestly increased survival. In patients with AML undergoing chemotherapy, high expression of α4β1 integrin correlated with disease relapse and decreased survival, whereas α4β1-negative patients had relapse rates of 0% and survival rates of 100%[11]. Later work by this group showed that *ex vivo* abrogation of α4β1-mediated adhesion in specimens from patients with AML could overcome CAM-DR[22]. These results were consistent with a much larger study of 175 specimens from patients with AML who underwent induction chemotherapy with cytarabine and anthracyline. That study not only demonstrated α4β1 integrin-mediated drug resistance in patient specimens *ex vivo*[23] but also found significantly increased α4 integrin expression in secondary compared with newly diagnosed AML. This intriguing result suggests that expression of α4 integrin may have been selected during disease treatment, highlighting the importance of integrin-mediated adhesion in MRD.

Work linking CAM-DR to MRD in AML is consistent with extensive *ex vivo* patient data from a wide range of malignancies. Adhesion of patient specimens from both haematopoietic[11,13,22–24] and epithelial[25] tumour cells to immobilized ECM components induces CAM-DR. It is not surprising, then, that increased specific integrin expression by tumour cells is associated with poor prognosis in both solid and haematopoietic tumours[25–30]. Furthermore, ECM protein expression in the microenvironment of epithelial tumours *in vivo* also correlates with poor outcome[31,32], as does tumour cell expression of the integrin signalling intermediate focal adhesion kinase[33]. Collectively, these results link CAM-DR with MRD and intuitively link EMDR with the development of acquired drug resistance.

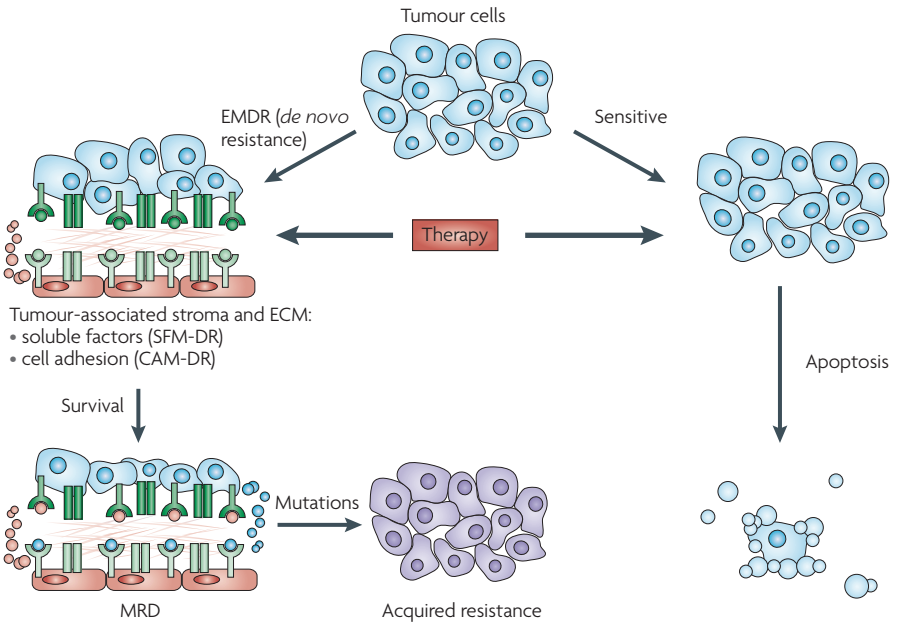

Figure 2 | **EMDR contributes to MRD and acquired drug resistance.** Factors that are present in tumour microenvironments induce environment-mediated drug resistance (EMDR) by two primary mechanisms: soluble factor-mediated drug resistance (SFM-DR) and cell adhesion-mediated drug resistance (CAM-DR). Most tumour cells succumb to therapy, but the interaction of a subset of tumour cells with microenvironmental factors allows them to survive the insult of therapy in a quiescent, protected state, resulting in minimal residual disease (MRD). Over time, genetic instability inherent in cancer cells combined with the strong selective pressure of therapy leads to successive, random genetic changes that cause the gradual development of more complex, diverse and permanent acquired-resistance phenotypes. These persistent tumour cells eventually cause disease recurrence and are much less likely to respond to subsequent therapy after acquired resistance develops. Therapeutic strategies that disrupt EMDR pathways would reduce the level of MRD and therefore reduce the emergence of acquired resistance. ECM, extracellular matrix.

Molecular profiling of patient specimens. Molecular profiling of MRD in patients would help to guide preclinical work and validate targets for further drug development by highlighting both intrinsic and extrinsic pathways of *de novo* drug resistance. For example, several groups have demonstrated that low expression of promoters of apoptosis and proliferation in ALL and AML tumour cells isolated from bone marrow is associated with higher levels of MRD in patients and, therefore, treatment resistance[34–36]. Because tumour cells were collected from patients at diagnosis and before treatment in these studies, gene expression patterns that are predictive of treatment failure can be used to target pathways in an effort to circumvent, rather than treat, resistance. Ideally, molecular profiles of MRD should also be determined after therapy and before relapse, when profiles unique to MRD are probably enriched. However, this approach is challenging because of the difficulty in examining sufficient numbers of surviving tumour cells after primary treatment. Nevertheless, the work by Flotho *et al.* showed that decreased expression of genes involved in proliferation in surviving ALL tumour cells 19 days after the initiation of induction therapy also correlated with MRD[37], consistent with expression profiles of naive tumour cells before therapy.

Information obtained from PCR-based approaches that are typically used to measure gene expression is limited, because these approaches identify only the transcriptional changes that occur in tumour cells, but

many pathways that mediate EMDR rely on post-translational mechanisms, such as protein degradation or subcellular localization, that are rapidly induced by integrin-mediated adhesion to microenvironmental factors. Therefore, preclinical experiments using *in vitro* cell culture and *in vivo* mouse models of EMDR have been used both to explore resistance signalling pathways identified by molecular profiling and to identify key factors contributing to EMDR that cannot be studied easily using patient specimens (TABLE 1). Importantly, findings from molecular profiling in patient MRD specimens are consistent with mechanistic studies in *in vitro* cell culture models of EMDR. These studies have determined that interactions with microenvironmental factors modulate the expression of regulators of apoptosis and proliferation in tumour cells to induce the EMDR phenotype.

Tumour–stroma coalition

The influence of stromal cells on tumour survival is often seen as the result of a passive relationship, but recent data have demonstrated that this relationship is more complex than previously thought[38]. Tumour cells and their stroma are exposed to the same physical and biological factors in the microenvironment. Therefore, tumour-associated stroma cells must also adapt to the stresses imposed by this harsh environment, including hypoxic and acidic conditions, as well as the insult of therapy. These physical factors can also contribute to resistance by restricting drug bioavailability or generating selective pressure that leads to the gradual development of genetic mutations or epigenetic changes, resulting in acquired drug resistance. We focus on the mechanisms by which biological factors produced by stroma, such as ECM and soluble factors, mediate EMDR. These physical and biological factors cause both malignant cells and their surrounding stroma to become increasingly abnormal during tumour progression and to develop a cooperative relationship that not only increases proliferation and survival of the two cell populations[39–42], but also leads to EMDR. In fact, recent work has demonstrated that stromal gene expression signatures can be a stronger predictor of clinical outcome in both haematological and epithelial malignancy than other factors[39,43]. Although various cell types, such as fibroblasts, endothelial cells and immune cells, associate with tumours and probably contribute to therapy resistance, the bulk of tumour stroma is composed of fibroblast-like cells. For this reason, this cell type has been the focus of therapy resistance studies to date and is the focus of this discussion. All microenvironments, from both solid and haematopoietic tumours, have common components that contribute to MRD and resistance to diverse therapies in a wide range of malignancies.

Cell adhesion-mediated drug resistance. Early work by Fridman *et al.* demonstrated the importance of microenvironmental factors in tumour biology, using a mouse model of small-cell lung cancer (SCLC). They showed that disruption of tumour adhesion to ECM and stromal cells by co-injection of tumour cells with a peptide that corresponded to the integrin-binding domain of the ECM component laminin led to a dramatic decrease in tumour load[44]. Later work by Mori *et al.* found that preventive use of a blocking antibody to α4 integrin alone could reduce tumour burden and bone destruction and increase overall survival in a mouse model of the haematopoietic malignancy multiple myeloma[45]. As impressive as their initial findings were, the potential of this anti-adhesion approach was not realized until therapeutic blockage of integrin binding was combined with the conventional cytotoxic melphalan. This combinatorial approach reduced tumour burden substantially more than either treatment alone[45]. Similarly, Park *et al.* showed that blockage of β1 integrin binding reduced tumour volume and increased radiotherapy in a xenograft model of SCLC[46,47]. These results demonstrate that integrin-mediated interactions of both epithelial and haematopoietic tumours with microenvironmental components lead to therapy resistance *in vivo* and indicate that targeting this major EMDR pathway can increase the effectiveness of traditional therapies.

Investigations using simple *in vitro* models of EMDR have been useful for delineating the specific molecular mechanisms of CAM-DR. For example, Damiano *et al.* and Sethi *et al.* showed that adhesion of myeloma[1] or SCLC cells[31] to the ECM components fibronectin, collagen and laminin confers a transient

Box 1 | **EMDR influences the development of acquired resistance**

The use of tumour cell lines that have been selected for acquired resistance to various chemotherapeutic agents has proven to be a useful tool for delineating drug resistance pathways. To date, these resistant cell lines have mostly been developed by drug selection in the absence of environmental factors and therefore may not reflect drug resistance phenotypes generated *in vivo*. For example, Hazlehurst *et al.* discovered that myeloma cells that showed cell adhesion-mediated drug resistance (CAM-DR) undergo DNA damage similarly to cells in suspension, whereas acquired-resistance phenotypes undergo less damage[13]. Hodkinson *et al.* confirmed these results in a small-cell lung cancer model of CAM-DR by showing that adhesion to extracellular matrix components does not affect chemotherapy- or radiotherapy-induced DNA damage or repair[51]. Therefore, DNA damage-inducing agents that are commonly used in chemotherapy may actually increase the DNA mutation rate in protected cancer cells, possibly leading to genetic changes that ultimately result in drug resistance.

Early work by Goldie *et al.* attempted to mathematically predict the probability that drug resistance phenotypes would develop during the course of tumorigenesis by analysing spontaneous mutation rates. They found that the probability and heterogeneity of populations with drug-resistant phenotypes increased drastically in the presence of a stem cell niche[15,102]. One could predict that environment-mediated drug resistance (EMDR), by maintaining a pool of protected cells, would have a similar effect on the generation of acquired-resistance phenotypes. Understanding this phenomenon is of crucial importance because it is one of the main obstacles to effective cancer treatment.

To determine whether EMDR could influence the development of acquired drug resistance, Hazlehurst *et al.* used a simple cell culture model to compare acquired drug resistance phenotypes that were allowed to develop either in the absence or presence of integrin-mediated adhesion. Intriguingly, they found that the presence of a single microenvironmental factor, the extracellular matrix component fibronectin, during the development of acquired drug resistance in a histiocytic lymphoma cell line results in levels of acquired resistance more than twofold higher than those developed in the absence of any microenvironmental factors[103]. These results demonstrate that EMDR not only protects tumour cells from therapy while they develop more complex acquired resistance phenotypes, but also directs the development of more highly resistant phenotypes. Future experiments of this nature should use more complex cell culture and *in vivo* tissue recombination models that more accurately reflect the complexity of *in vivo* microenvironments to study the mechanisms by which EMDR influences the development of acquired resistance.

Table 1 | **Experimental models of EMDR**

Model	Observed phenotype	Description of model	Refs
Isolated tumour cells	Susceptibility to cytotoxics, radiation and receptor-mediated cell death	Monoculture of tumour cells	1,3–6,13, 22–25,31,32, 44–48,51,52,96
CAM-DR	Transient protection from cytotoxics, radiation and receptor-mediated cell death; increased levels of acquired resistance develop with chronic drug exposure	Tissue culture plates coated with immobilized ECM components (fibronectin, collagen and laminin)	1,3–5,13,22–25, 31,32,44–48, 51,52,96,103
	Supports *in vitro* expansion of primary haematopoietic tumour cells and their stroma by mimicking the architecture of the bone marrow microenvironment, providing a system for *ex vivo* drug efficacy studies	3D matrix of fibronectin and collagen supported in Matrigel and supplemented with patient serum	46–47,108
SFM-DR	Dynamic paracrine interaction between tumour cells and stroma is required to produce soluble factors that induce resistant phenotypes	Conditioned medium harvested from stroma grown in monoculture or co-culture with tumour cells is applied to tumour cells; alternatively, membrane (transwell) allows soluble factors to diffuse between cell populations while preventing direct contact of tumour cells with stroma	53,58,79,80
SFM-DR and CAM-DR	Resistance and cell cycle arrest in tumour cells. Response to therapy more closely reflects the *in vivo* experience when tumour cells and their stroma are grown in a 3D matrix of ECM constituents that is more representative of conditions present in *in vivo* microenvironments	Tumour cells grown in co-culture on a monolayer of stromal cells or in co-culture with a 3D matrix of ECM components; includes resistance induced by both direct cell contact (CAM-DR) and soluble factors secreted in response to tumour–stroma interaction (SFM-DR)	6,50,53, 78,90,92, 98–101,104,108
Xenograft and *in vivo*	Integrin and soluble factor antagonists increase survival and inhibit tumour progression as monotherapy; EMDR antagonists enhance traditional therapy	Tumour cells and stromal fibroblasts are manipulated in tissue culture and engrafted into mice	11,22, 44–47,101
Clinical trials	EMDR-specific compounds demonstrate modest anti-tumour activity as monotherapy, but extensive preclinical data suggest that they would increase the effectiveness of chemotoxics	Efficacy of integrin antagonists and statins, as studied in patient trials, focus on blocking angiogenesis and tumour adhesion to stromal cells and ECM in the microenvironment; also, studies are exploring antagonists of soluble factors, such as small-molecule inhibitors of chemokine receptors	82–86,93,97

3D, three-dimensional; CAM-DR, cell adhesion-mediated drug resistance; ECM, extracellular matrix; EMDR, environment-mediated drug resistance; SFM-DR, soluble factor-mediated drug resistance.

de novo drug-resistant phenotype (TABLE 1). Interestingly, the mechanisms of EMDR discovered in these models are consistent with expression patterns that are predictive of treatment failure in patients with ALL or AML — they also involve the modulation of regulators of apoptosis and proliferation. For example, Shain *et al.* showed that β1 integrin-mediated adhesion induces resistance to the physiological mediator of programmed cell death FAS (also known as CD95) in leukaemia and myeloma cell lines. This resistance correlated with the cellular redistribution of the anti-apoptotic protein CASP8 and FADD-like apoptosis regulator (FLIP; also known as CFLAR) from the cytoplasm to cell membranes[5]. Similarly, Hazlehurst *et al.* demonstrated that proteasomal degradation of the pro-apoptotic protein BCL2-interacting mediator of cell death (BIM) is induced by β1 integrin adhesion and contributes to drug resistance in leukaemia cells[48]. Finally, G1 cell cycle arrest induced by β1 integrin adhesion of myeloma cells is associated with drug resistance and rapid (less than 2 hours) post-translational upregulation of p27 (encoded by *CDKN1B*) and downstream inactivation of cyclin-associated kinase activity[48]. Disruption of adhesion caused an equally rapid decrease in p27 expression and the reversion of adhesion-mediated resistance, which was mimicked by the artificial reduction of its expression with small interfering RNA (siRNA) in adhered cells, causally linking p27 levels to resistance. Later work by Lwin *et al.* and Fu *et al.* showed that adhesion-mediated increases in p27 expression in non-Hodgkin B cell lymphoma and hepatocellular carcinoma cell lines is mediated by proteasomal degradation of S phase kinase-associated protein 2 (SKP2), a subunit of the ubiquitin ligase SKP1–CUL1–F-box (SCF), which, in turn, regulates p27 expression by targeting it for proteasomal degradation[6,49]. All these mechanisms ultimately require the post-translational regulation of protein expression. This is consistent with the work of Hazelhurst *et al.* who showed that, compared with acquired drug resistance, CAM-DR by itself does not induce gene transcription significantly[13], a fact that might explain the rapid induction that is characteristic of this phenotype. To date, specific mechanisms mediating the CAM-DR phenotype have been identified largely in cell culture models of haematological malignancies[4–6,48], but this phenotype has also been described in cell culture models of a wide variety of epithelial and endothelial malignancies[31,50–52]. More work is necessary to identify the specific mechanisms of integrin-mediated drug resistance that are common to diverse malignancies.

Soluble factor-mediated drug resistance. Nefedova *et al.* reported that conditioned medium from stromal cells provided protection only if it was collected from cells grown in co-culture with myeloma cells[53]. This indicates that a dynamic interaction between tumour cells and their stroma is required to produce the soluble factors that mediate drug resistance. Consistent with this observation, interleukin 6 (IL-6)

and stromal cell-derived factor 1 (SDF1), the most widely studied mediators of SFM-DR, are known to mediate resistance to various chemotoxics in *in vitro* EMDR models of haematological and epithelial cancer[50,54–59] and are produced at higher levels in tumour-associated stroma than in normal bone marrow stroma[60–62]. Recently, Perez *et al.* extended these findings by using immortalized stromal fibroblasts and conditioned medium from patient bone marrow stroma to show that paracrine interaction between myeloma cells and stroma is also required to protect myeloma cell lines from the ligation of the death receptor for TNF-related apoptosis-inducing ligand (TRAIL)[58]. IL-6 was found to contribute to this effect by increasing the expression of the anti-apoptotic protein FLIP. Earlier work by Catlett-Falcone *et al.* demonstrated that IL-6-induced signal transducer and activator of transcription 3 (STAT3) signalling protects myeloma cells from FAS-mediated apoptosis by upregulating transcription of the anti-apoptotic molecule BCL2-like protein 1 (B2CL1; also known as BCL-X_L)[55]. Therefore, mechanisms of SFM-DR and CAM-DR protect myeloma cells from immunological mediators of cell death (TRAIL and FAS) and chemotoxics by similar mechanisms that depend on the modulation of molecules that control apoptosis.

Cooperative signalling and matrix remodelling. CAM-DR and SFM-DR are phenotypes observed in *in vitro* models of EMDR, but *in vivo* they probably cooperate in the overall EMDR phenotype. This is because tumour-stimulated production of soluble factors by the stroma increases EMDR not only by directly upregulating anti-apoptotic molecules, but also by inducing increased integrin expression and/or affinity for their ligands on tumour cells. For example, SDF1 increases β1 integrin-mediated adhesion of myeloma[63] and SCLC[50] cells, leading to drug resistance in ECM-adhered tumour cells[50]. SDF1 is known to increase integrin affinity for ECM components and membrane-bound ligands by a process called 'inside-out' integrin signalling, which induces a conformational change in the extracellular domain of β-integrins. Not only do soluble factors increase integrin affinity by inside-out signalling, but the converse is also true: integrin-mediated adhesion increases the activation of cytokine signalling pathways. Shain *et al.* recently demonstrated that adhesion of myeloma cell lines to immobilized fibronectin through β1 integrin amplifies IL-6-induced STAT3 signalling, and previous work by Kettritz *et al.* showed a similar phenotype associated with cytokine-induced nuclear factor-κB (NF-κB) signalling in primary neutrophils[64,65].

The tumour–stroma cooperativity also increases CAM-DR by modulating the composition of the ECM in their microenvironment in both epithelial and haematopoietic tumours. For example, Sherman-Baust *et al.* showed that overexpression of collagen VI in ovarian cancer correlates with tumour grade and that adhesion of these tumour cells to collagen VI *in vitro* mediates CAM-DR[32]. Similarly, the expression of collagen IV was higher in bone marrow from patients with multiple myeloma than in normal controls[66]. The modulation of integrin and ECM expression by cancer cells and their stroma is important because adhesion is a major mechanism of EMDR. An emerging picture in which tumour cells and their stroma communicate by upregulating ECM, integrins and soluble factors is becoming even more complex when we consider that cytokine and integrin signalling pathways modulate the activity of each other. Further research is needed to better understand how cooperative integrin–cytokine signalling influences EMDR.

Protective quiescence. Adhesion of metastatic epithelial tumour cells[67,68] and haematopoietic tumour cells to microenvironmental components, such as the ECM or stromal cells, through receptors including vascular cell adhesion protein 1 (VCAM1)[48,53] leads to a state of tumour dormancy that is associated with CAM-DR and MRD. For example, β1 integrin ligation on haematopoietic tumour cells leads to cell cycle arrest[6,48,53,69]. Also, although breast cancer cells require β1 integrin adhesion for proliferation at the primary tumour site[70,71], the work of Goodison *et al.* and Naumov *et al.* has demonstrated, using mouse models of breast cancer micrometastasis, that metastatic microenvironments can induce a quiescent state in epithelial breast tumours[67,72,73]. Collectively, these results indicate that haematopoietic and epithelial tumours may actually show similar behaviour in protective microenvironments. Importantly, the results in mouse models are consistent with those obtained by Pantel *et al.* in breast, gastric and colorectal cancer bone marrow specimens, in which micrometastases were found in 34% of 532 patients and these micrometastases overwhelmingly showed markers of dormancy[74].

Differences in the way adhesion of epithelial tumour cells influences the cell cycle at primary and metastatic sites suggest that it is the context of other factors in the microenvironment, such as soluble factors or specific ECM components, during adhesion that determines whether a cancer cell is proliferative or dormant. Nevertheless, work in *in vitro* cell culture models indicates that, in epithelial tumour cell lines derived from various tissues, β1 integrin-mediated adhesion to ECM components leads to cell cycle arrest through p21 and p27 upregulation[75–77]. Therefore, metastatic epithelial cells and haematological tumour cells can respond similarly to microenvironments by becoming dormant. Dormancy has important implications for EMDR, because upregulation of p21 or p27 by adhesion has been shown to mediate cell cycle arrest and contribute to CAM-DR in both haematopoietic[48,78] and epithelial[68] tumour cell lines.

The quiescent state induced by adhesion can be counteracted by stroma-derived factors such as IL-6, which prompts cell cycle progression of adhered cells[65]. This implies that models of tumour dormancy may not be as simple as they seem. For example, Shain *et al.* found that, when myeloma cells were adhered to immobilized fibronectin, they could still proliferate in response to IL-6 stimulation although they retained the CAM-DR phenotype[65]. Moreover, Bisping *et al.* and Dankbar *et al.* used patient specimens to show that fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), respectively, produced by myeloma cells induced IL-6 secretion by stromal cells, and vice versa[79,80]. Taken together, these findings suggest that a paracrine amplification loop is elicited by the dynamic interaction between myeloma and their stroma that allows tumour cells to maintain a low level of proliferation while they are adhered. IL-6 mediates both proliferation and EMDR in myeloma cells[55,58,59], indicating a dual role for this cytokine in the microenvironment. Therefore, it is theoretically possible that a few dormant haematological or metastatic epithelial tumour cells could survive treatment in protective microenvironments and later proliferate after acquiring more permanent resistance mechanisms and

altering their microenvironment, leading to MRD outgrowth and disease recurrence. This idea is supported by *in vivo* studies using single-cell murine models of metastasis that demonstrated that single epithelial tumour cells can remain dormant in metastatic locations but retain their ability to proliferate when transplanted to their tissue of origin[67,72,73].

Therapeutic strategies

There are three categories of possible anti-EMDR therapeutic targets: extracellular ligand–receptor interactions, downstream pathways in tumour cells and downstream pathways in tumour stroma. The first category includes integrin antagonists that were previously developed to combat various inflammatory and autoimmune diseases[81], but increased recognition of the important role of integrins in mediating EMDR has led to their recent inclusion in clinical trials as anti-tumour agents. Results from recent clinical trials reflect data from earlier *in vivo* mouse models — integrin antagonists show limited efficacy as monotherapy for solid and haematopoietic tumours[82–86]. However, preclinical data in mouse models suggest that these compounds would prevent EMDR in combination therapy with chemotoxics[45–47,69]. Furthermore, clinical trials in inflammatory diseases[81] and preclinical experiments designed to overcome EMDR in *in vivo* mouse models[46] have indicated that integrin antagonists have low toxicity at high doses. To date, clinical trials have not included anti-integrin therapy with traditional chemotherapy, and integrin antagonists have primarily been used as single agents in recurrent, drug-resistant diseases. Therefore, future work should continue to capitalize on previous clinical experience by combining currently available small-molecule integrin antagonists with traditional therapy in clinical trials to prevent CAM-DR and CAM-RR in newly diagnosed patients and to limit MRD, thereby preventing the emergence of acquired drug resistance.

Targeting downstream pathways in tumour cells. The second type of target is exemplified by an even older class of drugs that has also been repurposed to combat EMDR. This strategy was justified by the work of Hazlehurst *et al.*, who found that genes in the cholesterol biosynthesis pathway are overexpressed in myeloma cells expressing both CAM-DR and acquired drug resistance phenotypes[13]. Follow-up on this work by Schmidmaier *et al.* has demonstrated that statins, inhibitors of cholesterol biosynthesis, can be repurposed to combat CAM-DR in malignancies. These compounds and their derivatives can overcome drug resistance by direct extracellular integrin antagonism[87,88] and by inhibition of small GTPases such as Ras, Rho and RAP1 that are dependent on the intermediates of cholesterol biosynthesis[89,90]. GTPases are known to be crucial mediators of adhesion strengthening by regulating focal adhesion formation and modulating integrin affinity for its ligands[91,92]. The precise role of these proteins in mediating CAM-DR has not been defined, but they are probably required to maintain cell adhesion contacts.

The first Phase II clinical trial showed that simvastatin could overcome drug resistance in refractory myeloma by blocking HMG-CoA reductase, a key intermediate of cholesterol biosynthesis[90,93]. A more recent report by Sondergaard *et al.* did not find that treatment with simvastatin improved bone turnover in a small cohort of patients with advanced myeloma who had previously undergone heavy treatment[86]. Importantly, this finding contradicts extensive preclinical work in both *in vitro* and *in vivo* models of bone metabolism that concluded that statins inhibit bone resorption, a hallmark of cancers that localize to bone and an indication of a co-opted bone marrow microenvironment[94,95]. Crucially, the study by Sondergaard *et al.* was carried out in the absence of other chemotherapy[86], whereas Schmidmaier *et al.* combined statin treatment with chemotoxics[93].

Work in *in vitro* models of EMDR has also led to the inclusion of a newer compound in clinical trials that targets downstream EMDR pathways in myeloma cells. These models have demonstrated that several tumour cell CAM-DR pathways (p27, BIM and NF-κB) are directly regulated by the proteasome[4,6,48,49,96], which is inhibited by bortezomib. Although trials with this compound have previously focused on the treatment of refractory myeloma, one recent trial included the compound with standard melphalan and prednisone chemotherapy for initial treatment of patients who are ineligible for high-dose melphalan therapy[97]. This large trial showed that the addition of bortezomib to the treatment regimen increased time to progression by 45%, with a complete response rate of 30% compared with 4% without bortezomib[97]. We contend that anti-EMDR strategies would be more effective if administered at the time of diagnosis, in conjunction with traditional therapies, as opposed to when tumours have developed acquired resistance following treatment.

Box 2 | **Targeting tumour-associated stroma as a treatment strategy**

Stroma-induced signalling pathways associated with the tumour microenvironment are increasingly being targeted by therapeutic approaches intent on combating environment-mediated drug resistance[2], but little is known about how tumour therapy affects its stroma. To date, most studies have not sought to understand how stroma responds to therapy and how this response influences the development of resistance in tumours.

The demonstrated importance of the tumour–stroma coalition in tumour survival and in the development of resistance to therapy raises an obvious question: why not treat tumour stroma directly to combat disease? Moshaver *et al.* demonstrated that chemotherapy of bone marrow stromal cells decreased their ability to protect primary acute myeloid leukaemia (AML) cells from chemotherapy[104]. Using stromal fibroblasts that were pretreated with chemotoxics, they showed that treatment of stroma reduced the ability of primary AML and AML cell lines to proliferate and survive subsequent exposure to chemotherapy, suggesting that these cells must also need to develop resistance to therapy to ensure tumour survival. Work by Spiotto *et al.* suggests that targeting tumour stroma with immunotherapy *in vivo* could be an effective strategy by showing that bystander elimination of subpopulations of antigen loss variant tumour cells by cytotoxic T cells was possible only when parental tumour cells express sufficient amounts of antigen to be cross-presented by tumour stroma, allowing stromal cells themselves to be targeted for killing by T cells[105]. Later work by Zhang *et al.* from the same group showed that irradiation or chemotherapy could also increase cytotoxic T cell killing of established tumours by causing enough antigen to be released from tumour cells to target antigen-presenting stroma for destruction[106]. They later verified the important role of stroma in this effect by showing that cytotoxic T cell killing of only major histocompatibility complex (MHC)-restricted tumour stroma causes long-term inhibition of tumour growth[107]. Collectively, these data illustrate the important role of stroma in tumour survival and resistance to therapy and suggest that directly targeting stroma and stroma-mediated pathways might be an effective means of tumour therapy.

Targeting stroma-derived paracrine factors. Preclinical work with tumour cell lines and patient specimens in *in vitro* EMDR cell culture models has demonstrated that targeting stroma-mediated paracrine-resistance pathways with specific receptor tyrosine kinase inhibitors can overcome EMDR. Lin *et al.* showed that blockage of paracrine IL-6 production by stroma through specific inhibition of VEGF receptor tyrosine kinase activity can overcome the protective effect of stromal cells on dexamethasone-induced apoptosis in myeloma cells[98]. Bisping *et al.* reported similar results using an indoline-derivative inhibitor of the receptor tyrosine kinase for FGF that also blocks the production of IL-6 by bone marrow stromal cells[79,99]. This inhibition not only led to the apoptosis of patient myeloma cells *ex vivo*, but also attenuated myeloma cell adhesion and proliferation and increased the efficacy of chemotherapeutic agents. This approach is promising because it blocks the stroma-derived IL-6 that is produced in response to tumour cells and therefore disrupts the paracrine amplification loop.

Receptor antagonists are also being developed to block stroma-derived soluble factors extracellularly. For example, Burger *et al.* demonstrated that a peptide antagonist of the chemokine SDF1 resensitizes primary chronic lymphocytic leukaemia cells to cytotoxics when co-cultured with bone marrow stroma[100]. Recent work by Zeng *et al.* showed that AMD3465, a second-generation small-molecule inhibitor of the SDF1 receptor (CXCR4), could overcome resistance to kinase inhibitors and chemotherapy in a mouse model of AML. Moreover, they found that blockage of stroma-derived SDF1 prolonged survival[101]. The success of small-molecule inhibitors of the SDF1 pathway in preclinical models has led to ongoing clinical trials. Importantly, both IL-6 and SDF1 also mediate drug resistance in epithelial malignancies[50,56]. The emergence of therapeutic approaches that disrupt communication between tumours and their stroma provides evidence of the increasing recognition of the importance of this relationship in the development of drug resistance in malignancy (BOX 2).

Conclusions

Preclinical studies using *in vivo* tumour models have shown that the disruption of EMDR pathways can increase the efficacy of primary therapies in combinatorial treatment strategies. Traditionally, clinical trials seeking to determine the efficacy of new therapies are generally performed after patients relapse from primary therapy. We contend that this approach is inherently flawed for testing agents that might be able to prevent EMDR, because EMDR is a primary contributor to MRD following primary therapy. In recurrent disease following primary therapy, cancer cells have already developed complex acquired resistance mechanisms and no longer require EMDR pathways for survival. As a form of *de novo* resistance, EMDR shields cancer cells from the effects of the initial therapy, allowing the development of more permanent mechanisms of resistance in the context of the powerful selective pressure of therapy. Therefore, therapeutic strategies should seek to circumvent EMDR during the initial treatment to prevent the emergence of acquired resistance. Furthermore, the development of molecular imaging techniques designed to monitor signalling pathways involved in EMDR would provide crucial information about the influence of the tumour microenvironment in the clinical drug response and the emergence of drug resistance.

Cancer cells and their associated stroma coexist in an evolving ECM and soluble factor milieu that is moulded by their interaction. Reciprocal integrin- and soluble factor-mediated signalling interactions between these two groups of cells induce

Box 3 | **Building a mathematical model of EMDR**

Cancers are complex, multiscalar, dynamical systems that show extensive spatial heterogeneity, phenotypic diversity and temporal evolution. In addition to tumour subpopulations, cancers contain tumour-associated mesenchymal cells that strongly influence tumour growth and physiology. Although such systems are difficult to model mathematically, they are impossible to understand intuitively.

Building mathematical models of cancer requires an understanding of both the underlying biology and the biological question. For example, general population or molecular dynamics are typically modelled with ordinary differential equations. When there are spatial components in the dynamics, partial differential equations are used. The life history of individual cells can be obtained using cellular automata models, which are often modified using ordinary differential equations or partial differential equations to account for environmental factors that affect or are influenced by cellular activity.

As an example, we briefly present a model of tumour–mesenchymal interactions in treated and untreated cancers. The general strategy in initial model building is simplicity. That is, rather than including every possible factor in the complex dynamics, the model begins with just a few components (lumped phenomenological terms) that broadly summarize the observed system behaviours. Once a simple model is established, components of interest can be added by expanding the lumped terms into component parts.

Assume there are *n* subpopulations of tumour cells (*i*) and a single mesenchymal (*M*) population. The change in the size of each population at each time step can be expressed as follows:

$$P_i(t+1) = P_i(t) \times (1 + [\gamma_i \times G_{ii} \times G_{iM}])\ i = ,\ldots.n\ j = 1,\ldots\ n\ i \neq j \quad (1)$$

P_i is the probability that any cell sampled in the tissue of interest will be a member of the *i*th tumour subpopulation. Similarly, P_M is a measure of the population size of tumour-associated mesenchymal cells. The term γ represents the replication rate, and *G* is a function that represents the positive and negative interactions among different tumour populations (G_{ij}), the effects of the mesenchymal cells on each tumour population (G_{iM}) or between the effects of tumour cells on mesenchymal cells (G_{Mi}).

To examine the effects of therapy, we add a 'death function', *d*(*t*), so that, following administration of chemotherapy, the tumour populations will proliferate linearly in γ_i and *G*, but decline linearly owing to therapy-induced cytotoxicity. The effects of chemotherapy can be mitigated by phenotypic resistance. In addition, a phenotypically sensitive cell can be rendered transiently resistant owing to environmental effects. This can be expressed mathematically as follows:

$$P_i(t+1) = P_i(t) \times (1 + [\gamma_i \times G]) \times (1 - d(t)),\ d(t) = a(t)\beta(P_M, P_i)\sigma_i \quad (2)$$

a(*t*) is the therapy dose (or intratumoral concentration), σ_i is the phenotypic sensitivity of the population *i* to the therapy and *β* is the environmental sensitivity, that is environment-mediated drug resistance (EMDR). β, in turn, is dependent on the relative density of both the tumour cells (P_i) and the mesenchymal cells (P_M). The combination of both phenotypic resistance and EMDR will allow, for example, modelling of both the EMDR process itself and the conceptually more difficult transition from minimal residual disease to clinical recurrence with resistant phenotypes.

a transient EMDR phenotype in tumour cells, protecting them from therapy until more complex acquired drug resistance phenotypes can develop. Previously, research in this area has relied on tissue culture and *in vivo* models that have been extremely valuable tools for unravelling the basic mechanisms of EMDR. However, in recent years an increasingly dynamic picture of the tumour microenvironment has developed that suggests that cancer cells co-evolve with other cells present in their microenvironment. These models are informative in revealing the details of specific signalling pathways, but their use should be directed by a systems biology approach that can integrate the data they generate into a comprehensive theoretical model that reflects the complexities and long time frames operative in *in vivo* tumour microenvironments. We propose that a mathematical approach would help us to better understand how this highly complex and constantly evolving microenvironment induces EMDR by providing a conceptual framework from which hypotheses can be generated. Outcomes measured using experimental models can then be used to validate the 'fluid' theoretical model. In this way, mathematical models can be used to develop hypotheses and guide research much more rationally than static, reductionist approaches allow (BOX 3). A better understanding of the intricacies of this phenomenon is of crucial importance, because it is one of the major obstacles to effective cancer treatment.

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DATABASES

National Cancer Institute Drug Dictionary: http://www.cancer.gov/drugdictionary/
bortezomib | cytarabine | cytosine arabinoside | dexamethasone | melphalan | prednisone | simvastatin

UniProtKB: http://www.uniprot.org
B2CL1 | BIM | CXCR4 | FAS | FLIP | IL-6 | $p27^{kip1}$ | SDF1 | SKP2 | STAT3 | TRAIL | VCAM1

FURTHER INFORMATION

William S. Dalton's homepage: http://www.moffitt.org/Site.aspx?spid=0F04FE7484014C88B2BB1F23A8D93FEA&SearchType=Researcher

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