E-Cadherin Upregulation as a Therapeutic Goal in Cancer Treatment

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Abstract: We review the role of E-cadherin in cancer progression, and its therapeutic restoration as a strategy to suppress metastasis. We subsequently discuss E-cadherin upregulating drugs, proposing a schema for restoring E-cadherin by targeting its epigenetic and transcriptional regulators. These pathways will likely provide significant future treatment breakthroughs against cancer metastasis.

Key Words: E-cadherin, cell adhesion, epithelial-mesenchymal transition, epigenetics, transcriptional repressors, chemotherapy, metastasis, cancer.

INTRODUCTION

A key goal of recent research has been the identification of therapeutic targets with the potential to inhibit cancer metastasis which is widespread at presentation and carries a grave prognosis. Loss of tumor tissue integrity and organization are essential pre-conditions for tumor dissemination, and the loss of epithelial cell-cell adhesion molecule E-cadherin is one of the key factors involved in this early stage of metastatic progression. E-cadherin downregulation is one of the most commonly reported phenotypes in advanced and metastatic epithelial cancers, and *in vitro* studies have demonstrated that decreased E-cadherin dependent cell-cell adhesion predisposes to cell motility and tumor cell dissemination. In addition, E-cadherin is also involved in the several pathways that play key roles in tumorigenesis including β-catenin/Wnt and EGFR pathways. These lines of evidence implicate a crucial role of E-cadherin inactivation in the development and progression of human cancer.

Crucially, unlike the majority of tumor and metastasis suppressor genes which are irreversibly inactivated by mutation or deletion in cancer cells, the *CDH1* gene remains intact in the majority of human cancers but is silenced through hypermethylation of its promoter region and overexpression of its transcriptional regulators, particularly Snail. This provides the intriguing possibility of therapeutic re-induction of E-cadherin expression in cancer cells. Supporting the efficacy of this mechanism, experimental restoration of E-cadherin in cancer cell lines *in vitro* has been shown to reduce tumorigenicity, cancer cell growth and apoptosis. In the past decade, there has been a widespread research effort exploring the possibility of targeting E-cadherin, with the goal of developing novel therapeutic agents to inhibit metastatic spread of local cancers.

E-CADHERIN STRUCTURE AND FUNCTION

E-cadherin is a cellular adhesion molecule that functions as the basic adhesive subunit of intercellular adherens junctions (AJs) which provide epithelial polarity and tissue integrity. It was the first member of the cadherin superfamily to be identified, a large family of at least 80 specific types that are characterized by their ability to form calcium dependent intercellular homophilic bonds. E-cadherin is a classical (Type I) cadherin, the other four subgroups being categorized as Type II cadherins, desmosomal cadherins (localized to desmosomes), protocadherins (found in the central nervous system) and cadherin-related proteins. E-cadherin creates adhesive junctions between cells by forming strong homotypic bonds with E-cadherin molecules on adjacent cells which are vital for the correct organization of epithelial tissue. Structurally, mature E-cadherin is a 120kDa single-span transmembrane glycoprotein which has at its N-terminus five extracellular cadherin (EC) domains, which provide the adhesive surface responsible for cadherin function. The distal N-terminal EC1 domain is responsible for the specificity of cadherin-cadherin homotypic dimerization, however all five EC domains of E-cadherin are involved in the formation of the strong, dynamic bonds between adjacent cells (reviewed in [1]). To provide the structural rigidity required for strong E-cadherin-dependent bonds at the AJ, the intracellular C-terminal domain of functional E-cadherin is anchored to the cytoskeleton *via* an interlinking armadillo-family molecule, either β-catenin or γ-catenin (plakoglobin). The β- or γ-catenin in turn binds to anchor protein α-catenin, which provides the molecular bridge between E-cadherin/catenin complex and the actin cytoskeleton. The resulting E-cadherin cell-cell bond links the cytoskeletons of adjacent cells and provides a major proportion of the mechanical strength of epithelial surfaces. A summary of the function of E-cadherin in normal epithelial cells is shown (Fig. (**1A**)). Both β- and γ-catenin have additional regulatory functions: when dissociated from the cadherin-catenin complex, they translocate to the nucleus and co-activate oncogenic Wnt pathway genes through an interaction with TCF/Lef transcription factors. This suggests an additional role for E-cadherin in suppressing the activation of these pro-oncogenic pathways, which has been extensively reviewed by Hajra and Fearon [2], and lies beyond the scope of this review.

E-CADHERIN IN DEVELOPMENT AND CANCER

Epithelial-Mesenchymal Transition (EMT)

During embryogenesis, the precisely co-coordinated spatiotemporal expression of cadherin molecules plays a domi-

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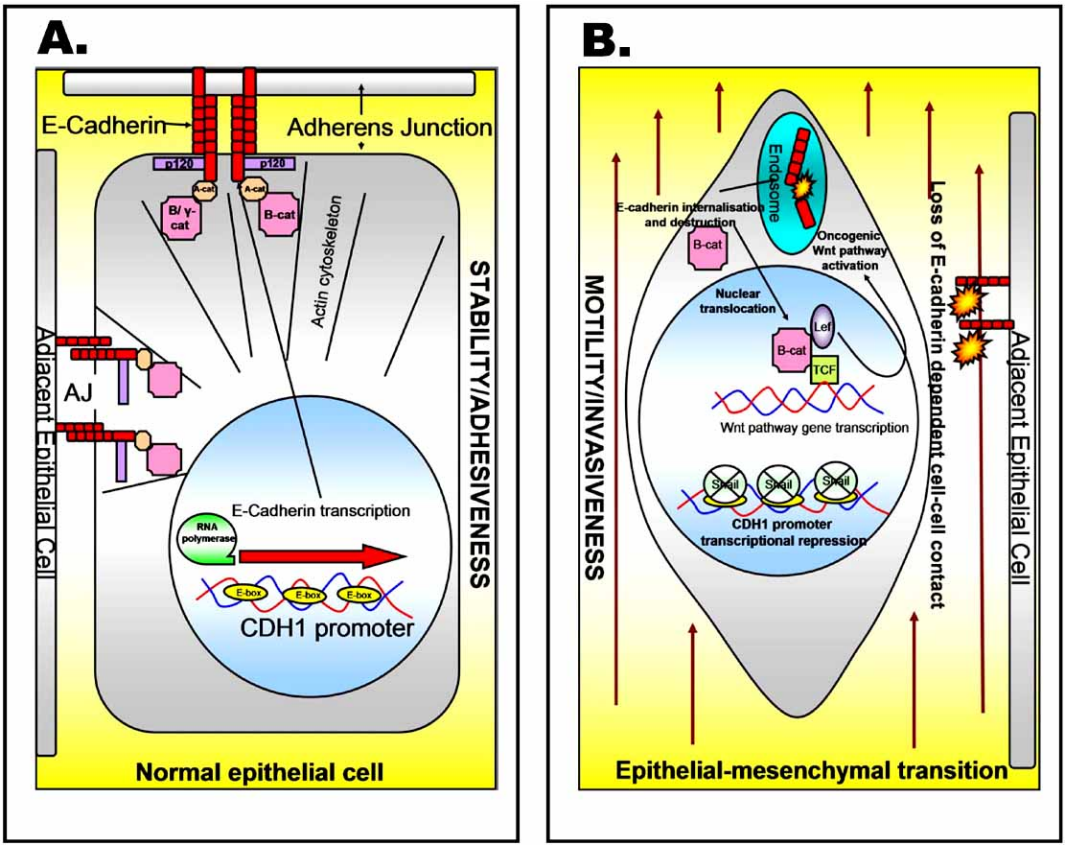

Fig. (1). Schematic illustration of E-cadherin regulation in epithelial cells. A. Localization and function of E-cadherin in normal epithelial cells. E-cadherin forms strong, stable cell-cell bonds at the Adherens Junction (AJ) that link to the actin cytoskeleton *via* α and β/γ-catenins. Normal transcriptional activity of E-cadherin enables cell polarization and maintenance of epithelial organization. **B**. Epithelial-Mesenchymal Transition. Cells undergoing EMT downregulate E-cadherin expression through transcriptional repression of the *CDH1* gene. E-cadherin is lost from the cell membrane and is catabolized in the endosomal compartment, disrupting the adherens junctions. The loss of cell-cell contact is associated with a motile, fibroblastic morphology, and furthermore the failure to sequester β and γ-catenin at the cell membrane allows them to translocate to the nucleus to activate the Wnt pathway which may contribute to the oncogenic process. EMT allows cancer cells to migrate and invade.

nant role in determining the morphology of the developing cell mass. E-cadherin is the first cell adhesion molecule to be expressed in the embryo, as early as the eight cell morula stage, hence its original name, ovumorulin. Several experiments have demonstrated that E-cadherin is essential for the initial compaction and eventual organization of the embryo, and experimentally induced E-cadherin deficiency (in knockout mice or following antibody treatment) is embryonic lethal at an early stage [3]. While its expression is universal in early embryological development, during gastrulation a subpopulation of cells undergoes epithelial-mesenchymal transition (EMT), in which cells lose their epithelial morphology and adopt an invasive, motile phenotype with fibroblastic morphology (Fig. (**1B**)). The hallmark of EMT is the downregulation of E-cadherin. The mesenchymal cells resulting from the EMT form, amongst other structures, the neural crest, the cardiac cushion and the palate, each of which is essential for fetal viability. Typically during EMT, although with some exceptions, the E-cadherin downregulation is associated with the reciprocally gained expression of the mesenchymal adhesion molecule N-cadherin, in a process known as "cadherin switching". EMT also occurs during pathological processes in adult tissues, for example during wound healing, in which epithelial cells are required to undergo EMT and migration to restore the integrity of the epithelial barrier. The coordinated alteration of E-cadherin expression at different stages of development indicates the presence of a complex and highly efficient regulatory mechanism. This regulation is critical for tissue homeostasis, as it allows organization of epithelia and suppresses invasiveness and motility of human cells with proliferative potential. It therefore comes as little surprise to discover that in cancer, a disease typified by disorganization and invasiveness, EMT is now recognized to be a key stage in the development of metastatic disease (reviewed in [4]).

Pathological Evidence: E-Cadherin is Downregulated in Common Cancers

The first evidence suggesting the involvement of E-cadherin repression and EMT in cancer was the 1989 discovery that E-cadherin was downregulated in highly metastatic ovarian cancer cells [5]. Since that time, evidence from over 100 studies involving every major cancer type has shown that, with few exceptions, E-cadherin downregulation is a common event in human cancers. According to 2005 cancer

statistics, the commonest three causes of cancer death in the USA were lung, prostate and colorectal cancers in men and lung, breast and colorectal cancers in women [6]. Using these four cancers as an example, we comprehensively reviewed the literature concerning the expression of E-cadherin in clinical specimens, and the compiled data is represented in Table **1**. There is clear evidence linking adverse grade, stage, metastasis, invasiveness and prognosis of these cancers with reduced or aberrantly expressed E-cadherin, clearly implicating E-cadherin as a central player in the suppression of cancer spread. Furthermore, E-cadherin expression is disrupted and related to the above indices in a diverse range of other cancers, including but not limited to pancreatic cancer [7, 8], esophageal cancer [9, 10], hepatocellular carcinoma (HCC) [11, 12], and bladder cancer [13, 14]. Regardless of its etiology, the widespread prevalence of E-cadherin abnormalities suggests an important role in cancer pathogenesis, and implies that E-cadherin may be a therapeutic target in cancer treatment.

CDH1 Gene Mutations and Cancer

Although the loss of gene expression in cancer cells is frequently due to gene mutation or deletion, in most tumors thus far described E-cadherin expression is reduced, heterogeneous or localized to the cytoplasm rather than completely lost, indicating deregulation rather than deletion of the E-cadherin gene. E-cadherin was initially linked to cancer when its 16q22.1 locus was noted to undergo loss of heterozygosity (LOH) in hepatocellular and breast cancers with high metastatic potential [69, 70], and subsequently a *CDH1* mutation was identified in an invasive gastric cancer cell-line [71]. Strong genetic evidence for the causal role of E-cadherin in cancer pathogenesis was provided by the identification of a familial cancer syndrome associated with germline *CDH1* mutations, namely the highly infiltrative Hereditary Diffuse Gastric Cancer (HDGC) [72]. This syndrome has a penetrance of approximately 80% and a mean onset age of 38 years, and also predisposes individuals to colorectal, breast and prostate cancers albeit with lower frequency. As well as a lower age of onset, HDGC has a distinctive histopathology of unpolarised, discohesive cells without glandular architecture which do not express E-cadherin [73]. The existence of a *CDH1*-related syndrome suggests that its somatic mutations may also be involved in sporadic tumorigenesis. In fact, this has been proven to be the case in significant numbers in only certain subtypes of cancer, namely sporadic diffuse gastric cancer and lobular breast cancer (41% and 32% of cases respectively) [74, 75]. Sporadic diffuse gastric cancer is similar to HDGC pathologically, and invasive lobular breast cancer (ILC) is characterized by the complete and almost universal absence of E-cadherin expression [76], which occurs early at the *carcinoma in situ* stage [54]. It is completely distinct from invasive ductal breast carcinoma (IDC), in which E-cadherin is downregulated late and heterogeneously in association with invasion and metastasis. This is similar to the pattern seen in lung, prostate and colorectal cancers (see Table **1**) in which there is little evidence of genetic alterations of the *CDH1* gene. This is critical, since the success of E-cadherin upregulating therapy depends on the presence of functional yet repressed *CDH1* in the cancer cell genome.

Epigenetic Silencing of E-Cadherin in Cancer

In contrast to the occurrence of CDH1 mutations in certain very specific cancers, it has become increasingly apparent in recent years that promoter hypermethylation of *CDH1* is a common event in multiple cancers. It occurs due to the aberrant activity of DNA Methyltransferase (DNMT) on CpG islands found within or adjacent to almost half of the gene promoters in the human genome, converting the cytosine nucleotides to 5-methylcytosine. In non-pathological states, these promoter associated CpG islands are unmethylated, regardless of the transcriptional status of the gene in question, whereas CpG islands not associated with a promoter are heavily methylated. This methylation is preserved in mitotic cells by an active replication process, and is therefore stably inherited by progeny, including in cancer cells. CpG methylation induces gene silencing through a complex interplay involving methylated DNA binding proteins (MBPs) and their co-repressors histone deacetylase inhibitors (HDACi) and histone methyltransferase (HMT) which alter the histone conformation in such a way as to prevent gene transcription (the "histone code").

In cancers, DNA methylation patterns are broadly abnormal, with generalized hypomethylation contrasting with intense hypermethylation at certain promoter-associated CpG regions linked to tumor suppressor genes. In 1995, Yoshiura *et al.* discovered that the *CDH1* promoter region was hypermethylated in a number of cancer cell lines and that this was associated with decreased or lost E-cadherin protein expression [77]. Since that time, *CDH1* promoter hypermethylation has been a common finding in various human cancers and is usually associated with reduced expression of E-cadherin (summarized in Table **2**). The broad spectrum of cancers in which *CDH1* is hypermethylated, together with its association with poor prognostic indicators, underscores the importance of E-cadherin suppression in human cancers. The presence of epigenetic *CDH1* silencing also at least partially explains the discrepancy between the reduced E-cadherin protein levels observed in clinical tumor specimens and the low frequency of mutations or deletions of the gene. Furthermore, promoter hypermethylation has been shown to be the most common "second hit" of the *CDH1* gene in HDGC and in lobular breast and diffuse gastric cancers. As well as implicating E-cadherin suppression as a key element in disease progression, the prevalence of this abnormality also provides a therapeutic target for the upregulation of E-cadherin.

Transcriptional Regulation of E-cadherin

In a proportion of tumors, the E-cadherin gene is not mutated or deleted, nor is the promoter hypermethylated, yet E-cadherin expression is known to be suppressed. This suggests the presence of the aberrant activity of E-cadherin transcriptional repressors, which operate *via* a direct interaction with the E-Cadherin *CDH1* gene promoter region. The human *CDH1* gene promoter contains positive regulatory CAAT- and GC-rich regions that are recognized by CAAT-binding proteins and AP2 factors [129]. Additionally, the human promoter contains three E-box sequences, E-box1, E-box3 and E-box4, the latter of which is located downstream to the transcription initiation site. Each of the E-boxes play an important role in E-cadherin transcriptional control, and

Table 1. Summary of E-Cadherin Expression in Clinical Specimens of 4 types of Common Human Cancers

Cancer Origin	Sample Size	Cancer Type	% E-Cad Down-Regulation	Correlated with					Ref.
				Stage	Metastasis	Grade	Prognosis	Invasion	
Lung	52	Advanced NSCLC	n.s.		Y	Y	Y		[15]
	111	Previously-operated NSCLC	37-80	Y	Y	Y	Y		[16]
	18	BPC	22		Y				[17]
	81	NSCLC	63-88	Y	Y			Y	[18]
	28	NSCLC	36	N	N	N	N	N	[19]
	331	Lung Cancer	42	Y	Y	Y	N	Y	[20]
	88	NSCLC	61.4			Y			[21]
	141	Lung cancer	60			Y	N		[22]
	193	Lung cancer	10	Y	Y	Y	Y	Y	[23]
Prostate	92	Primary /metastatic	50	Y	?	Y			[24]
	89	Primary	32-76	Y	Y	Y	Y	Y	[25]
	67	Primary tumors/ LN mets	n.s.			Y			[26]
	99	Primary	56	Y	Y	Y	Y	Y	[27]
	67	Primary	n.s.				?		[28]
	53	Primary /metastatic	29-73		Y				[29]
	76	Primary	40		N	Y	Y	Y	[30]
	1220	Primary	13	Y	N	N		N	[31]
	112	Primary	25	Y	Y	Y		Y	[32]
	44	Localized primary	53						[33]
	58	Primary	64-83	Y		Y			[34]
	20	Primary	n.s.						[35]
	16	Primary	63-87	N		Y			[36]
Colorectal	68	Primary/metastases	63-88	Y	Y	Y		Y	[37]
	5	Poorly-differentiated primary	100			Y			[38]
	107	Primary/metastases	14-50	N	N	Y		N	[39]
	100	Primary	57	Y	Y	Y	Y	Y	[40]
	68	Primary	38						[41]
	57	Primary	46	N		Y			[42]
	118	Primary/metastases	44			Y			[43]
	60	Primary	52						[44]
	100	Primary	29		N	N			[45]
	43	Primary (Rectal only)	0-46		Y	Y			[46]
	63	Primary	47.5		Y				[47]
	48	Primary/metastatic	n.s.		Y				[48]
	120	Primary			Y			Y	[49]

(Table 1. Contd….)

Cancer Origin	Sample Size	Cancer Type	% E-Cad Down-Regulation	Correlated with					Ref.
				Stage	Metastasis	Grade	Prognosis	Invasion	
Breast	20	Breast Cancer	45						[50]
	25	ILC	100						[51]
	61	54 IDC and 7 ILC	100			Y			[52]
	208	Breast Cancer	100	Y		Y			[53]
	362	Breast Cancer	52-100				Y		[54]
	96	DCIS	n.s.			Y			[55]
	156	6 LCIS, 150 DCIS	0-100						[56]
	32	Breast Cancer	n.s.						[57]
	218	Breast Cancer	91	N	N	Y	Y		[58, 59]
	142	Breast Cancer	58	Y	Y	Y	Y	Y	[60]
	120	Breast Cancer	19-64				Y		[61]
	171	Invasive Breast Cancer	44		Y		Y		[62]
	20	Inflammatory breast cancer	0	N	N	N	N	N	[63]
	174	Breast Cancer	66-74	N	N	Y	N	N	[64]
	66	Breast Cancer	35-71		Y		Y		[65]
	1665	Non-lobular Primary	n.s.	Y	Y	Y	Y	Y	[66]
	207	Primary/metastases	n.s.				Y		[67]
	86	Breast Cancer (detected by mammo-gram)	14-64	Y		Y			[68]

Note: NSCLC:Non-Small Cell Lung Cancer; BPC: Bronchopulmonary Carcinoma; ILC: Invasive Lobular Breast Cancer; IDC: Invasive Ductal Breast Cancer; DCIS: Ductal Carcinoma *In Situ*; LN: Lymph Node; Y:Yes; N: No; n.s: Not Specified; ?: Results inconclusive/not statistically significant.

Table 2. Frequency of E-Cadherin Promoter Methylation in Human Cancers

Cancer Type	No. of Cases	% *CDH1* CpG Hypermethylation	Correlated with E-Cadherin Expression	Ref.
NSCLC	107	18%	n/a	[78]
	75	75%	n/a	[79]
	224	58%	Yes	[80]
Prostate	5	100%	Yes	[81]
	10	80%	Yes,	[82]
Colorectal	156	93%	n/a	[83]
	29	100%	Yes	[84]
	185	5.7%	n/a	[85]
	58	0%	n/a	[86]

(Table 2. Contd….)

Cancer Type	No. of Cases	% *CDH1* CpG Hypermethylation	Correlated with E-Cadherin Expression	Ref.
Esophageal	31	84%	Yes	[87]
	20	80%	Yes	[88]
	41	66%	n/a	[89]
Liver	24	46-67%	Yes	[90]
	24	33%	Trend	[91]
	29	17%	Trend	[92]
	79	21.5%	Yes	[93]
	60	33.3%	Yes	[94]
	72	43%	n/a	[95]
	51	8-52.9%	No	[96]
Bladder	47	43%	Yes,	[97]
	51	84%	Yes	[98]
	69	30.4%	n/a	[99]
	111	30-60%	Yes	[100]
	81	35%	Yes	[101]
	71	72%	Trend	[102]
Gastric	26	81%	n/a	[103,104]
	58	58-65%	Yes	[105]
	36	78%	n/a	[106]
Oral Squamous Cell	52	17%	Yes	[107]
	18	94%	Yes	[108]
	109	64- 71%	Yes,	[109]
	48	85%	n.s.	[110]
	51	35%	Yes	[111]
	79	n.s.	No	[112]
Kidney	34	67%	Yes,	[113]
	100	11%	No	[114]
Nasopharyngeal	15	60%	n/a	[115]
	29	52%	Yes	[116]
Leukemia	18	78% (CLL)	Yes	[117]
	77	32-55% (AML & ALL)	Yes	[118]
	61	56% (AML)	Yes	[119]
	60	13% (AML)	n/a	[120]
	56	21.4% (myeloma)	n/a	[121]
Testicular	28	0-100%	n/a	[122]
Cervical	30	60%	Yes	[123]

(Table 2. Contd….)

Cancer Type	No. of Cases	% *CDH1* CpG Hypermethylation	Correlated with E-Cadherin Expression	Ref.
Endometrial	107	15.6-81.8%	Yes	[124]
Head and neck	32	2%	n/a	[125]
Laryngeal	76	40- 77%	n/a	[126]
Mucoepidermoid	46	72%	Yes	[127]
Thyroid	46	83%	Yes	[128]
Astrocytoma	53	32%	n/a	[129]

Note: CLL: Chronic Lymphatic Leukemia; AML: Acute Myeloid Leukemia; ALL: Acute Lymphatic Leukemia.

point mutations in E-box1 or 3 promote strong induction of E-cadherin promoter activity in E-cadherin deficient cell lines [130]. The E-box elements of the promoter are also specifically preserved in E-cadherin deficient cells, including tumor cells and fibroblasts [130]. These experiments confirm the existence of dominant E-box binding DNA-binding repressor proteins, which have been characterized and are known to include zinc finger proteins Snail, Slug, ZEB1 and Smad interacting protein (SIP1), and bHLH factors Twist and E47 [131]. Significantly, each of these proteins plays a key role in the EMT of development. Snail homologs are evolutionarily conserved in vertebrates and invertebrates, and the Snail protein was first identified in *Drosophila melanogaster*, in which it was shown to be essential for mesodermal formation [132]. It has since, with its structural homologue Slug, been shown to be essential for the initiation of EMT [133], and *Snail* knockout mice are embryonic lethal, failing to correctly complete gastrulation [134]. Zfh-1 genes *ZEB1* and *SIP1* are homologous to *Drosophila* zfh-1 gene, the loss of which induces spatial defects in the process of gastrulation [135]. *TWIST* knockout mice also have deficiencies in mesenchyme formation, although confined specifically to the head region [136]. These correlations with gastrulation and EMT indicate their importance in the regulation of E-cadherin expression.

During tumor metastasis, the physiological EMT is hijacked by cancer cells allowing them to downregulate E-cadherin and consequently to invade, migrate, intravasate and disseminate. As well as *CDH1* deletion and epigenetic silencing, aberrant overexpression of its transcriptional repressors is also widely associated with cancer pathogenesis. Snail has been shown to be overexpressed in a number of cancer cell lines including oral squamous cell carcinoma [137], melanoma cells [138] and HCC cells [139]. In human cancer specimens, Snail expression is correlated with E-Cadherin downregulation in gastric and breast cancers [140, 141] but not in one study in esophageal squamous cell carcinomas, in which an interesting association between Snail expression and *CDH1* hypermethylation was noted [142]. Snail expression has been further associated with tumor grade in invasive ductal breast cancer and hepatocellular carcinoma [143-145], and it was strongly and independently predictive of poor survival in two studies of 34 and 464 pathological breast cancer specimens [140, 146]. With the very recent development of specific murine Snail antibodies, it has been found to be overexpressed specifically at the invasive edge of tumors at the tumor-stroma interface, suggesting a role in invasive infiltration [147]. Other E-cadherin repressors including zinc finger proteins Slug and E47 have been shown to induce similar but not identical EMT-like changes to that of Snail when overexpressed, suggesting separate but not mutually exclusive roles for each in carcinogenesis [148]. Slug expression has been inversely correlated with E-cadherin expression as well as poor survival indices in breast cancer [149], esophageal squamous cell carcinoma [150] and lung adenocarcinoma [151], although this correlation was weaker than that of Snail in ductal breast cancers [143, 101]. It is also associated with increasing tumor grade in primary breast cancers and colorectal cancer without association to E-cadherin downregulation [152, 153]. SIP1 expression was shown to correlate with E-cadherin repression and poor prognosis in oral squamous cell carcinoma [154], and intestinal-type gastric cancer [141], and to suppress E-cadherin following conditional exogenous expression in MDCK cells, inducing invasiveness [155]. Twist has been established mainly to correlate with the increased expression of N-cadherin rather than decreased E-cadherin, suggesting its involvement in the cadherin switch [141], although siRNA to TWIST upregulated E-cadherin in prostate cancer cells, a disease in which TWIST is upregulated in 90% of pathological specimens [156]. δEF1/ZEB1 is known to be overexpressed in aggressive uterine cancers [157] and breast cancer specimens, in which it is associated with E-cadherin downregulation [158]. It also suppresses E-cadherin when ectopically expressed in breast cancer cells, while siRNA inhibition of ZEB1 de-repressed E-cadherin and restored cell-cell adhesion [158]. In colorectal cancers, however, it did not correlate with disease progression, Snail expression or E-cadherin expression, suggesting its involvement in a distinct subset of hormone-related cancers [159]. These lines of evidence indicate that E-cadherin restoration may be induced *via* the inhibition of its transcriptional repressors in cancer cells.

Therefore of the several cancer-related transcriptional repressors of E-cadherin, Snail protein is the most widely described. Affinity binding studies support this observation, in that Snail binding to the E-pal sequence is at least one or two orders of magnitude stronger than that of the next most

frequently identified repressors, E47 or Slug [160]. In fact, in addition to their critical role in repressing E-cadherin, Snail proteins induce all of the changes that correspond to the EMT, including the upregulation of pro-invasive matrix metalloproteins MMP1, MMP2, and MMP7 and mesenchymal markers vimentin and fibronectin [161]. This suggests a particular importance of Snail protein in antimetastasis therapy, since as well as its effects on E-cadherin expression, it has broadly pro-invasive properties, and is frequently overexpressed in invasive malignancies and cancer cell lines. Additionally its expression is influenced by multiple cell signaling pathways, which as we will discuss, makes it amenable to pharmacological targeting.

THERAPEUTIC UPREGULATION OF E-CADHERIN

Evidence of Inhibitory Effect of E-Cadherin Upregulation in Tumor Invasion

One of the key questions for the E-cadherin targeting therapy is whether upregulation of E-cadherin alone is sufficient to suppress the invasion and metastatic ability of cancer cells. The initial experimental evidence suggesting that E-cadherin played a causative role in suppressing invasion was elicited by transfecting E-cadherin deficient L-fibroblasts with full length F9 cell E-cadherin cDNA. The fibroblasts acquired strong cadherin-like calcium-dependent aggregating ability and underwent a morphological transition to a tightly interconnected colony compared with the motile, invasive phenotype of the parental cells [162]. Subsequently it was discovered that E-cadherin cDNA transfection into MDCK and murine mammary carcinoma cells reduced their ability to invade according to *in vitro* assays, which was reversible upon addition of antibodies to E-cadherin [163]. In the same article, the author transfected antisense E-cadherin cDNA into non-invasive transformed E-cadherin positive cells, resulting in partial E-cadherin downregulation and decreased invasive ability. Similar abolition of invasiveness has been obtained by transfecting E-cadherin cDNA into A549 lung cancer cells [164], bronchial carcinoma cells [165, 166], Dunning rat prostate cancer cells [167] and ovarian cancer cells [168], in each case invoking phenotypic changes reminiscent of the MET. In at least two further cases, E-cadherin transfection was associated with increased sensitivity to chemotherapy: to taxol in CHO cells [169], and EGFR-inhibitor gefitinib in lung cancer cells [170]. Likewise, the treatment of E-cadherin expressing endometrial carcinoma cells with E-cadherin antibody HECD-1 increased their invasiveness by 307% whereas it did not affect that of E-cadherin negative cell populations [171]. Additionally, E-cadherin antibody treatment disrupted the formation of multicellular spheroids of colorectal carcinoma cells in 3D culture [172], however on this occasion *loss* of E-cadherin was related to increased sensitivity to chemotherapeutic agents 5-FU, taxol, vinblastine and etoposide. Repression of E-cadherin expression by siRNA also potentiated invasive ability in gastric cancer cells, although the maximum effect was delayed until eleven days post-transfection, suggesting the involvement of complex regulatory alterations [173]. In pancreatic cell-line JHP-1, siRNA to E-cadherin induced an immediate increase in invasiveness without affecting proliferation [174]. Finally, introduction of dominant negative E-cadherin into epidermoid carcinoma cells induced EMT [175], and in squamous carcinoma cells increased migration 12-fold, induced nuclear β-catenin sequestration and increased MMP secretion [176, 177]. The increase in invasiveness associated with decreased E-cadherin is also found in wild type cells which have not undergone experimental manipulation: primary culture of bronchial carcinoma cells produced cells that either did or did not express E-cadherin; the latter was revealed in multiple assays to be more invasive and aggressive. Together these studies provide *in vitro* evidence to suggest that restoration of E-cadherin expression is sufficient to suppress invasion ability of cancer cells.

Subsequent *in vivo* experiments provided confirmation of the efficacy of E-cadherin restoration therapy. For example, when E-cadherin cDNA transfected MDA-MB231 breast cancer cells were injected into the left ventricle of nude mice, a dramatically reduced bone metastasis formation ability was observed compared with cells expressing low levels of E-cadherin [178]. Furthermore, when eight oral squamous cancer cell lines with differential expression of E-cadherin were implanted into SCID mice, the depth of invasion of the resulting tumor was inversely proportional to the expression of E-cadherin, although two E-cadherin negative cell-lines failed to grow [179]. In conclusive evidence that E-cadherin is causally involved in tumor invasion *in vivo*, Perl *et al.* performed a series of experiments on transgenic mice expressing a *Rip1Tag2* insulin promoter-stimulated tumorigenic SV40T antigen, which lead to pancreatic β-cell adenomas and invasive carcinomatous progression in 26% of mice. In an ingeniously designed experiment, they crossed these Rip1Tag2 mice with transgenic mice that expressed E-cadherin under the same promoter, producing upregulation of E-cadherin in the β-cells, and consequently reducing the number of mice that bore tumors to 8%. On the other hand, Rip1Tag2 mice engineered to express a dominant negative E-cadherin had invasive and even metastatic cancers in 50% of cases. Interestingly, the latter mice did not demonstrate tumor formation in the absence of SV40T transformation, demonstrating that loss of E-cadherin alone is not sufficient to provoke tumorigenesis [180]. The evidence therefore indicates that E-cadherin upregulation is a feasible method of suppressing tumorigenesis, invasiveness of cancer cells and metastasis.

Methods of Inducing E-cadherin Expression

The targeting of transcriptional repressor proteins has received a lot of recent attention, and in a recent review of the topic by Melnick *et al.*, a three level schema for developing novel treatments was proposed, classified by their proximity of action to the target gene, i.e. *CDH1*[181]. They proposed that since transcriptional repressors often involve co-repressor proteins and co-enzymes in a "repressor complex," each of these stages could be targeted with decreasing specificity. Since E-cadherin repression is known to occur by both epigenetic and transcriptional regulation, we adapt this model to create a four level model for the targeting of the repressed *CDH1* gene (Fig. (**2**)). As we will describe, the majority of the compounds that induce E-cadherin expression fit into this framework.

Level 1: Targeting epigenetic suppression

Level 2: Targeting transcriptional repressors of *CDH1*

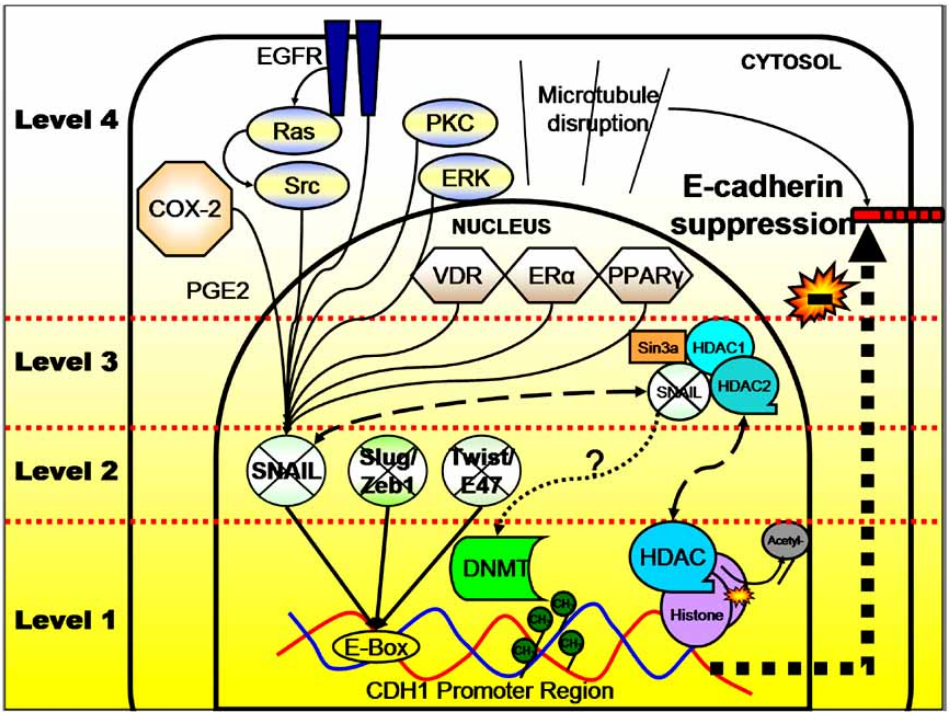

Fig. (2). Four Level Schema of E-cadherin Induction by Targeting Drugs. E-cadherin upregulation may be induced by drugs targeted at four operational levels. Level 1 is epigenetic regulation of DNMTs and HDAC which aberrantly silence *CDH1* in cancer cells. Level 2 is the direct inhibition of transcriptional regulator proteins including Snail, Slug, ZEB1, TWIST and E47. Level 3 is the targeting of Level 2 protein co-repressors, which include HDAC. Level 4 is the targeting of cell signaling cascades which converge on the common pathways of Levels 1-3. The overall effect of these processes in cancer cells is the aberrant repression of E-cadherin leading to the invasive phenotype of the EMT as shown in Fig. (**1B**).

Level 3: Targeting enzymes in the repressor complex

Level 4: Targeting cell signaling cascades that are effected by the repressor complex

Level 1: Targeting Epigenetic Silencing of CDH1

DNA Methyltransferase Inhibitors (DNMTi)

There are two key stages at which the gene silencing can potentially be targeted: the prevention or reversal of DNMT activity or the inhibition of histone modifications, and each of these methods has been shown to effectively upregulate E-cadherin. DNMTs have complex structures, and consist of a C-terminal catalytic domain and an N-terminal regulatory domain linked by a repetitive GlyLys region [182]. At least three functional DNMT enzymes exist including the commonest, DNMT1, and DNMT3a and DNMT3b [182]. It is thought that the former is mainly involved in maintaining DNA methylation, while *de novo* CpG methylation is thought to arise under the control of DNMT3a and DNMT3b, which have equal affinity for hemi-methylated and non-methylated deoxycytidine residues in DNA [183]. Anti-metastatic therapy targeting *CDH1* hypermethylation has the goal of inhibiting of DNMT1 to prevent the propagation of previously methylated DNA, although inhibition of DNMT3a and 3b theoretically has preventive value.

DNA methyltransferase inhibitors have been available for as long as 40 years, and hence are relatively well studied in tumor samples. The original nucleoside DNMT inhibitor to be described was 5-azacytidine, derived from the nucleoside cytosine. Common to this group of compounds, its mechanism of action is through incorporation into the DNA helix and methylation by DNMT enzymes, it then forms an irreversible covalent link with DNMT and traps the enzyme in complex with the DNA strand, leading to a rapid depletion of cellular DNMT [184]. This deficiency during the course of DNA replication leads to a global reversal of CpG methylation, which is then stably inherited by progeny. Since 5-azacytidine is a nucleoside, it must be converted to a nucleoside triphosphate and undergo catalytic conversion to the deoxyribose form before incorporation into genomic DNA. To prevent the incorporation of 5-azacytidine into RNA, which predisposes to ribosomal dysfunction, 5-aza-2'-deoxycytidine was developed, which also has been demonstrated to demethylate DNA more potently than 5-azacytidine (Fig. (**3**)) [184].

In terms of their activity on E-cadherin, both 5-azacytidine and 5-aza-2'-deoxycytidine are able to restore its expression and revert cancer cells to the epithelial phenotype in cell lines including breast [77], prostate [77], esophageal [88], gastric [185], leukemia [117], and renal cell carcinomas [112]. In an *in vivo* model of breast cancer using cell-line MDA-MB-435S, 5-aza-2'-deoxycytidine was able to restore E-cadherin expression and suppress metastasis formation as well as primary growth, possibly through E-cadherin upregulation [186]. It also increased cellular adhesion in gastric cancer cells, although since their "metastatic" model relied on intraperitoneal injection of cell suspensions, the increase in tumor adhesion actually enabled peritoneal implantation,

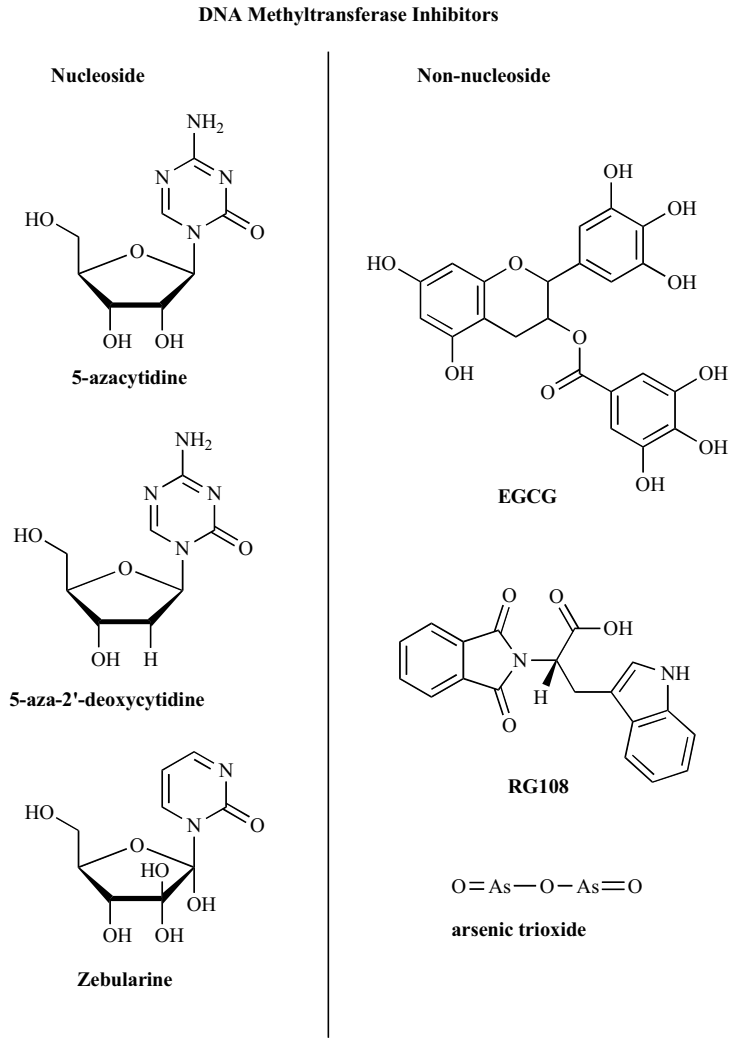

Fig. (3). Representative structures of DNA methytransferase inhibitors.

an adverse outcome which may not be present in spontaneous cancers [185]. A common problem in these compounds is their adverse toxicities that include myelosuppression and high-grade nausea and vomiting, therefore several novel nucleoside DNMTs have been developed including Zebularine, which has shown the ability to shrink tumors in mice and is less toxic than the conventional agents, although its effects on E-cadherin expression have yet to be studied [187].

Since the incorporation of atypical nucleosides into the DNA strand is associated with severe adverse effects, there has been a great deal of interest in developing enzyme blocking agents that interact directly with the DNMTs, i.e. non-nucleoside DNMTi (for structure detail, see Fig. (**3**)). Recently, the tertiary structure of human DNMTs was discovered, leading to the identification of small molecule inhibitor RG108 during high throughput *in silico* testing [188]. As yet, due to the novelty of this compound, very few studies on human cancer cells have been performed and the ability of RG108 to upregulate E-cadherin is unknown, however it is an attractive candidate for further investigation. One non-nucleoside DNMTi that has received a lot of recent attention is the green-tea derived compound, (-)-epigallocatechin-3-gallate (EGCG), which has been demonstrated to induce the expression of E-cadherin in human HepG2 hepatoma cells [189], and oncogenic-Ras transformed intestinal cells [190], decreasing tumor cell proliferation in the latter study. Oral therapy with EGCG increased E-cadherin expression in a mouse model of intestinal cancer, and also increased *in vitro* expression of E-cadherin in colon cancer cells [191]. EGCG has been demonstrated to inhibit DNA methyltransferase in human cancer cell lines and examination of its chemical structure in molecular modeling studies has demonstrated a close fit in the active enzyme site of DNMT1 [192]. A further suggested DNMT inhibitor is arsenic trioxide, which induced DNA hypomethylation in Fisher 344 rats [193], and has been demonstrated to reverse methylation of *CDH1* in human hepatocellular carcinoma cells *via* a suppression of DNMT1 transcription, inducing the re-expression of E-cadherin [194].

DNMTi drugs have been frequently proposed to be useful drugs for inclusion in combinatorial regimes, and two studies have demonstrated synergistic combinations to 5-aza-

2'deoxycytidine with docetaxel in lung, breast and prostate cancer cells [195], and the HDACi depsipeptide in Raji lymphoma cells [196] and breast cancer cells [197]. Both combinations induced additive upregulation of E-cadherin and consequent suppression of tumor growth. As we previously discussed, CpG methylation works in concert with a variety of cofactors to induce gene silencing, and once such area that has received a great deal of recent attention is the histone modification proteins, HDAC and HMT, since histone modification appears to be the final effector of the hypermethylation signal. In fact, regimes targeting both steps of this two-stage process have been proposed as effective novel treatment strategies in several malignancies, most notably hematological cancers, and are undergoing early clinical trials [198].

Histone Deacetylase Inhibitors (HDACi)

In addition to their synergy with DNMTi, HDACi have been shown in preclinical studies to selectively target cancer cells with high specificity. Their various effects include the induction of apoptosis and cell cycle arrest, and suppression of tumor angiogenesis, metastasis and invasion at least partially through their potent capacity to upregulate E-cadherin [196]. There are at least 18 human HDAC molecules, separated into four classes based on their homology to yeast HDACs, subcellular localization and chemical activities, although their individual functions remain elusive. Class I HDAC are mainly localized in the nucleus, whereas Class IIa may shuttle between the nucleus and cytoplasm and Class IIb are localized in the cytoplasm, and it is the inhibitors of the nuclear species of HDAC that have most frequently been proven to induce E-cadherin re-expression. The chemical structures of effective HDACi are shown (Fig. (**4**)).

The first HDACi to be identified was dietary component butyrate, which was first demonstrated to be a differentiating agent before subsequently being shown to induce histone hyperacetylation. Butyrate, along with valproic acid, is selective for Class I and IIa nuclear HDAC. The link between butyrate and E-cadherin upregulation was first discovered in breast cancer cell-lines YMB-S and ZR-75-1S, in which it induced cell-cycle arrest and increased cell-cell adhesion which was reversible upon addition of E-cadherin antibodies,

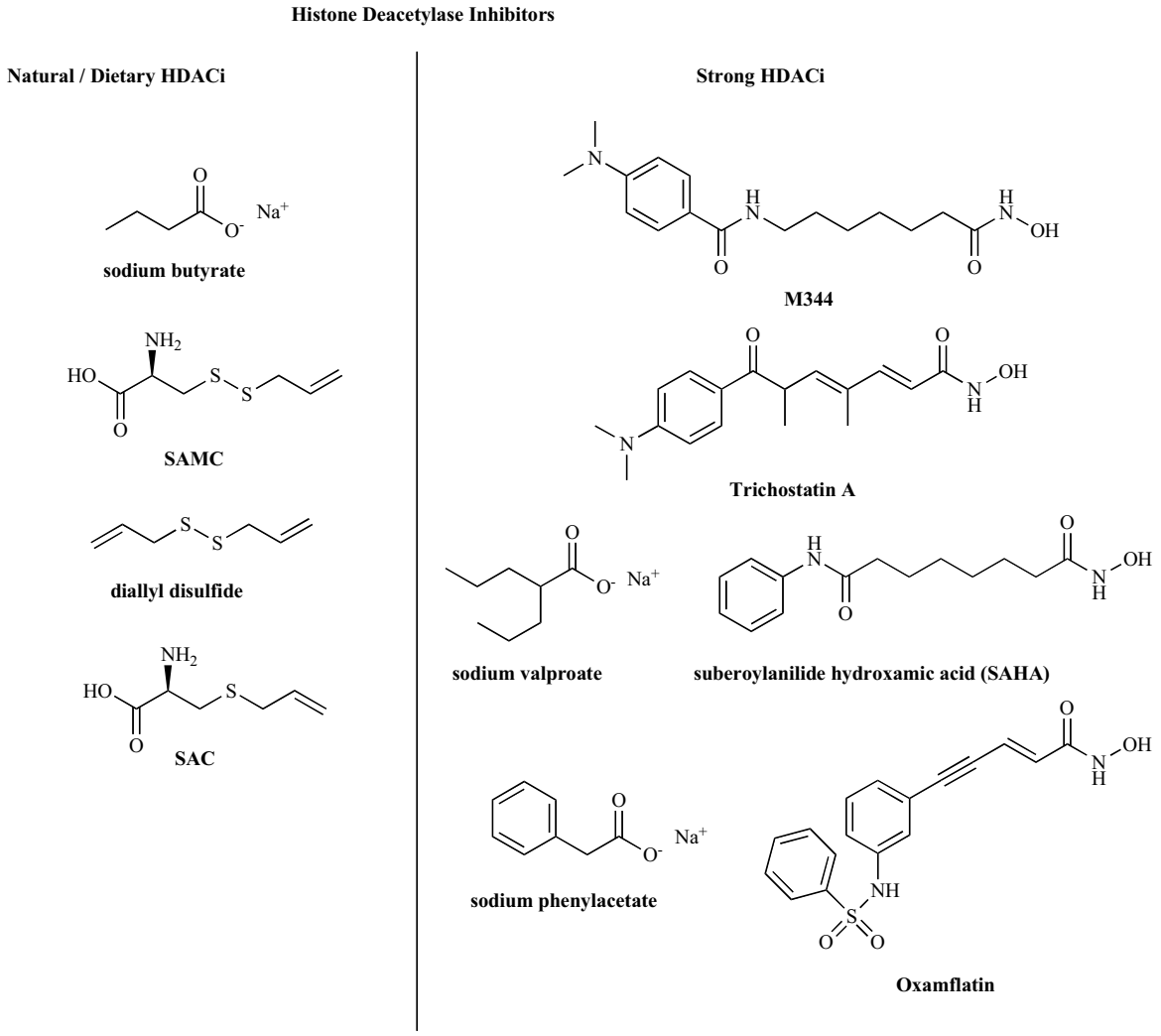

Fig. (4). Representative structures of histone deacetylase inhibitors.

although E-cadherin protein level itself was not found to be elevated, suggesting a functional alteration [199]. Subsequently, butyrate pro-drug tributyrin was found to upregulate E-cadherin expression by 100% in colon cancer cells [200], and similar E-cadherin induction was induced by sodium butyrate treatment in HCC cells [201], and endometrial carcinoma cells [202]. In colorectal carcinoma cells, butyrate is associated with the functional restoration of E-cadherin-catenin complexes at the cell membrane, as well as enhanced cell-cell interaction and reversal of invasive phenotype [203]. Other Class I-specific inhibitors that have been shown to stimulate E-cadherin expression include depsipeptide [196]. A number of other strong HDACi exist, specifically Trichostatin A and suberoylanilide hydoxamic acid (SAHA), which have potent HDACi activity in all three categories, and they are capable of stimulating E-cadherin upregulation in endometrial carcinoma cell lines [202]. Valproate, a frequently used mood stabilizing and anti-epileptic drug with HDACi properties, was also demonstrated to have strong E-cadherin restorative properties in the same study, and additionally was able to limit tumor growth *in vivo*. The same authors discovered that novel HDACi M344 had similar E-cadherin upregulating properties, although it is not known which group of HDAC this experimental compound targets [204].

In addition to these well-described HDAC compounds, a number of other compounds that upregulate E-cadherin probably do so through their HDACi effect. Sodium phenylacetate, which is known to have HDACi properties, upregulated E-cadherin expression in deficient breast cancer cell-lines and produced G0/G1 cell cycle arrest [205]. Dietary garlic compound diallyl disulfide (DADS) has been shown to catabolize to S-allylmercaptocysteine (SAMC) and S-allylcysteine (SAC) *in vivo*, and the latter two metabolites have HDACi activity [206, 207]. Recently, SAMC and SAC have been demonstrated to induce E-cadherin upregulation and suppress invasiveness of advanced prostate cancer cells *in vitro* [208], and recent unpublished data from our laboratory suggests that they may have potent anti-metastatic activity *in vivo*. Dietary HDACi, which also include butyrate, are a particularly interesting area of research, since humans who consume high levels of them are theoretically predisposed to epigenetic alterations including globally reduced histone acetylation [206]. Although the effects of long term HDACi consumption are unknown, high levels of garlic consumption have been correlated with low rates of gastric and prostate cancer in several epidemiological studies, although there is insufficient evidence at present to attribute this to their HDACi activity.

Level 2: Targeting Transcriptional Repressors of CDH1

As discussed previously, E-cadherin transcriptional repressors, most notably Snail, are frequently overexpressed in cancer cells and potently repress E-cadherin expression. At present there are no small molecule pharmacological compounds available to target these molecules, partially because their crystal structure has yet to be elucidated. A number of experiments have however established the potential efficacy of Snail inhibition by using genetic manipulation of *Snail* to demonstrate the reversibility of its role in the E-cadherin loss and cellular invasiveness of cancer. This link was first discovered with the observation that epithelial cells ectopically expressing Snail protein adopted tumorigenic and oncogenic properties [209]. *Snail* knockout from various malignant cell lines by siRNA or antisense *Snail* cDNA transfection induced re-expression of E-cadherin [130, 138, 210], whereas ectopic Snail expression repressed E-cadherin and induced EMT in ovarian cancer cells and colorectal cancer cells [211, 212]. Poignantly, in an intricately performed experiment using a mouse model of HER2/neu-induced breast cancer, Snail protein was found to be upregulated 9-fold in tumor recurrences and associated with EMT. Furthermore, xeno-grafting *Snail*-transfected breast cancer cells into mice massively increased the risk of tumor recurrence after removal (17/20 in transfected group *vs*. 0/20 of empty vector transfectant controls), and was accompanied by the corresponding loss of E-cadherin, denoting the specific role for Snail in oncogenic E-cadherin suppression [140]. Experimental knockout of Snail by treating colorectal cancer-prone MIN mice with antisense phosphorodiaminate morpholino oligomer to Snail induced E-cadherin increase and significantly reduced the number of spontaneous tumors identified (22% *vs*. 54%) [210]. Conversely, mice engineered to express Snail protein at 20% higher than normal levels had normal anatomy, but showed an increase in both epithelial and mesenchymal tumors [213]. The evidence therefore suggests that pharmacological compounds designed to inhibit Snail protein activity could potentially have an advantageous impact on cancer metastasis and consequently mortality, therefore a priority in this research would be the elucidation of its tertiary structure.

Level 3: Targeting Enzymes in the Repressor Complex

The mechanism of action of *CDH1* transcriptional repressors was originally thought to be simple mechanical obstruction of the RNA polymerase machinery; however recent studies have demonstrated that the truth is far more complex. In fact, Snail proteins form a repressor complex at the *CDH1* gene promoter in association with Sin3A and two histone deacetylase inhibitors, HDAC1 and HDAC2 [214]. This is analogous to the silencing mechanism of CpG hypermethylation, which also induces HDAC activity *via* MeBPs, suggesting that HDAC enzymes are in fact the common effectors of the epigenetic and transcriptional pathways of repression, acting as both Level 1 and Level 3 inhibitors of E-cadherin expression. Since HDACi and DNMT show synergistic inhibition of the CpG methylation repressor complex [196], it is also likely that a combination of HDACi and Snail inhibitors would be a particularly potent method of inducing E-cadherin expression by targeting the Snail repressor complex at two levels. This would have the added benefit of E-cadherin gene-specificity, which is lacking with the former approach. As well as their association with HDACi, E-cadherin transcriptional repressors are increasingly suspected to induce DNA hypermethylation *via* direct or indirect interaction with DNMT. In at least one study, CDH1 CpG hypermethylation was directly correlated to Snail expression [142], hinting at the presence of transregulation. Although further study is needed in this area, defining the presence of an interplay between these two pathways would greatly assist our understanding of *CDH1* gene silencing, potentially enabling the development of specific and efficacious E-cadherin upregulating drug combinations.

Level 4: Targeting Cell Signaling Cascades

Although to this point we have mainly focused on discussing regulation of E-cadherin at the level of direct gene control, there are a number of effective E-cadherin upregulating agents that operate through upstream pathways which converge on the *CDH1* promoter. Although one would suspect that such compounds would be poorly specific and therefore ineffectual in the treatment and prevention of cancer, in fact the opposite is true, since many of the pathways involved in E-cadherin suppression are themselves also involved in the pathogenesis of invasive cancer through a variety of other mediators. These include the cyclo-oxygenase 2 (COX-2) pathway, the epidermal growth factor receptor (EGFR) pathway, nuclear hormone receptors including the Vitamin D receptor (VDR), estrogen receptor (ER) and peroxisomal proliferator-activated gamma receptor (PPARγ) pathways as well as their cell signaling cascade intermediaries. This category of drugs is particularly important due to the number of pre-existing pharmacological compounds that are capable of modifying their activity. As we will describe, many of these compounds have anti-cancer effects characterized by increased E-cadherin expression and suppression of cellular invasiveness.

COX-2 Inhibitors

COX enzymes catalyze arachidonic acid to prostaglandins. There are three COX enzymes in humans, of which only COX-2 is inducible. COX-2 is overexpressed in a number of cancers, including lung, prostate, colon and breast cancer in proportion to disease stage, and COX-2 inhibitors have been demonstrated in experimental models of lung cancer to suppress metastasis. The anti-metastatic effect of COX-2 inhibition is very interesting, and recent evidence suggests the involvement of E-cadherin. COX-2 expression is inversely proportional to E-cadherin expression in transitional cell carcinomas [215], and NSCLCs [216]. The interplay between COX-2 and E-cadherin was confirmed by the exogenous expression of COX-2 in renal cell carcinoma cells, which eliminated E-cadherin expression, while the reverse was true on exogenous expression of antisense COX-2 [217]. The mechanism for this repression was recently elucidated by Dohadwala *et al.*, who demonstrated that COX-2 overexpression inhibits E-cadherin expression *via* the direct upregulation of Snail and ZEB1 and induction of their binding to the *CDH1* promoter, inducing a motile, invasive phenotype and eliminating cell clumping [216]. Significantly this was reversible on addition of selective COX-2 inhibitor celecoxib, supporting the anti-metastatic role of COX-2 inhibitors. A variety of NSAID COX-2 inhibitors have been shown to induce E-cadherin expression. Aspirin and sulindac were the first COX-2 inhibitors shown to upregulate E-cadherin, and were found to simultaneously repress invasiveness including MMP2 downregulation in Hep G2 cells [218]. Also, etodolac was able to induce E-cadherin expression in a non-expressing gastric cancer cell line [219], sulindac derivate IND12 was able to induce E-cadherin expression in Ras-transformed MDCK cells [220], and unselective COX inhibitor indomethacin significantly upregulated E-cadherin in colon cancer cells [221]. Their structures are shown in Fig. (**5**). It is likely that all COX-2 inhibitors share this E-cadherin upregulating activity, since E-cadherin repression has also been demonstrated following treatment of cancer cells with COX-2 metabolite PGE2 [216]. COX-2 inhibitor trials are currently underway for the treatment of NSCLC, the results of which are awaited. However, although COX-2 inhibitors demonstrated significant tumor preventive activity in previous clinical trials in colorectal cancer, they also induced a well publicized increase in adverse cardiovascular events which was of sufficient severity to preclude their use in this function at the present time [222].

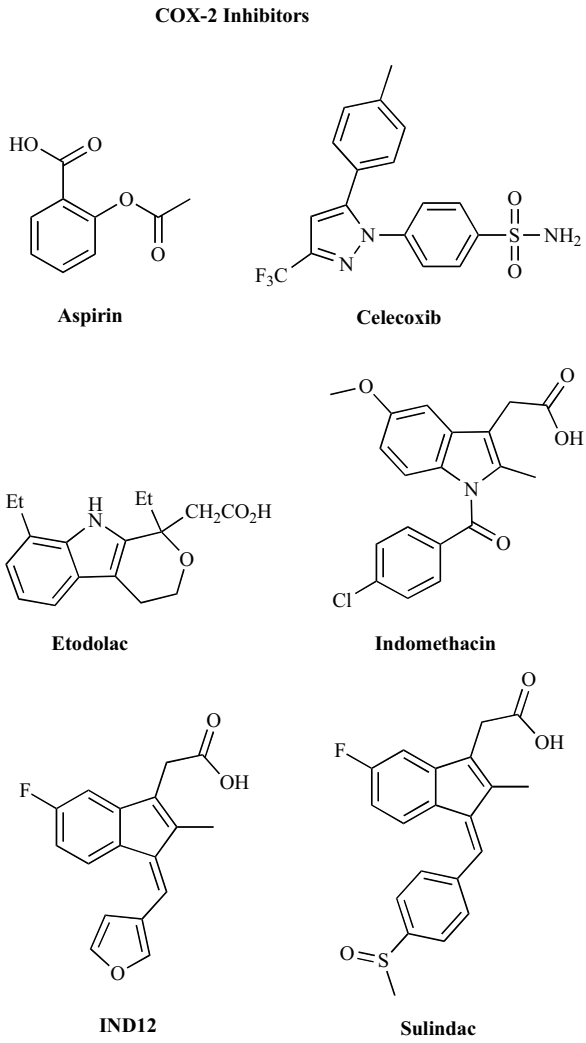

Fig. (5). Representative structures of COX-2 inhibitors.

EGFR Inhibitors

EGFR signaling is known to be altered in a variety of cancers and its activation is known to induce oncogenesis. There are several subtypes of EGFR, including ErbB1, ErbB2, ErbB3 and ErbB4, which are ligands for EGF-like growth factors, which include EGF, TGFα and amphiregulin. Once the ligand is bound the EGFR dimerizes and induces a highly complex signaling cascade through the MAPK/Erk, AKT/PKB, JAK/STAT and PLCγ pathways (reviewed in [223]). Although the effects of EGFR activation are widespread, transient activation induces the redistribution of

Caveolin-1 from the cell surface to the endocytic compartment, with the effect of E-cadherin inactivation and internalization. Chronic EGF activation also suppresses E-cadherin expression, and eventually induces EMT, a process recently demonstrated to be dependent on Caveolin-1 activity, which induces potent upregulation of Snail and consequently downregulation of E-cadherin [224]. Consequently, one method of inducing EMT reversal and E-cadherin upregulation in EGFR-expressing cells is *via* the inhibition of EGFR-dependent Snail induction. Previous evidence supports this notion, with ErbB1 receptor antibodies upregulating E-cadherin expression in EGFR positive breast and lung cancer cell lines respectively [225, 226]. Furthermore, specific ErbB2 antibody trastuzumab, which is licensed for use in treating Her2 (ErbB2) receptor positive breast cancer, also induced E-cadherin upregulation accompanied by decreased cellular invasiveness [227]. Moreover, EGFR (ErbB1 and ErbB2) destabilizing agent geldanamycin (Fig. (**7**)) induced E-cadherin expression in NSCLC and melanoma cells, and increased cell-membrane sequestration of proteasome-resistant mutant β-catenin, suppressing its activity on TCF/Lef Wnt pathway activation [228, 229]. An additional proposed mechanism of E-cadherin regulation by EGFR is that they interact directly at the cell membrane, although evidence supporting this hypothesis is scarce [230, 231]. Together these results indicate that one mechanism of action of the high-efficacy EGFR inhibitors is to reverse EMT *via* pathways that center on *Snail* suppression, which at least partially explains their clinically proven ability to suppress metastasis. EGFR inhibitors are an increasingly important area of anti-cancer treatment, with EGFR antibodies such as cetuximab, and receptor tyrosine kinase (RTK) inhibitors such as gefitinib demonstrating highly promising results in the treatment of NSCLC, and Her2/neu receptor antibody trastuzumab having high efficacy in breast cancer.

Targeting Nuclear Hormone Receptors (NHR): Estrogen, Progesterone, Steroids

The estrogen signaling pathway has also been demonstrated to regulate E-cadherin in breast cells *via* its activity on the Snail gene. Ligand-binding of the NHR estrogen receptor alpha, ERα, activates transcription of MTA3, which then complexes with the Mi-2/NuRD complex which has HDAC and chromatin remodeling properties, one of the key effects of which is the repression of Snail transcription [232]. Indeed ER, MTA3, Snail and E-cadherin expression are correlated as would be predicted by this sequence of events, in that ER loss leads to loss of MTA3, expression of Snail, and loss of E-cadherin in breast cancer patients [232]. The clinical significance of this data is however equivocal, since estrogen antagonist tamoxifen was shown to upregulate E-cadherin, while estradiol treatment repressed its expression in breast cancer cells [233], and additionally ER positive tumors respond well to anti-estrogen treatment, suggesting that the ER is more likely to repress E-cadherin in breast cancers. This said, one study showed that 8-prenylnaringenin (8-PN), a phytoestrogen found in hops and beer stimulates E-cadherin-dependent aggregation and growth of MCF tumor cells in suspension [234], and in prostate cancer cells, testosterone metabolite 5α-androstane-3β,17β-diol binds estrogen receptor, increases E-cadherin and inhibits migration [235]. Other hormones also may have some effect on E-cadherin expression, for example in the MDA-MB-231 breast cancer cell-line engineered to express progesterone receptor, E-cadherin expression was upregulated upon addition of progesterone [236]. Furthermore, a single article demonstrated that corticosteroid dexamethasone repressed the invasiveness of fibrosarcoma cells, accompanied by a drastic increase in E-cadherin expression, although the precise mechanism was not examined [237]. The ligand structures are shown (Fig. (**6**)).

Targeting Nuclear Hormone Receptors: PPARγ

Peroxisomal proliferator-activated gamma receptor (PPARγ) is a nuclear hormone receptor which is activated by peroxisomal proliferators, which include long chain polyunsaturated fatty acids, arachidonic acid metabolites (not PGE2) and components of oxidized low density lipoproteins, as well as by the thiazolinedione (TZD) class of drugs and certain NSAIDs. It is expressed at low levels in human malignancy and its activation has been described to induce anti-proliferative, pro-apoptotic, anti-angiogenic and pro-differentiation alterations in tumor cells in culture (reviewed in [238]). The mechanism by which it achieves its differentiation effects are not well characterized, but the TZD PPARγ ligand is known to upregulate E-cadherin expression and induce its localization to the cell membrane in colorectal and pancreatic cancer cell lines, accompanied by β-catenin sequestration [239, 240]. Despite these described pro-differentiation effects and other anti-cancer properties, results of PPARγ studies *in vivo* and in clinical experiments on liposarcoma, prostate and breast cancers have been equivocal, with some results demonstrating beneficial results and others finding no benefit. Additionally, TZD itself was withdrawn from the market due to hepatic side effects, although structural homologs rosiglitazone and pioglitazone remain on the market as hypoglycemic drugs (structures shown in Fig. (**6**)). At present there is no evidence to recommend their use in cancer therapy, although elucidating the mechanism of their E-cadherin upregulating property remains a priority.

Targeting Nuclear Hormone Receptors: Vitamin D Receptor

The prevalence of several cancers has been shown to inversely correlate to sunlight exposure (the source of >90% of Vitamin D), suggesting a role for Vitamin D in the prevention of cancer [241]. Vitamin D receptor is expressed in tumor cells and its activation produces growth arrest, apoptosis and terminal differentiation in cancer cell lines. Additionally a number of cancer cell lines aberrantly possess the parathyroid-specific ability to convert the physiological form of vitamin D, 25-hydroxyvitamin D, into its bioactive form 1, 25-dihydroxyvitamin D (calcitriol). One of the important alterations associated with VDR activation is the upregulation of E-cadherin, which is associated with decreased invasiveness and inhibition of cell growth in prostate, breast and colorectal cancer cell lines following activation by 19-norhexafluoride Vitamin D3 and calcitriol (Fig. (**6**)) [242-244]. Unfortunately the clinical application of calcitriol and many of its >1000 analogues is limited by their hypercalcemic effects which induce renal impairment and mental state alteration. Consequently a huge amount of effort has been placed

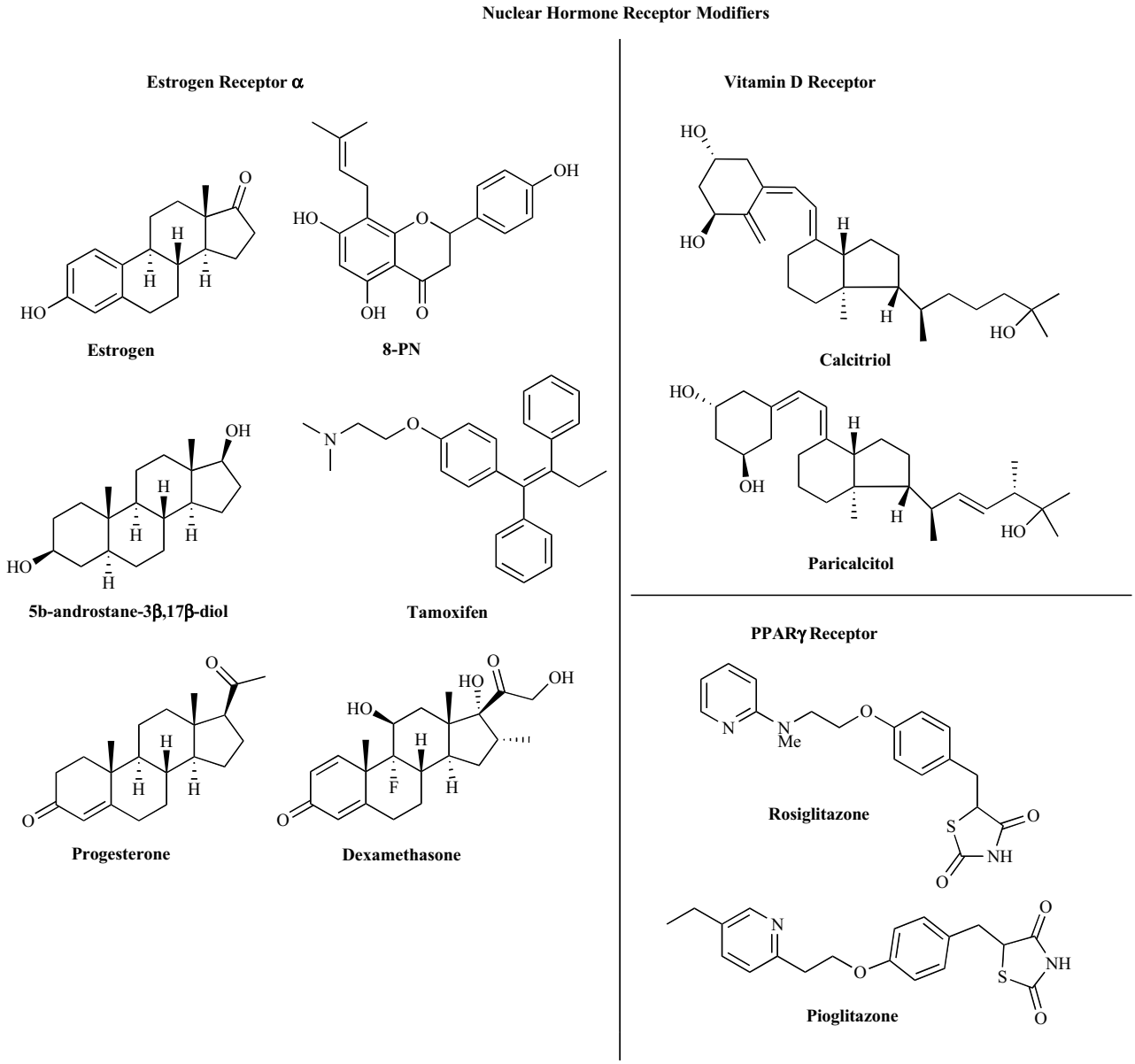

Fig. (6). Representative structures of nuclear hormone receptor modifiers.

in developing novel Vitamin D analogues which lack this effect (reviewed in [245]). Although their effect on E-cadherin indicates a specific role for Vitamin D analogues in antimetastasis therapy, caution should be applied, since in colon cancer cells it has been shown that Snail binds to the *VDR* gene and represses its expression. This suggests that cells in which Snail is upregulated may be resistant to the invasion-suppressive effects of VDR activation [243]. Clinical studies support this notion, including evidence that Snail is inversely correlated with VDR expression in clinical samples [246], therefore further evidence is required in this area before the role of VDR analogues can be fully defined.

Receptor Signal Transduction Inhibitors and E-Cadherin

In addition to targeting of cell-surface signaling and nuclear transcription factors, there are several compounds that upregulate E-cadherin *via* their effects on signal transduction molecules (Fig. (**7**)). *Ras* was one of the first oncogenes to be discovered and is involved in signal transduction from the cell surface to the nucleus including signals from growth factor receptors and G-protein coupled receptors. It is involved in cancers of many types including the majority of colon and pancreatic cancers (reviewed in [247]). Ras activation downregulates E-cadherin, and it has been shown that this occurs through the activation of Snail-family transcriptional repressors [248]. Consequently, it was recently demonstrated that Ras farnesylation inhibitor FTI-277 significantly increased E-cadherin expression and stabilization as part of the AJ complex, and additionally had a potent antimetastatic effect *in vivo* on liver metastases following splenic inoculation of epithelial carcinoma cells [249]. The same authors as well as another group also demonstrated that se-

Growth Factor and Cell Signaling Cascade Inhibitors

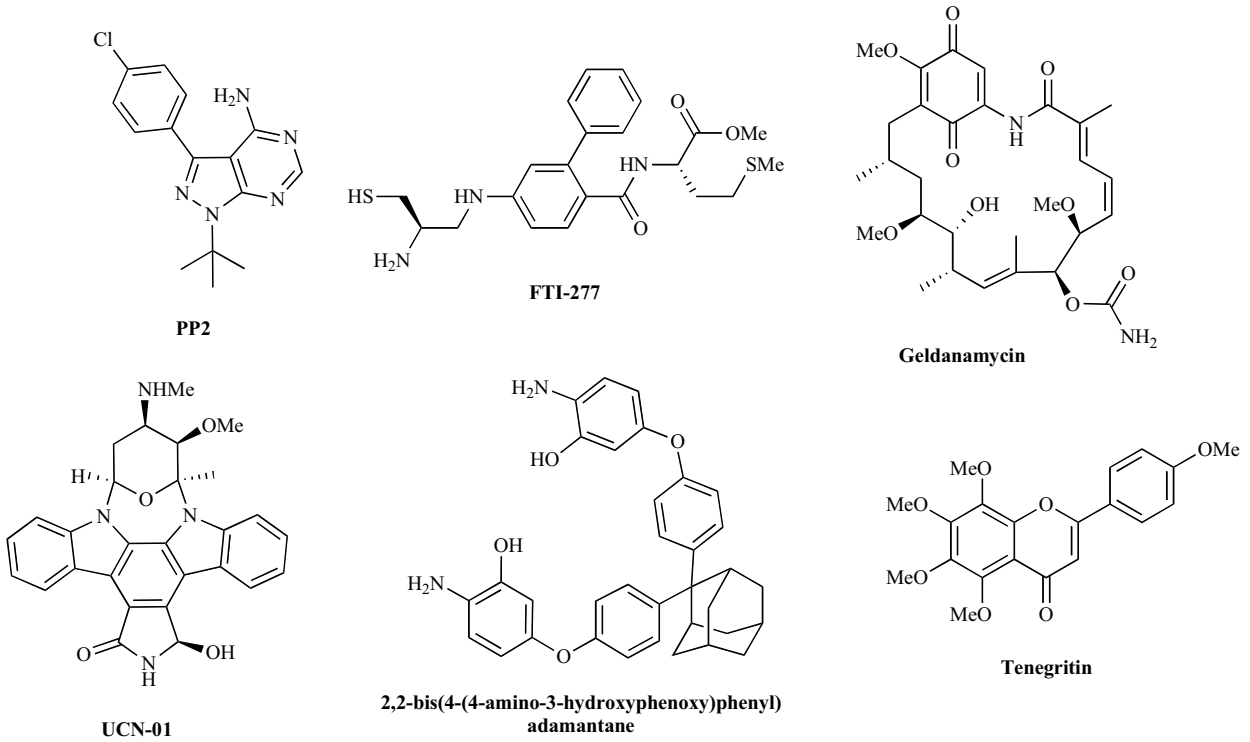

Fig. (7). Representative structures of growth factor and cell signaling cascade inhibitors.

lective Src tyrosine kinase inhibitor PP2 also induces E-cadherin at protein and mRNA level and potentiated cell clustering in colorectal, breast and hepatocellular carcinoma cells [250, 251]. *Src* overexpression has been demonstrated to induce invasive phenotype and E-cadherin downregulation, and although the mechanism by which it achieves this is not precisely understood, it is likely to involve the activation of Snail [252]. PKC is also involved in growth factor signal transduction, and broad spectrum PKC inhibitor 7-hydroxystaurosporine (UCN-01) decreased invasiveness in glioblastoma cells in conjunction with E-cadherin restoration [253]. Its application in humans is precluded by its limited bioavailability due to excessively strong binding to plasma proteins, although multiple other promising compounds are under development at present [254]. The citrus methoxyflavone molecule tangeretin also upregulates E-cadherin and has significant anti-invasive effects on breast cancer cell lines [255]. Recent data indicate that it achieves its therapeutic effects through the highly specific inhibition of ERK, which is a cell signaling intermediary located downstream of Src and Ras [256]. Although this molecule shows highly promising anticancer effects in a number of cell models, its use in breast cancer will be limited by its interference with the anticancer activity of tamoxifen [257]. Finally, the complex adamantine derivative 2,2-bis(4-(4-amino-3-hydroxyphenoxy)phenyl) adamantane (DPA) irreversibly arrest cells at G0/1 associated with increased E-cadherin expression, and demonstrates tumor growth inhibition *in vivo* in colorectal cancer xenografts [258]. The mechanism by which it achieves this is not currently known, but is likely to involve the inhibition of intracellular signaling pathways.

E-Cadherin Inducing Drugs of Unknown Mechanism

With increasing research interest in targeting E-cadherin for the treatment of human cancer, several new compounds have recently been demonstrated to upregulate E-cadherin through mechanisms that remain to be identified. For example, Indole-3-carbinol (I3C), which is a phytochemical agent found in cruciferous vegetables. It induced E-cadherin upregulation in breast cancer cells and both *in vitro* and *in vivo* indices of metastasis, including suppression of lung metastases in a mouse model of metastatic breast cancer [259, 260]. Chlorophyllin, a water soluble derivative of plant pigment chlorophyll, which has anticancer and cancer preventive properties, induces the expression and membrane translocation of E-cadherin in colorectal cancer cells by an unknown mechanism and has a promising lack of toxicity [261]. Xanthohumol, a prenylated chalcone derived from hops (but not present in beer), quenched the invasiveness of breast cancer cells by inducing E-cadherin in an effect that was reversible upon addition of E-cadherin antibodies [262]. Although the speculated mechanism was through effects on COX-2 or estrogen receptor, the mode of action requires clarification. Curcumin is another dietary agent that upregulates E-cadherin in melanoma cells, and decreases invasiveness *in vitro* and metastasis *in vivo*. It is obtained from the rhizome of Curcuma longa L. and has widely described anticancer and antiangiogenic properties, due to a broad inhibitory spectrum on cell signaling molecules including NF-κB, JNK and PKC, which are intermediaries in growth factor signaling, probably including upstream regulation of *Snail* transcription (reviewed in [263]). A further natural com-

pound that induces E-cadherin expression is norcantharidin, an isolate from blister beetles (non-dietary!) with known anticancer and pro-apoptotic properties, which in this study included a potent anti-metastatic effect both *in vitro* and *in vivo* in colorectal cancer cells, although its mechanism remains elusive [264]. The structures of these compounds are shown (Fig. (**8**)).

Several conventional chemotherapeutic drugs such as microtubule stabilizing taxane chemotherapy agents paclitaxel and docetaxel have been shown to induce E-cadherin upregulation in colorectal, prostate and melanoma cells [265-267], and additionally, docetaxel upregulates E-cadherin expression at the cell membrane on *in vivo* PC-3 prostate cancer xenografts (Fig. (**9**)) [266]. The mechanism by which it achieves this has not been elicited, however evidence suggests that microtubules are responsible for both the concentration of E-cadherin at the AJ [268], and the internalization and disassembly of AJ-associated E-cadherin [269], suggesting that microtubule disruption may increase E-cadherin by enabling the accumulation of E-cadherin at the cell membrane without providing the motor activity necessary to remove it. Accordingly, the former authors demonstrated that E-cadherin mRNA was not increased following treatment of human colonic epithelial cells with docetaxel or paclitaxel, although since non-tumor derived cells were used, the possibility of tumor-specific activity cannot be excluded. Interestingly, E-cadherin upregulation also sensitizes cancer cells to the effects of taxol, although the mechanism by which it achieves this effect is unclear [172]. Other chemotherapy agents also induce E-cadherin expression. The anthracycline doxorubicin, a topoisomerase II inhibitor chemotherapeutic agent (Fig. (**9**)), induces E-cadherin expression in breast cancer cells [270], although not in prostate cancer cells. The mechanism by which it induces E-cadherin upregulation is unclear [271]. Interestingly, radiotherapy is also known to induce upregulation of E-cadherin in the A549 lung cancer cell-line [272], and pre-operative chemoradiotherapy treatment of Barrett's esophagus induced the upregulation of E-cadherin [273]. The prevalence of E-cadherin upregulation following destructive cytotoxic or irradiation therapy in cancer cells suggests that E-cadherin upregulation may have an additional role in cancer cell death, further supporting its use as a therapeutic target, and perhaps explaining its chemosensitization effect. Although E-cadherin upregulation is unlikely to be the major therapeutic mechanism of these conventional treatment strategies, knowledge of this particular property may help to design combinatorial regimes with other E-cadherin inducing agents to produce maximal anti-metastatic effects. Furthermore the suggestion that E-cadherin may be involved in treatment-related cancer cell death indicates that specific E-cadherin upregulating agents may have synergistic cytotoxicity with currently available chemotherapy regimes.

CONCLUSIONS

Although the role of E-cadherin in epithelial tissue organization has long been suggested, its participation in the metastatic progression of human cancers has only been firmly established over the last decade. Based on the evidence reviewed in this article, we conclude that epigenetic inactivation of E-cadherin is one of the most common features of advanced metastatic cancers (Tables **1,2**), and there exists a broad body of evidence to support its active involvement in the pathogenesis of cancer metastasis. Accordingly, results from a number of *in vitro* studies demonstrate the possibility of inhibiting metastatic growth of cancer cells through restoration of E-cadherin expression. The limited *in vivo* studies on animal models also provide supporting evidence to sug-

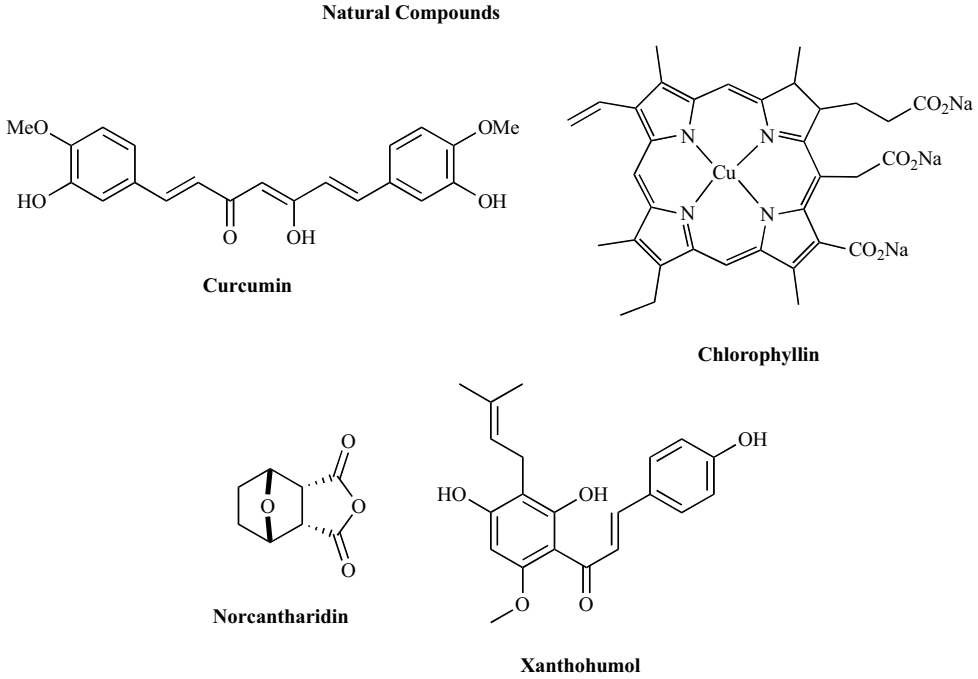

Fig. (8). Representative structures of natural compounds that upregulate E-cadherin.

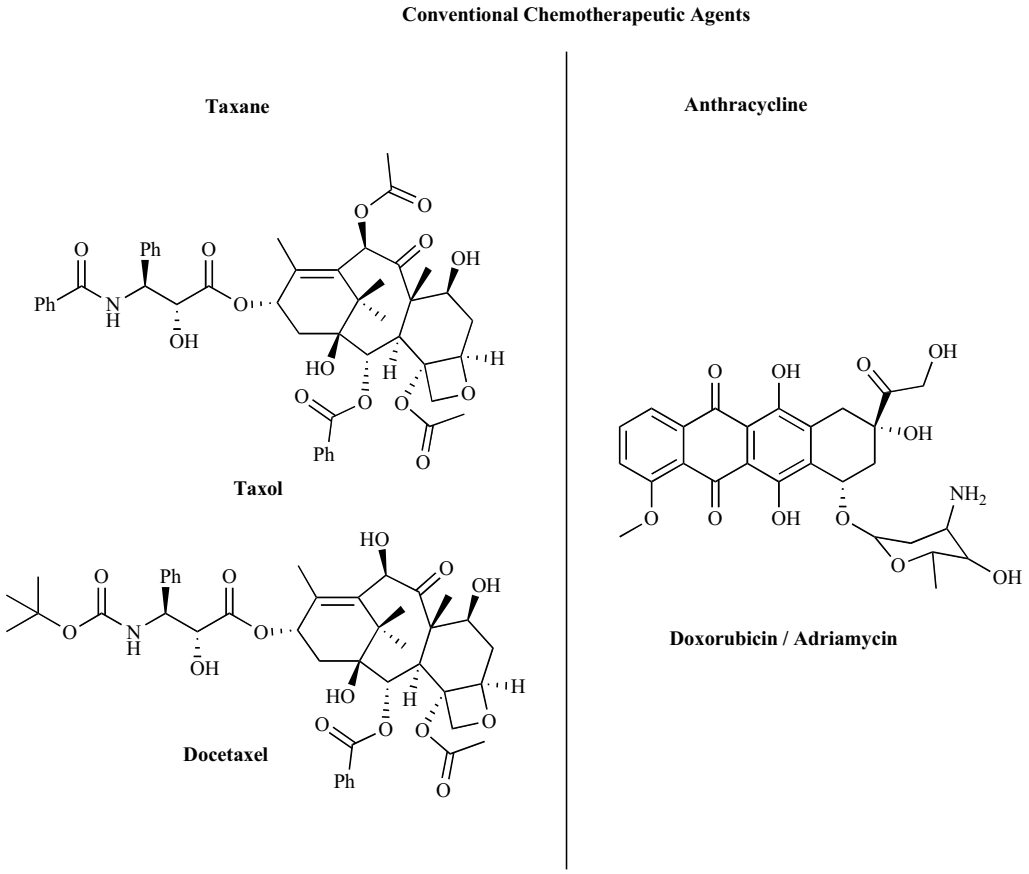

Fig. (9). Representative structures of conventional chemotherapeutic agents that upregulate E-cadherin.

gest that E-cadherin is a novel potential therapeutic target against advanced cancer. Although no drugs have been developed specifically to target the E-cadherin pathway, a significant number of currently available anticancer compounds have been demonstrated to function through induction of E-cadherin expression *via* various mechanisms of action. The group of drugs that has received the most attention is the epigenetic drugs which act through reversal of methylation-induced gene silencing. Although this category of drugs clearly has anticancer efficacy, the adverse effects observed in recent clinical trials are a drawback, and are probably at least partially due to their non-specific reversal of gene silencing. A potentially more lucrative target in terms of E-cadherin-specific pharmacological upregulation would seem to be the *CDH1* transcriptional repressors such as Snail. The evidence from studies utilizing genetic manipulation of *Snail* indicates its central role in E-cadherin downregulation, invasion and metastasis as well providing evidence of the successful restoration of E-cadherin expression by Snail-inhibitors. It is therefore tempting to suggest that Snail may be a critical drug target for upregulating E-cadherin. However, since these E-cadherin repressor proteins have some overlap in function, suppression of their expression or activity may be compensated for in the malignant cells through either upregulation of other E-cadherin suppressor genes or induction of *CDH1* promoter hypermethylation. A potential solution for this problem is the application of combination therapies incorporating Snail inhibitors and DNMTi or HDACi to specifically target the repressor complex of E-cadherin at two stages. Previous evidence from two-stage inhibition of methylation-induced gene silencing suggests that such strategies are likely to be the most effective mechanism of inducing E-cadherin expression and preventing or reversing the EMT of cancer metastasis. At present there are no specific Snail inhibitors available for experimentation, and consequently it remains to be seen whether they will also produce non-specific upregulation of genes other than E-cadherin which may be detrimental to the clinical situation. As well as these promising treatment strategies, we have discussed the evidence that the anticancer effect of several natural dietary compounds such as EGCG, I3C and SAMC is associated with their ability to induce E-cadherin expression, and this indicates the availability of a group of novel E-cadherin inducers with proven tolerable toxicity. Further chemical and biological analysis may generate additional compounds that produce specificity with low incidence of adverse effects in cancer patients. In summary, with overwhelming evidence implicating E-cadherin as a therapeutic target for the treatment of metastatic cancer, the next goal in this field will be the development of pharmacological agents or combinations with high specificity and tolerable toxicity which act through E-cadherin upregulation to improve both survival and quality of life for advanced cancer patients.

ABBREVIATIONS

8-PN = 8-prenylarenin

AJ = Adherens Junction

COX-2 = Cyclo-oxygenase 2

DADS = Diallyl disulfide

DPA = 2,2-bis(4-(4-amino-3-hydroxyphenoxy)phenyl) adamantine

DNMT = DNA Methyltransferase

EC = Extracellular cadherin

EGCG = (-)-epigallocatechin-3-gallate

EGFR = Epithelial Growth Factor Receptor

EMT = Epithelial-mesenchymal transition

ER = Estrogen receptor

HCC = Hepatocellular carcinoma

HDGC = Hereditary diffuse gastric carcinoma

HDACi = Histone deacetylase inhibitor

bHLH = Beta helix-loop-helix

HMT = Histone methyltransferase

I3C = Indole 3 carbinol

IDC = Invasive ductal breast cancer

ILC = Invasive lobular breast cancer

LOH = Loss of heterozygosity

MBP = Methyl domain binding protein

MET = Mesenchymal epithelial transition

MMP = Matrix metalloproteins

NSCLC = Non-small cell lung cancer

NHR = Nuclear hormone receptor

PGE2 = Prostaglandin E2

PPARγ = Peroxisomal proliferator-activated receptor γ

RTK = Receptor tyrosine kinase

SIP1 = Smad-interacting protein 1

SAHA = Suberoylanilide hydoxamic acid

SAC = S-allylcysteine

SAMC = S-allylmercaptocysteine

TZD = Thiazolinedione

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