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Project Title: Biological significance and targeting HER family members and cyclin-dependent kinases in gastric cancer

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Abbreviations

ACS: American cancer society
AJCC: American Joint Committee on Cancer
ALK: anaplastic lymphoma kinase
CDK: cyclin-dependent kinase
DMEM: Dulbecco's Modified Eagle Medium
DMSO: dimethyl sulfoxide
ECD: extracellular domain
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EMA: European medicines agency
ERK: extracellular-signal-regulated kinase
FBS: foetal bovine serum
FISH: fluorescence in situ hybridisation
FLT3: FMS-like tyrosine kinase 3
GC: gastric cancer
GCCL: gastric cancer cell line
HER: human epidermal receptor
IC50: half-maximal inhibitory concentration
IGF-IR: insulin-like growth factor receptor
IHC: immunohistochemistry
MAPK: mitogen-activated protein kinase
MEM: minimum Essential Medium
MET: mesenchymal-epithelial transition factor
MFI: mean Fluorescence Intensity
OS: overall survival
PDGFR: Platelet-derived growth factor receptors
PFS: progression-free survival
PI3K/Akt: phosphatidylinositol 3’ kinase- Akt
RPMI: Roswell Park Memorial Institute
RTK: receptor tyrosine kinase
SRB: sulforhodamine B
STAT3: signal transducer and activator of transcription 3
TKI: tyrosine kinase inhibitor
WHO: World Health Organisation
EBV: Epstein-Barr virus
GEJ: gastroesophageal junction

Abstract

Introduction: In 2018, gastric cancer was the 5th most commonly diagnosed cancer and the 3rd leading cause of cancer deaths globally, highlighting the urgent need for the development of more effective and less toxic therapeutic agents for patients with this disease. In the past three decades, increased expression and activation of the human epidermal growth factor receptor family members have been reported in a wide range of human cancers, and in some studies have also been associated with a poorer prognosis. In other studies, the expression of HER family members, other heterologous growth factor receptors (e.g., IGF-IR) and tumour heterogeneity have been associated with the poorer response to therapy with the HER inhibitors.. Therefore, the major aim of this project was to investigate the effect of various targeted agents on proliferation and migration of tumour cells, the two hallmarks of human cancer, and whether there were any associations between the expression levels of various biomarkers and the response to the treatment.

Methods: The effects of 18 agents targeting different members of the HER family, cyclin dependent kinases (CDKs), other growth factor receptors or cell signalling molecules on growth and migration of a panel of human gastric cancer cell lines (GCCLs) were determined by sulforhodamine B colourimetric assay and scratch wound healing assay respectively. The relative expression of all four members of the HER family members and three other heterologous growth factor receptors (i.e., IGF-IR, C-MET and ALK7) in human GCCLs were determined using flow cytometry. Linear regression analysis was employed to determine whether there was an association between the expression level of the growth factor receptors and response to these agents.

Results: Of all growth factor receptors studied, EGFR had the highest level of expression in all five human GCCLs with the mean fluorescence intensity (MFI) ranging from 469 (AGS) to 32 (HGC27), respectively. All GCCLs were also HER2 positive with the MFI value ranging from 66 (AGS) to 18 (MKN-1), respectively. With the exceptions of the cytotoxic drugs paclitaxel and docetaxel, the CDK 1/2/5/9 inhibitor dinaciclib was found to be the most effective agent and inhibited the growth of all GCCLs with an IC50 value ranging from 4nM (HGC27) to 7.6nM (MKN1). Of various types of the HER inhibitors examined, all human GCCLs were most sensitive to treatment with the irreversible pan-HER TKIs (e.g. afatinib) than to treatment with other types of HER inhibitors such as the reversible EGFR specific TKI erlotinib or the irreversible HER2 specific TKI TAK165. A significant association was only found between the IGF-IR expression and the response to treatment with the NVP-AEW742 (IGF-IR inhibitor), the IGF-IR expression and response to paclitaxel and docetaxel, and between the EGFR expression and response to treatment with the PDGFRα/β TKI crenolanib. Scratch wound healing assay demonstrated different significant results with each of the five tested agents on different cell lines and demonstrated significantly higher efficacy with afatinib and the CDK inhibitors Dinaciclib and AT7519.

Conclusion: The results support the need for further studies of the relative expression, predictive value and prognostic significance of such antigens in patients with stomach cancer as well as the therapeutic potential of these agents in such patients.

1.0. Introduction

1.1. Background

At present, the majority of global deaths are due to non-communicable diseases, one of which is cancer. In most of the countries, cancer is ranked as the first leading cause of death in people aged below 70. The global map by World Health Organisation (WHO) which shows the ranking of cancer as a cause of death in 2015, puts cancer as the first or second leading cause of death in 91 countries (Figure 1). According to the Global Cancer Observatory (GLOBOCAN) 2018 statistics, there were an estimated 18.1 million new cases of cancer and 9.6 million cancer-related deaths worldwide, respectively (Bray et al., 2018). This project is focused on one of the common cancer types, stomach cancer, also referred to as gastric cancer (GC).

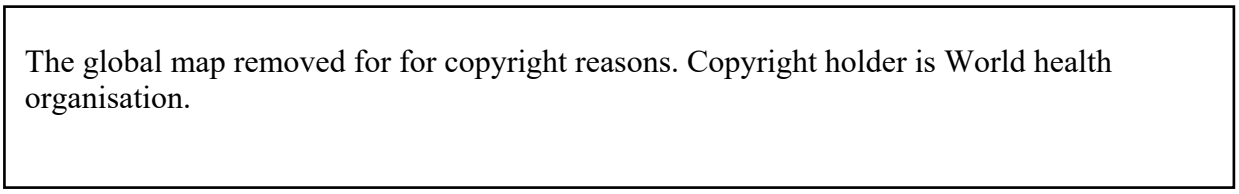


Figure 1. The global map presented by WHO demonstrates the ranking of cancer as a cause of premature mortality in people aged between 0-69 years of age in 2015 (Bray et al., 2018).

1.2. Gastric cancer

1.2.1. Epidemiology

According to GLOBOCAN 2020, GC is ranked as the fifth most prevalent cancer (1.08 million new cases) and the third cause of cancer-related mortalities (768,793 deaths) worldwide (GLOBOCAN, 2020). There has been a substantial decrease in the rates of GC incidences since 1975 when GC was ranked as the most prevalent cancer (Ferlay et al., 2015). However, despite the decrease in the incidence rates, GC remains as one of the common malignant cancers worldwide, with the 5-year survival rate of 32% in the USA. This low 5-year survival rate reflects the fact that most people with this cancer are diagnosed at later stages which leads to shorter survival rates after diagnosis (American cancer society, 2021). Although there has been a decrease in mortality rates in late 20th century, especially in developed countries, there are still high rates of mortality in distinct areas such as East Asia, South America and Eastern Europe more specifically in countries such as Korea, Russia, Japan, Portugal and Iran (Forman et al, 2006, Farmanfarma et al., 2020). One of the factors leading to this decline was the introduction and popularisation of refrigerators, which led to a rise in the levels of fruits and vegetable intake and a decrease in salt intake (Palli 2000, Potter et al. 1997). Improved sanitation and the development of effective antibiotics also attributed to this reduction.

1.2.2. Aetiology and prognosis

GC is classified as a heterogeneous disease and can be caused by both various environmental and genetic risk factors (Figure 2). Apart from the mainly investigated aetiology in GC which will be discussed in the following section, other general risk factors include male sex, exposure to radiation, older age, low socioeconomic status, obesity, gastro-oesophageal reflux disease, and intake of statins and non-steroidal anti-inflammatory drugs (Karimi et al. 2014).

Helicobacter pylori infection

One of the leading causes of the GC is an infection by *H. pylori;* this Gram-negative bacterium accounts for 78% of all GC cases and 89% of noncardia GC (WHO, 2014, Farmanfarma et al., 2020). In 1994, WHO classified *H. pylori* infection as a type I carcinogen (Anwar et al., 1994). Interestingly, in countries such as India and Thailand, despite the high levels *of H. pylori* infection rates, GC incidences are not high. This is known as the "Asian Enigma" phenomenon (Miwa et al., 2002), which suggests possible reasons for this low incidence rate such as different dietary programmes, genetic factors and the severity of *H. pylori* infection (El-Omar et al., 2000).

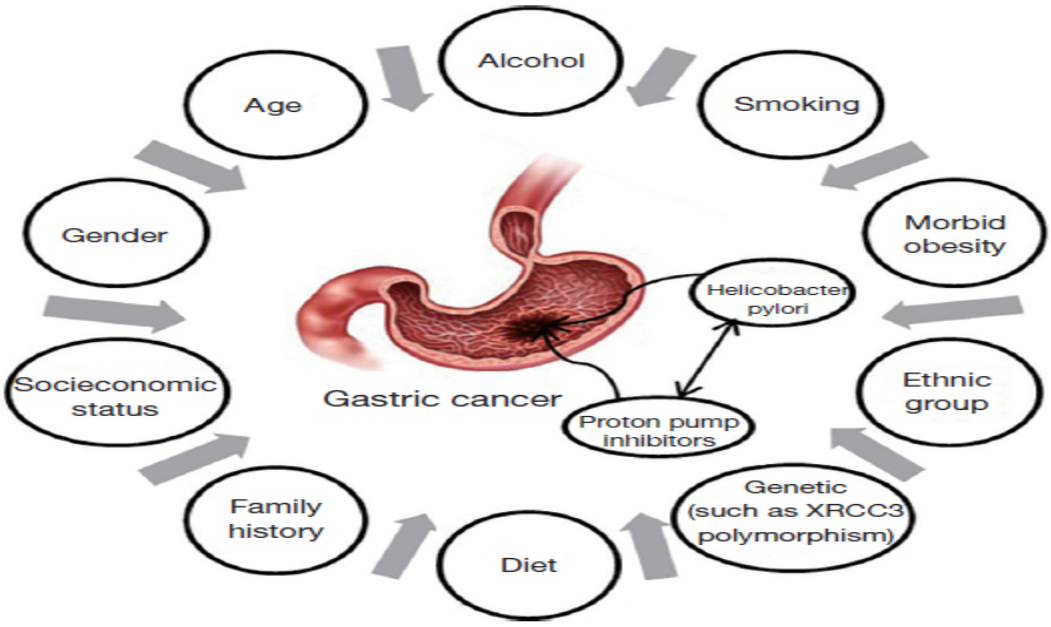


Figure 2. Risk factors in GC (Coronel-Castillo et al., 2018).

Dietary factors

Investigations on the relationship between dietary factors and the development of GC have been carried out. According to these investigations, consumption of fruits and vegetable have been reported to be effective in preventing and protecting against GC. In contrast, diets high in processed, salty, preserved foods and grilled meats have been associated with a high incidence of GC (Wiseman, 2008).

These factors have been further studied, and the most important one is considered to be salt consumption which has been shown to increase the risk significantly in a large cohort study in a Korean populations (Woo et al, 2014).

In another study conducted by Gaddy et al, (2013) the mechanism of action of high salt intake on the development of GC was studied. According to the findings from this study, high levels of NaCl lead to colonisation by *H. Pylori* in patients with gastritis, exacerbating the condition and subsequently increasing the risk of gastric carcinogenesis.

Smoking and alcohol consumption

Smoking and alcohol intake has been considered as an important risk factor in GC development. Various studies demonstrated statistically significant results of increased risk among smokers and heavy alcohol users. One example of these studies is the large Chinese cohort study of 18,244 men with over 20 years of follow up. The results demonstrated 80% increase in the risk of GC among smokers with no alcohol consumption (HR=1.81,95% CI 1.36-2.41). Drinking alcohol also increased the risk statistically among all subjects (HR=1.46, 95% CI 1.05-2.04). Additionally, smoking has been shown to cause a 60% and 20% increase in the risk of developing GC in male and female smokers, respectively compared to non-smokers (Ladeiras, Pereira and Nogueira., et al, 2008).

Epstein-Barr Virus infection

Epstein-Barr Virus (EBV) is another infectious agent which has been commonly associated with GC. This universal virus is present in >90% of adults and plays a key role in the development of some malignancies like Hodgkin's lymphoma, Burkitt's lymphoma and many others. In the case of GC, about 8% of cases have EBV, but there is still insufficient data to show its particular aetiological role (Demetriou et al, 2012). According to the results from a meta-analysis, EBV-positive GC is distributed differently according to the age, sex and anatomic subsite, for example, it becomes less prevalent in men with increasing age (Camargo et al, 2011).

Hereditary and genetic factors

Compared to other health conditions, inherited GC is uncommon. However, genetic alterations are frequently observed in these patients. For example, if in a family, there are two cases of GC in the first or second-degree relatives that have established aetiology, and at least one is under 50 years of age, or three cases at any age, this would support the high chance of genetic predisposition to GC. In regards to the genetic factors, so far few genes have been identified as a risk factor for GC development. One of these is *Interleukin 1ß* gene which is an important gene contributing to the induction and intensification of an inflammatory response (Cheng et al, 2016). Some of the genomic features of GC inspired by cancer hallmarks are presented in Figure 3.

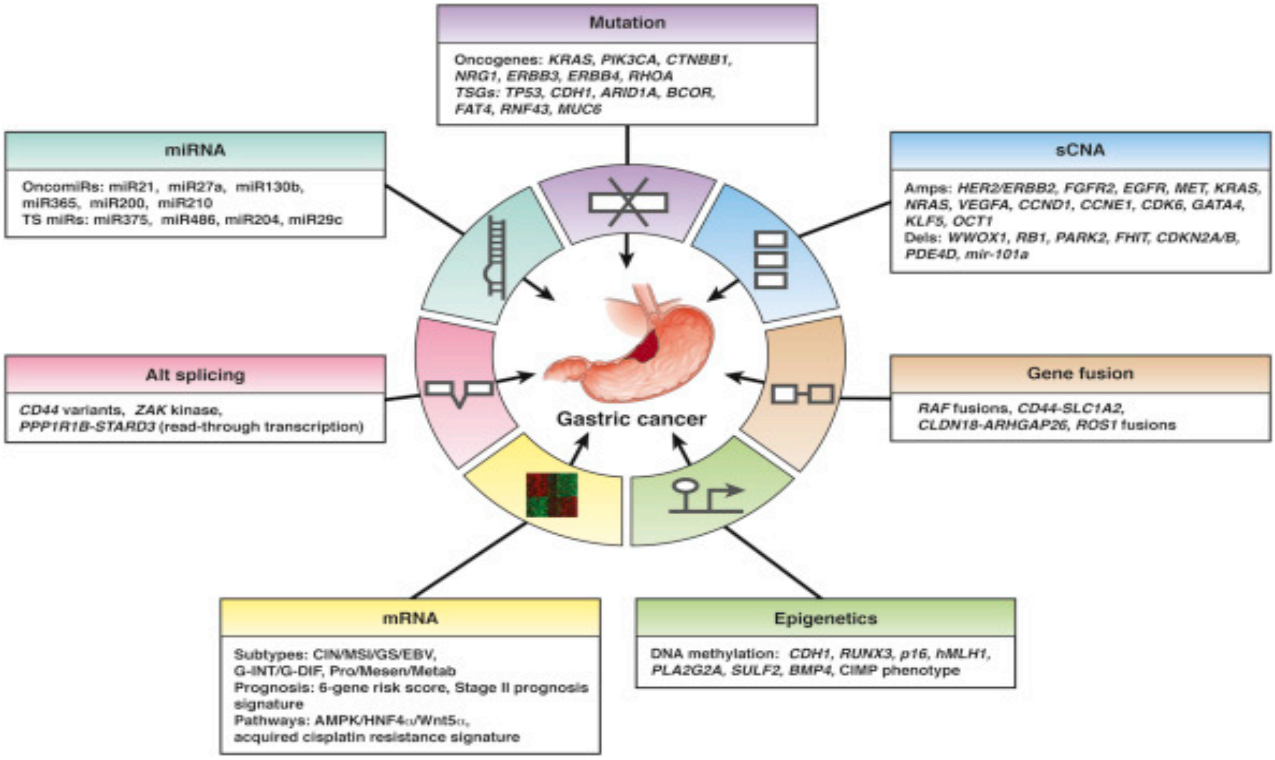


Figure 3. Genomic features of GC (Tan and Yeoh, 2015).

1.2.3. Classification of gastric carcinoma

The stomach is a muscular organ located in the upper area of the abdomen between the oesophagus and the duodenum. As shown in figure 4, this digestive organ has a J shape and is comprised of four anatomical divisions main: cardia, fundus, body, and pylorus. The cardia is the part connecting stomach and oesophagus and hence where the food enters first after leaving oesophagus (Tomar et al., 2013).

Around 90% of the GCs are adenocarcinomas. Adenocarcinomas occur in the mucosa of the stomach arising either from the cardia or other parts (noncardia GC). The other types include mucosa-associated lymphoid tissue, lymphomas and leiomyosarcomas (Karimi et al., 2014). Adenocarcinomas are further divided into diffuse (undifferentiated) and intestinal (differentiated) according to their histology as described in popular Lauren's classification (Lauren, 1965).

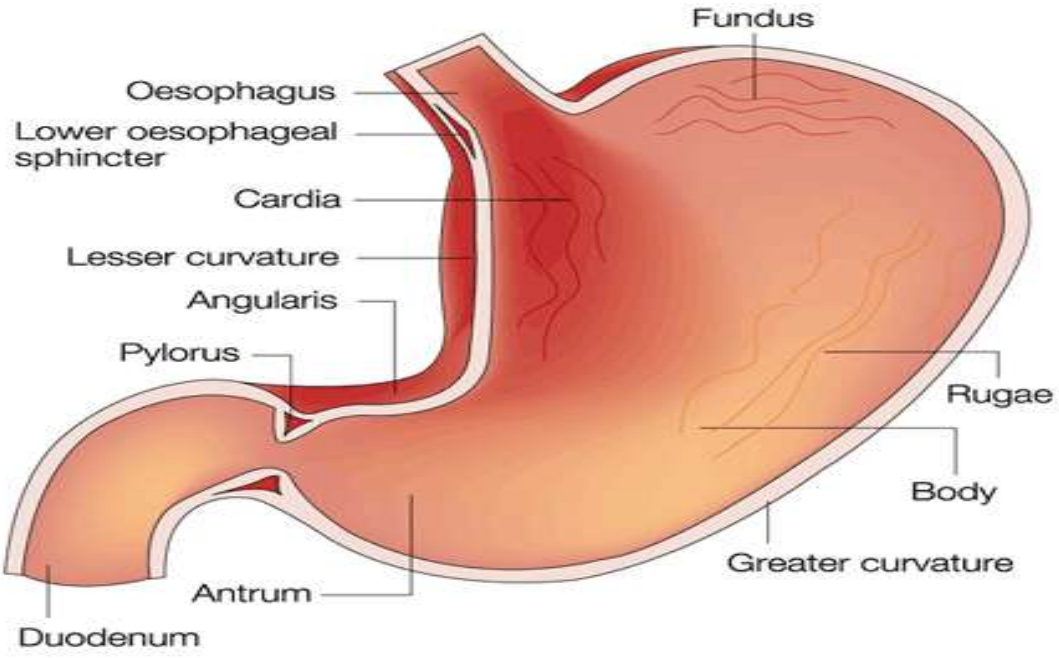


Figure 4. Anatomy of the stomach (Tomar et al., 2013).

When the gastric carcinoma is being analysed based on its anatomic position, complications arise when the tumour is found at proximal stomach or cardia, mainly when it also involves gastroesophageal junction (GEJ) (Figure 4). These difficulties are due to the shared histologic features and the absence of a universally agreed anatomic rationale for gastric cardia (Chandrasoma, 2000). To address this issue, a classification scheme was proposed by the International Gastric Cancer Association. According to this scheme, GCs are divided into three categories of Type I, type II and type III. Type I represents the tumours located at the distal oesophagus, Type II the tumours at cardia and type III the tumours at the stomach distal to cardia (Siewart 1998). However, this classification lacks a clear classification of the criteria for these anatomic locations.

Therefore, recently, the American Joint Committee on Cancer (AJCC) published their 7th edition of the TNM classification to facilitate the gastric carcinoma classification (Table 1). According to this scheme, if the tumour's epicentre relies on the lower thoracic oesophagus or GEJ or within the proximal 5cm of the stomach with the tumour mass reaching into GEJ, it is called oesophageal carcinoma. Nevertheless, if the tumour's epicentre is more than 5cm distal to the GEJ, or within 5 cm of GEJ but not extending into GEJ or oesophagus, it is classified as gastric carcinoma (Edge et al., 2010). GC, like other cancer types, is also classified as an early or advanced stage. When the tumour irrespective of its size is only confined to the mucosa and/or submucosa, with/without metastasis to the lymph nodes, it is classified as early-onset gastric cancer (EOGC). EOGCs encompass 10% of GCs, are mostly detected in females and occur at the ages of 45 years or below. GC patients diagnosed at adanced stage IV of the disease have the lowest 5-year survival rates. The 5-year

survival rates for patients diagnosed at different stages of GC between 2013-2017 in England are presented in Figure 5, highlighting the importance of diagnosing cancer at the earlier stage of the disease.

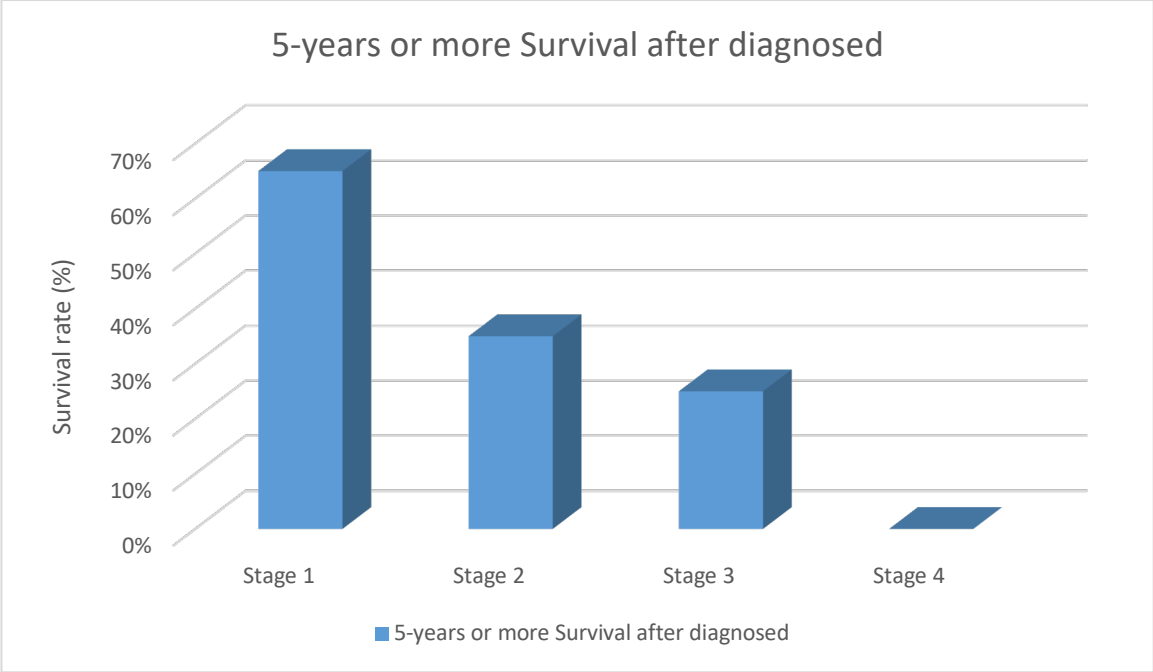


Figure 5. Survival statistics for each stage of stomach cancer in England; these figures are for people diagnosed between 2013 and 2017. There are no 5-year survival data for stage-4 stomach cancer, as most people don't live for that long after being diagnosed (Cancer research UK, 2020).

1.2.4. Screening and Diagnosis

Generally, the purpose of screening for GC is to detect cancer in its early stages. However, there are still controversies around the target population as some low-risk countries consider the mass screening on large masses of population costly and unwarranted. Therefore, in those countries, only people at higher risks may have the advantage of the GC screening. However, in countries with high prevalence such as Japan and Korea, mass screening is the standard practice for early diagnosis (Fock et al., 2008). As seen in the 2015 updated Japanese guidelines, all the people in Japan who are 40 years old or above are recommended to be screened every year with either endoscopy or upper gastrointestinal series with barium meal (Hamashima, 2018). For Korean residents, this practice is recommended bi-annually (Kim et al., 2016).

There are various diagnostic tests such as endoscopy, ultrasound endoscopy, contrast radiography, biopsies, blood tests, X-ray, computed tomography (CT or CAT) scan, magnetic resonance imaging (MRI), positron

emission tomography (PET) or PET-CT scan, and laparoscopy used for diagnostic purposes. Among all these, endoscopy is a standard technique being used widely for the diagnosis of GC. This technique enables the visual examination of the mucosa, followed by biopsy and histologic examination if required. It has a high detection rate and is cost-effective in countries where the incidence rate is high (Areia et al., 2013). However, there is still no evidence of its efficacy and cost-effectiveness in other populations (Choi et al., 2009). Contrast radiography is the second main screening to detect malignant ulcers and lesions. In this technique contrast medium such as barium is used, but unfortunately, the sensitivity of the barium to early GC is so low and may often give false negatives.

1.3. Treatment

There are various treatment options for GC, which will be discussed in the following sections. Such treatments are dependent on the classification, histological type, location, grading, and staging of such cancers.

1.3.1.1. Surgery

In Japan and Korea, endoscopic mucosal resection is being used as the standard of care for the EOGC in large flat and sessile shapes. However, in Western countries, because EOGC is not as common as advanced stage, the standard of care is the gastrectomy (Bollschweiler et al. 2014). For the early-stage localised tumour, surgical resection (R0) is often preferred. However, the problem with surgery as monotherapy is poor survival (i.e. 20-50% at five years). Perioperative or postoperative chemotherapy is usually used to improve the outcomes in these patients (Charalampakis et al., 2017).

Table 1. The gastric cancer pathologic staging system by American Joint Committee on Cancer (AJCC) TNM classification (American cancer society, 2017).

AJCC stage	Stage grouping	Stage description
0	TisN0M0	There is high grade dysplasia (very abnormal looking cells) in the stomach lining OR there are cancer cells only in the top layer of cells of the mucosa (innermost layer of the stomach) and have not grown into deeper layers of tissue such as the lamina propria (Tis). This stage is also known as carcinoma in situ (Tis). It has not spread to nearby lymph nodes (N0) or distant sites (M0).
IA	T1N0M0	The tumor has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa (T1).It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IB	T1N1M0	The cancer has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa (T1) AND it has spread to 1 to 2 nearby lymph nodes (N1). It has not spread to distant sites (M0).
	T2N0M0	The cancer is growing into the muscularis propria layer (T2). It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIA	T1N2M0	The cancer has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa (T1) AND it has spread to 3 to 6 nearby lymph nodes (N2). It has not spread to distant sites (M0).
	T2N1M0	The cancer is growing into the muscularis propria layer (T2) AND it has spread to 1 to 2 nearby lymph nodes (N1) but not to distant sites (M0).
	T3N0M0	The cancer is growing into the subserosa layer (T3).It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIB	T1N3aM0	The cancer has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa (T1) AND it has spread to 7 to 15 nearby lymph nodes (N3a). It has not spread to distant sites (M0).
	T2N2M0	The cancer is growing into the muscularis propria layer (T2) AND it has spread to 3 to 6 nearby lymph nodes (N2). It has not spread to distant sites (M0).
	T3N1M0	The cancer is growing into the subserosa layer (T3) AND AND it has spread to 1 to 2 nearby lymph nodes (N1) but not to distant sites (M0).
	T4aN0M0	The tumor has grown through the stomach wall into the serosa, but the cancer hasn't grown into any of the nearby organs or structures (T4a). It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIA	T2N3aM0	The cancer is growing into the muscularis propria layer (T2) AND it has spread to 7 to 15 nearby lymph nodes (N3a). It has not spread to distant sites (M0).
	T3N2M0	The cancer is growing into the subserosa layer (T3) AND it has spread to 3 to 6 nearby lymph nodes (N2). It has not spread to distant sites (M0).

	T4aN1M0	The cancer has grown through the stomach wall into the serosa, but it has not grown into any of the nearby organs or structures (T4a). It has spread to 1 to 2 nearby lymph nodes (N1) but not to distant sites (M0).
	T4aN2M0	The cancer has grown through the stomach wall into the serosa, but it has not grown into any of the nearby organs or structures (T4a). It has spread to 3 to 6 nearby lymph nodes (N1) but not to distant sites (M0).
	T4bN0M0	The cancer has grown through the stomach wall and into nearby organs or structures (T4b). It has not spread to nearby lymph nodes (N0) or to distant sites (M0).
IIIB	T1N3bM0	The cancer has grown from the top layer of cells of the mucosa into the next layers below such as the lamina propria, the muscularis mucosa, or submucosa (T1) AND it has spread to 16 or more nearby lymph nodes (N3b). It has not spread to distant sites (M0).
	T2N3bM0	The cancer is growing into the muscularis propria layer (T2) AND it has spread to 16 or more nearby lymph nodes (N3b). It has not spread to distant sites (M0).
	T3N3aM0	The cancer is growing into the subserosa layer (T3) AND it has spread to 7 to 15 nearby lymph nodes (N3a). It has not spread to distant sites (M0).
	T4aN3aM0	The cancer has grown through the stomach wall into the serosa, but it has not grown into any of the nearby organs or structures (T4a) AND it has spread to 7 to 15 nearby lymph nodes (N3a). It has not spread to distant sites (M0).
	T4bN1M0	The cancer has grown through the stomach wall and into nearby organs or structures (T4b). It has spread to 1 to 2 nearby lymph nodes (N1) but not to distant sites (M0).
	T4bN2M0	The cancer has grown through the stomach wall and into nearby organs or structures (T4b). It has spread to 3 to 6 nearby lymph nodes (N1) but not to distant sites (M0).
IIIC	T3N3BM0	The cancer is growing into the subserosa layer (T3) AND it has spread to 16 or more nearby lymph nodes (N3b). It has not spread to distant sites (M0).
	T4aN3bM0	The cancer has grown through the stomach wall into the serosa, but it has not grown into any of the nearby organs or structures (T4a) AND it has spread to 16 or more nearby lymph nodes (N3b). It has not spread to distant sites (M0).
	T4bN3aM0	The cancer has grown through the stomach wall and into nearby organs or structures (T4b) AND it has spread to 7 to 15 nearby lymph nodes (N3a).It has not spread to distant sites (M0).
	T4bN3bM0	The cancer has grown through the stomach wall and into nearby organs or structures (T4b) AND it has spread to 16 or more nearby lymph nodes (N3b). It has not spread to distant sites (M0).
IV	AnyTAnyNM1	The cancer can grow into any layers (Any T) and might or might not have spread to nearby lymph nodes (Any N). It has spread to distant organs such as the liver, lungs, brain, or the peritoneum (the lining of the space around the digestive organs) (M1).

1.3.1.2. Chemotherapy and radiation

For advanced stage or unresectable GC, chemotherapy is the first-line therapy. Cytotoxic agents like 5-fluorouracil (5-FU) combined with a platinum compound with the addition of docetaxel (in the US) and epirubicin (in Europe) are considered as the first-line treatment. In terms of the second-line treatment, often docetaxel, irinotecan and paclitaxel are recommended to enhance the survival (Arienti et al., 2019). Palliative chemotherapy is considered as the standard first-line treatment for patients diagnosed with advanced GC who are also in good functional status (Digklia and Wagner 2016). Most chemotherapeutic treatments of GC involve the combinations of the following cytotoxic agents: cisplatin, oxaliplatin, fluorouracil, capecitabine, docetaxel, epirubicin, irinotecan, paclitaxel (Arienti et al., 2019).

Radiation used on its own is not as effective as chemoradiation in terms of overall survival in advanced GC patients (Arienti et al., 2019). In patients with pathologic stage II-IIIC, chemoradiation in the form of post-surgery radiation with fluorouracil based chemotherapy has shown effective results by improving OS (Smalley et al. 2012).

However, tumour heterogeneity and resistance to chemotherapy and radiotherapy are some of the factors contributing to the response of short duration in many patients with GC. As a result, there is a need to discover novel therapeutic targets and to develop more effective and less toxic targeted agents (i.e. targted therapy of cancer).

1.4. Targeted therapy of human cancer

Over the past two decades, a new generation of cancer treatment has emerged as a promising alternative to conventional chemotherapy and radiotherapy. As the name applies, targeted treatments are designed in a way tho selectively deliver the pharmacological agents to particular genes or proteins and consequently block the cell proliferation, induceapoptosis or promote cell cyle regulation. Therefore, unlike chemotherapeutic agents, targeted therapies aim to reduce the cytotoxicity caused to normal tissues and increase the effectiveness of the pharmacological agent by releasing it on a specific site to the cancer cells. There are two main classes of targeted therapies in cancer; monoclonal antibodies and small molecule inhibitors (Padma, 2015). Growth factor receptors and cyclin-dependent kinases are examples of biomarkers commonly used in targeted therapy of different cancer types (Chou et al. 2020 and Baudino 2015)

1.4.1. Growth factor receptor cell signalling pathways and its role in human cancers

Since their discovery, Receptor Tyrosine Kinases (RTKs) have emerged as vital regulators of important cellular signal pathways like cell growth, migration, survival, proliferation, differentiation and apoptosis. There are 58 known RTKs categorised into twenty subfamilies which share a similar structure (Figure 6). All these receptors are activated when a ligand like a growth factor binds the specific receptors and induces receptor dimerisation or oligomerisation (Lemmon and Schlessinger 2011). Once activated, they transfer phosphate groups to tyrosine residues on their intracellular domain also known as autophosphorylation, and this initiates a variety of downstream signalling pathways.

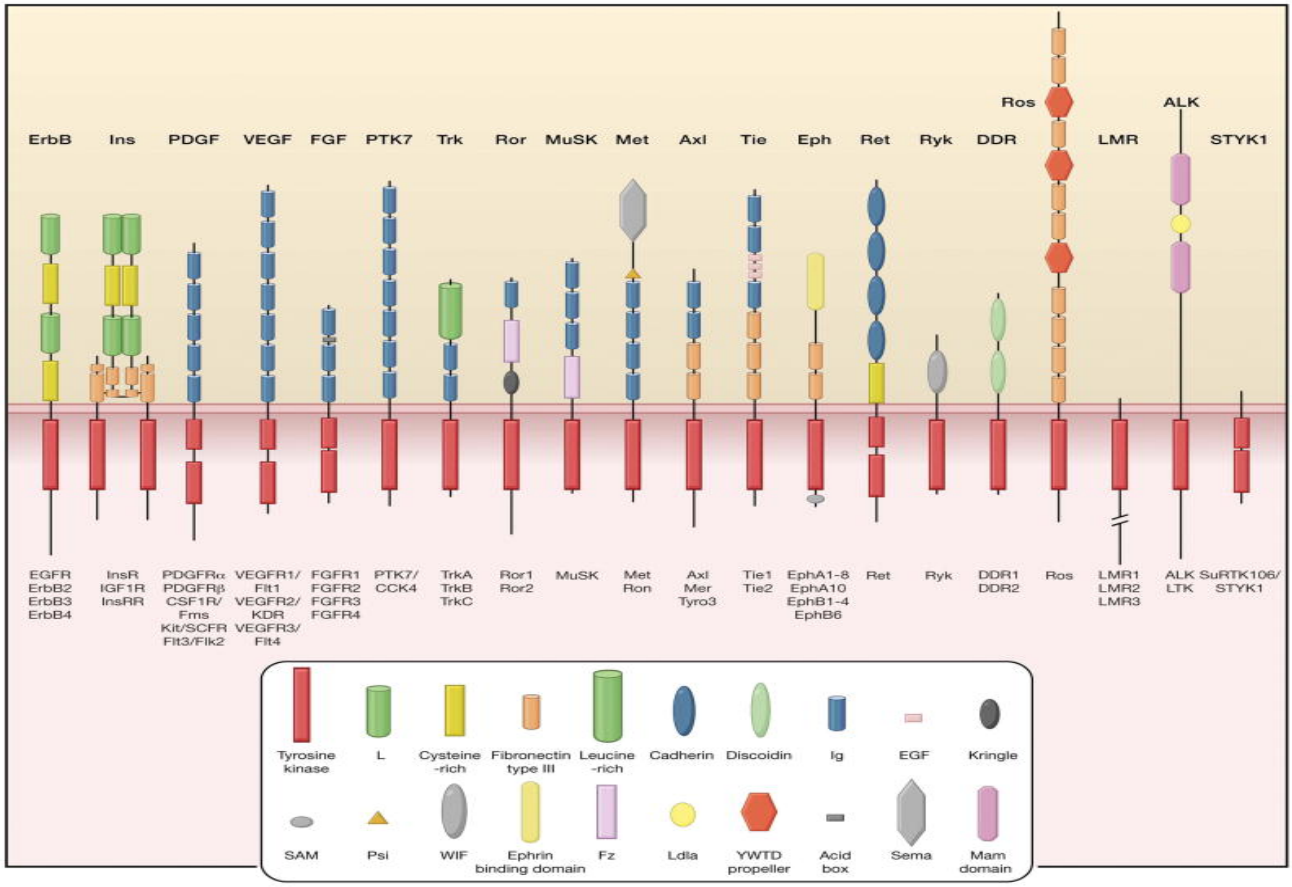


Figure 6. Different types of Receptor tyrosine kinases (RTKs) (Lemmon and Schlessinger 2011).

Of the RTKs, the human epidermal receptor (HER)/ErbB family have been of particular interest in cancer biology. The HER/ErbB family consists of four members; epidermal growth factor receptor (EGFR)/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4. All of these four members have a common structure even though they are found on different chromosomes (Carpenter, 1987).

EGFR is the first component of this family to be discovered (Cohen 1983), and it consists of three parts; an extracellular receptor domain, a lipophilic transmembrane region, and an intracellular domain which is covered in tyrosine kinase residues.

In healthy epithelial cells, the expression level of EGFR ranges between 40,000 to 100,000 receptors per cell. When a ligand binds to the EGFR, it leads to EGFR homodimerisation or hetero-dimerisation with another member of the HER family, receptor internalisation and the autophosphorylation of the intracellular EGFR tyrosine kinase domain. As seen in figure 7, phosphorylated tyrosine kinase residues then act as binding sites for signal transducers like Ras. Ras is an important component for stimulation and intracellular signal transduction (Franklin et al., 2002). Two vital signalling routes for the HER family are Ras-Rad mitogen-activated protein kinase (MAPK) pathway (Alroy and Yarden., 1997) and the phosphatidylinositol 3' kinase-Akt (PI3K/Akt) pathway (Burgering and Coffer., 1995). These pathways are responsible for regulating important critical processes like gene expression, cellular proliferation, angiogenesis and inhibition of apoptosis (Figure 7).

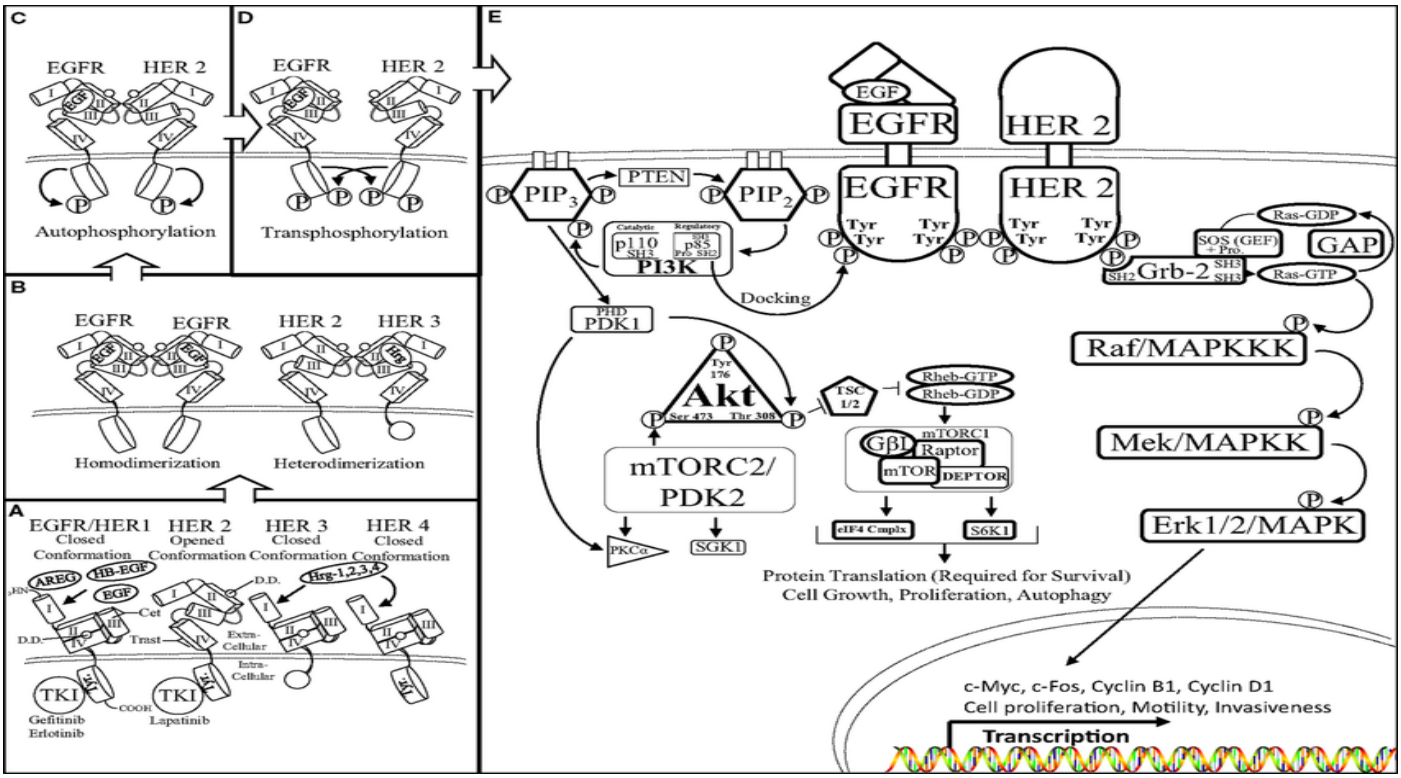


Figure 7. The representation of signal transduction and interplay pathways of HER (ErbB) family involved in cell differentiation and growth (Baker, Zlobin , and Osipo , 2014).

There are different ligands which bind to each of these monomeric receptors, interestingly enough, while seven growth factors bind to EGFR, seven to HER4, two to HER3, there are still no ligands identified to date which bind to HER2. Therefore, HER2 undergoes heterodimerisation with other HER members, such as EGFR and HER3, to become activated (Yarden, 2001); HER2-HER3 dimer is the strongest HER family dimer. Although HER3 lacks essential kinase activity unlike the other three members, it plays a vital role in promoting cell survival (Shi, 2010) and is a key inducer of the PI3K/Akt pathway (Arienti 2019). Also, as seen in two

breast cancer studies which feature HER2-driven cancers, the HER2-HER3 dimer has been confirmed fundamental for tumour development and survival (Vaught et al, 2012 and Holbro et al 2003).

As HER3 can not be inhibited by TKIs, various antibodies and substitute HER3 inhibitors have been sought as cancer therapeutics (Kawakami et al., 2014, Yonesaka et al., 2016, Harrington et al., 2016, Merrimack, 2016, Liu et al., 2016, Sala et al., 2012, Reynolds et al., 2017). HER4 is also an essential part of this family, responsible for mediating the transmembrane TK activation. Dissimilar to other members, numerous studies have proven antiproliferative and pro-apoptotic activity of HER4 (Okazaki et al, 2016., Wege et al., 2018, Tovey et al., 2006).

Aberrant expression and activation of the HER family members and the downstream cell signalling molecules have been shown to occur in a wide range of human cancers and to be associated with a poorer prognosis in many patients and become important targets for targeted cancer therapy (Chan, Rittenhouse and Tsichlis., 1999; Modjtahedi and Dean 1994, Khelwatty et al., 2014 and Ioannou et al., 2013). For example, gefitinib, a small molecule EGFR TKI, was the first approved targeted therapy for the treatment of patients with non-small cell lung cancer in 2009. However, there are now various licenced monoclonal antibodies in the EU such as bevacizumab, ramucirumab and osimertinib that are indicated for the targeted therapy of EGFR positive NSCLC patients (Zugazagoitia et al., 2017 and Cancer Research UK, 2021).

1.4.2. Expression pattern, prognostic significance and targeting of HER family members in GC

EGFR overexpression has been determined in 27%-64% of GC cases (Dulak et al, 2012 and Kim et al 2008). However, despite its well-known role as an oncogene, there is still no established consensus on the prognostic significance in GC patients. While numerous studies suggested a poor outcome association for the highly expressed EGFR patients (Chen et al. 2013, Kandel et al. 2014), other studies indicated the opposite. A 2013 meta-analysis analysed the findings from 5 studies, including 1,600 patients in total and concluded the non-significant correlation between the EGFR expression and GC prognosis (Hong, 2013).

HER2 overexpression varies according to the histologic subtype and the location of the tumour (9%-38%) (Baniak et al., 2016); the highest expressions are often seen in intestinal-type tumours (Gravalos 2008). Trastuzumab which is a humanised anti-HER2 monoclonal antibody combined with chemotherapy has been used for the first-line treatment of metastatic HER2-positive cancer of the stomach or GEJ (EMA. 2019). However, despite the promising response rates of monotherapy or combined treatment of trastuzumab and chemotherapy, most patients suffer from progression within one year because of the tumour developing resistance to trastuzumab. These cases are also correlated with poor prognosis and more aggressive disease (Bang et al, 2010).

In 1986, Sakai et al., for the first time, described the HER2 overexpression in GC using immunohistochemistry (Sakai et al., 1986). These findings were then followed by other studies, including that by Yano et al. which found HER2 overexpression in 23% of 200 resected tumours by using immunohistochemistry (Yano et al., 2004). Two approved methods to test for HER2 are immunohistochemistry (IHC) and fluorescence in situ hybridisation (FISH) (Wolff et al., 2007). In GC, due to some heterogeneity in HER2 genotype, results obtained from these two methods can have some discrepancies (Hofmann et al., 2008). In a validation study by Ruschoff et al., about 4.8% of the GC samples, which were moderately or strongly stained, showed heterogeneity (Ruschoff et al., 2010). Intratumoral heterogeneity is suggested to make a contribution to these discrepancies in HER2 testings.

The ToGA was a phase III randomised controlled trial which demonstrated the superiority of combining trastuzumab with chemotherapy for first-line treatment in HER2 positive GC patients by extending the median overall survival (OS) rate from 11.1 to 13.8 months (95% confidence interval[CI]). This combination became a standard treatment for these patients (Bang et al., 2010). Currently, despite all the studies to find potential biomarkers for prognostic and therapeutic purposes, HER2 is the only biomarker screened to identify GC patients who would be responsive to trastuzumab treatment (Modjtahedi et al., 2012).

According to clinicaltrials.gov, there are currently 27 clinical trials recruiting patients for investigating the therapeutic advantages of trastuzumab in combination with other agents in patients with GC (Table S1).

Various mechanisms were proposed to contribute to resistance to treatment with trastuzumab, one of which is the activation of HER3 signalling. HER2 and HER3 are both functionally incomplete on their own, but when they form a heterodimer together, it activates the PI3K/AKT signalling in HER2 positive cancers. Trastuzumab can block ligand-independent HER2-HER3 dimerisation by targeting the domain IV of HER2 extracellular domain (Junttila et al., 2009). However, trastuzumab's activity is disabled towards blocking this dimerisation, when it occurs in the presence of ligands as it is required to bind to domain II of HER2-ECD. This, in return, leads to uncontrolled ligand-dependent HER3 signalling and subsequent PI3K/AKT signalling which is proposed as an explanation for trastuzumab's restricted therapeutic activity (Garner et al., 2013).

HER3, which is expressed in 20.7% of GCs (He et al., 2015), is a biomarker of interest to investigate its involvement in these mechanisms. Recent studies suggest the therapeutic potential of co-targeting HER2 and HER3 using a combination of trastuzumab and anti-HER3 monoclonal antibodies in GCCLs (Wang et al., 2016). Moghbeli et al. have also shown that co-expression of HER2 and HER3 heterodimer to be of prognostic significance in patients with GC. However, unlike reports from other studies, there was no correlation between ErbB1 overexpression and poor prognosis (Moghbeli et al., 2019). HER4 expression levels have been found

in around 13.3% of GCs, but there have been no studies analysing the significance of the HER4 expression or its coexpression with other HER family members in patients with GC and this is a major focus of this project.

The hypothesis behind the co-targeting of HER family members in order to overcome this resistance is being investigated actively and as shown in table 2, there is one phase II study recruiting advanced trastuzumab refractory GC or GEJC patients in order to investigate the effect of combination therapy of afatinib and paclitaxel. Afatinib which is a pan-HER inhibitor targeting all four members of HER family is also used in our study as well as paclitaxel which is a chemotherapeutic drug.

Table 2. Active (recruiting or not recruiting) trials of afatinib, pan-HER inhibitor, in GC or GEJC patients (Clinicaltrials.gov, 2021).

Clinicaltrials.gov Identifier	Study title	Condition	Intervention	Phase and Status
NCT02501603	Afatinib, Paclitaxel, 2nd Line, Advanced GC	GC or GEJC	Afatinib and paclitaxel	Phase II Recruiting
NCT01522768	Afatinib and Paclitaxel in Patients with Advanced HER2-Positive Trastuzumab-Refractory Advanced Esophagogastric Cancer	Advanced trastuzumab refractory GC or GEJC	Afatinib and paclitaxel	Phase II Active, not recruiting

Recently, on January 15, 2021, Enheru (fam-trastuzumab deruxtecan-nxki) which is an antibody-drug conjugate became the second HER2 targeted medicine approved by the FDA for the treatment of adult patients with locally advanced or metastatic HER2-positive gastric or GEJ adenocarcinoma who have received a prior trastuzumab-based regimen. The approval of this drug for this indication was based on a randomised multicentre trial (DESTINY-Gastric01, NCT03329690) in 188 HER2 positive patients who had progressed on at least two prior therapies including chemotherapy and trastuzumab. The overall survival (primary endpoint) was 12.5 months (95% CI: 9.6, 14.3) versus 8.4 months (95% CI: 6.9, 10.7) in the control group who received irinotecan or paclitaxel (HR 0.59; 95% CI: 0.39, 0.88, p=0.0097). Additionally, the objective response rate (co-primary endpoint) was 40.5% (95% CI: 31.8, 49.6) versus 11.3% (95% CI: 4.7, 21.9) for the active and control group, respectively (FDA press release, 2021). The promising results of this new HER2-targeted therapy would make a big difference in the lives of GC patients who develop resistance to combination chemotherapy plus trastuzumab as first-line treatment. It is important to note that currently, Enhertu is only approved in the United States for this indication, however, it is very likely that marketing authorisation applications will be made in other countries as well considering the unmet clinical need in this field.

1.4.3. Other approved targeted treatments for gastric cancer

Although HER2-targeted therapies play an important role in the treatment of GC patients, not all patients are eligible for these treatments due to the low or negative HER2 expression levels. Therefore, different treatments are often used for these patients. One of these drugs is Cyramza (ramucirumab) which is a targeted humanised monoclonal antibody that specifically inhibits the activation VEGFR2 and its downstream signalling pathways. As demonstrated on figure 6, VEGF is another RTK and is the key mediator of angiogenesis (Cyramza Summary of Product Characteristics, 2020). This drug as a monotherapy or in combination with paclitaxel has been approved by both FDA and EMA for the treatment of adult patients with advanced GC or GEJ adenocarcinoma with disease progression after prior platinum or fluoropyrimidine chemotherapy (Lilly press release, 2014). Therefore, since 2014 when this drug was first approved for use this indication has played an important role as a second line therapy in these patients.

Immunotherapy by Keytruda (pembrolizumab) is another approved treatment in the US for previously treated patients with recurrent locally advanced or metastatic GC or GEJ cancer whose tumours express programmed death ligand 1(PD-L1). This drug used in the third-line settings is the first PD-1 checkpoint inhibitor approved by the FDA for these patients and has demonstrated promising results (ORR: 13.3%; (95% CI: 8.2, 20.0) in a multicentre, open label, single arm, KEYNOTE-059 trial that enrolled 259 patients with GC or GEJ adenocarcinoma who had previously received two lines of treatment such as fluoropyrimidine and platinum doublet and a HER2-targeted therapy for HER2 positive patients (FDA press release, 2017). Although the approval of this drug in third line settings will make a significant difference in the lives of progressive GC patients, there are studies investigating the efficacy of immunotherapy when integrated into the first line. Recent results from the ongoing KEYNOTE-059 trial demonstrated a promising ORR of 68.8% (95% CI 41.3–89.0) when PD-L1 positive patients received pembrolizumab in combination with chemotherapy. Generally, the responses from chemotherapy alone in these patients ranges between 30-35% which are noticeably lower than the combination therapy. These data may rationalise the integration of immunotherapy in earlier setting and consequently improve the response rates in GC patients (Brar and Shah, 2019).

1.4.4. Limitations of Current therapies in Gastric cancer

As highlighted above, a major limitation of current therapies in patients with GC is resistance to the current therapeutic interventions. For example, many patients may not respond to current therapies (i.e., may have primary-resistance) while in others the duration of response may be short following the development of acquired or secondary resistance (Bang et al, 2010). While the underlying contributing factors remain

unclear, tumour heterogeneity has been highlighted as one major contributing factor. Recently, in addition to the expression of other members of the HER family, the co-expression of other heterologous growth factor receptors such as c-MET, IGF-IR, ALK has been associated with resistance to treatment with the HER inhibitors (Takano et al., 2000, Li et al., 2014, Iveson et al., 2014, Abd-Elazeem et al., 2015). Therefore, it is essential not only to discover biomarkers of diagnostic, prognostic and predictive values for the response to therapy in patients with GC but also to develop more effective and less toxic therapeutic interventions using a combination of targeted agents. In the following sections, some of the targeted agents used in this study are briefly reviewed

1.4.5. Targeted therapy of cancer using Cyclin-dependent kinases inhibitors

Alterations in cell cycle proteins result in increased tumour proliferation, and this dysregulation is a hallmark of cancer. These proteins include the cyclins, the cyclin-dependent kinases (CDKs), and the CDK inhibitors. CDKs consist of a serine/threonine-specific catalytic core and make CDK/cyclin complexes upon binding with cyclins. Currently, there are more than 20 CDK families and all share a similar structure; an ATP-binding site (catalytic core), cyclin binding site and a T-loop motif (activating site) (Lim and Kaldis, 2013).

CDKs are divided into two groups of 'cell cycle' and 'transcriptional'; cell cycle CDKs regulate the cell cycle and transcriptional CDKs drive mRNA synthesis (Shapiro, 2006, Ewen 1994, Figure 8 Top).
While CDK/cyclin complexes drive and promote the cell cycle activation and progression, CKIs restrain this activity. The clinical benefit of these CKI in cancer therapeutics has gained noticeable attention. These inhibitors are divided into two groups of ATP-competitive and non-ATP c-competitive CDK inhibitors. ATP-competitive drugs tend to mimic the ATP structure by binding to the ATP-binding site of the CDK proteins. Whereas, the non-ATP competitive CKIs target the cyclin-CDK complexes. Currently, various pan-CDK inhibitors and selective CDK inhibitors such as CDK4/6-selective CKIs are under development as promising drugs for cancer therapies (Figure 8 bottom). CDK4 and CDK6 hold the central role in the signal transduction network by regulating the G1/S transition of cells also referred to as DNA synthesis (Bai et al., 2017).
In a study by Takano et al., 260 GC cases were analysed for their cyclin D1, D2, E and CDK4 overexpression. The IHC results obtained from these cases reported the overexpression of these cyclins and CDK protein in 34%, 30%, 44% and 48% of the cases, respectively (Takano et al., 2000).

Therefore, alteration and inhibition of this network have become an essential target in cancer therapies. One of these CDK4/6 CKIs is palbociclib which will be used in this project, along with two pan-CDK inhibitors, AT7519 and dinaciclib. At present three different CDKIs, have been approved for the treatment of hormone receptor-positive, HER2-negative advanced breast cancer patients (Spring et al., 2019).

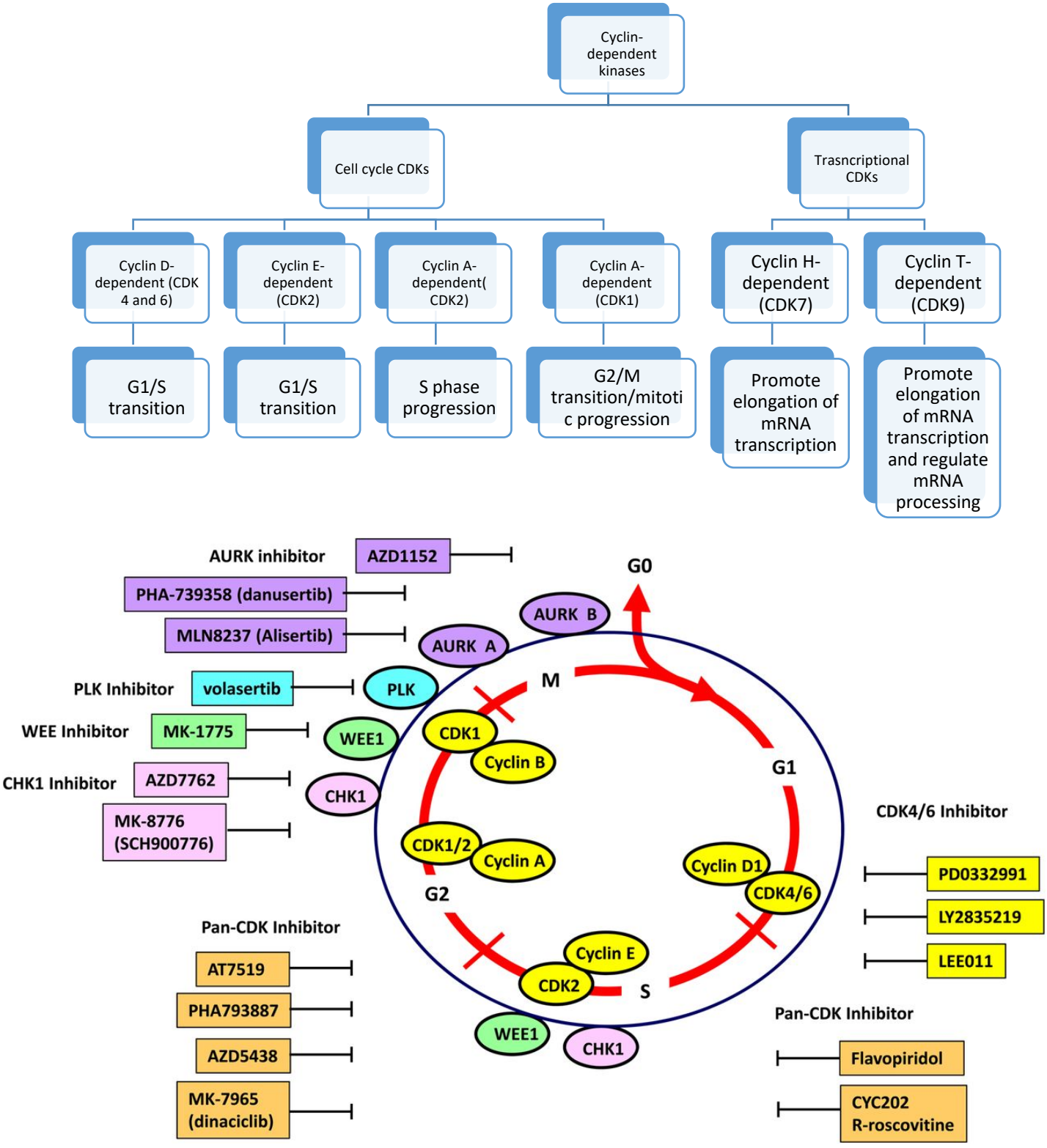


Figure 8. The role of each cyclin-CDK complex in each phase of this cycle (top) and targeting of cell cycle proteins in cancer (bottom) (Kenji and Tamura, 2015). Cell cycle is controlled by several key proteins, including CDKs (cyclin-dependent kinases) which combine with cyclin proteins, CHK1, WEE1, PLK (polo-like kinase) and AURK (aurora kinase). This figure shows that each compound selectively inhibits each cell-cycle checkpoint protein, respectively. The cell cycle, its proteins and inhibitors including the three FDA approved CDK4/6 inhibitors (Source: Kenji and Tamura, 2015).

1.4.6. Targeted therapy using insulin-like growth factor receptor (IGF-IR), C-MET and anaplastic lymphoma kinase (ALK) inhibitors

IGF-1 and IGF-2 are two tumour microenvironment ligands that when they bind to another transmembrane RTK called IGF-IR, promote tumour metastasis, angiogenesis and survival (Xu et al., 2016). IGF-IR activation has been reported in various cancer types, including pancreatic cancer (Karna et al., 2002) and non-small-cell lung cancer (Langer et al., 2014). In 2014, a study by Li et al reported that overexpression of IGF-IR is associated with lymph node metastasis in GC patients (Li et al. 2014). While the expression of IGF-IR has been associated with resistance to therapy with the EGFR inhibitors, there is currently no comprehensive study of the relative expression of IGF-IR and HER-family members in GC cells and their responses to the treatment with the IGF-IR and HER inhibitors.

Hepatocyte growth factor and its receptor C-MET have often been involved in the different phases of cancer cell growth, proliferation and metastasis. C-MET signalling has also been identified to have a possible effect on different pathways, such as blocking the β4-integrin and CD44 activation (Hasenauer et al. 2013). The C-MET expression has been reported in 26-74% of GC cases. In a phase II study, rilotumumab, a fully human IgG2 monoclonal antibody against HGF, plus epirubicin, cisplatin and capecitabine (ECX) was studied against placebo plus ECX and demonstrated greater activity than placebo (Iveson et al., 2014). However, these results were not consistent with the findings from the confirmatory phase III trial which demonstrated that this combination of rilotumumab and ECX was not effective in improving the clinical outcomes in MET-positive patients (Catenacci et al., 2017). Therefore, further research is necessary for this area.

ALK is a tyrosine kinase receptor and belongs to the insulin growth-factor receptor family. The high expression of ALK is found in the nervous system, while it is rarely expressed in normal tissues (Iwahara et al., 1997). Various mutations, for example, within chromosome or proteins, have been identified that lead to the expression of this biomarker in a subset of cancer cells such as anaplastic large-cell lymphoma (Morris et al., 1994) breast, colorectal and NSCLC (Lin et al., 2009). However, to date, very few studies have investigated the expression prognostic factors of ALK in GC. Alese et al. studied the ALK gene rearrangement in gastrointestinal tumours and reported rare expression of this gene in gastrointestinal cancer (Alese et al., 2015). Similar results were also reported by Abd-elazeem et al., which reported ALK expression in only 11.8% of GC cases. However, they also reported a positive correlation between ALK and HER2 expression (Abd-Elazeem et al., 2015). In an *in vitro* study by Ji et al., crizotinib which is a dual C-MET and ALK inhibitor was reported to have a possible anti-cancer effect in clinical settings when used in combination with other agents (Ji et al., 2018).

However, there is currently no comprehensive study of the relative expression of all four HER family members and these growth factor receptors in GC and the therapeutic potential of co-targeting such RTKs CDKs and other downstream cell signalling molecules in GC cells and this was a major goal of this project.

2. Aims of this MRes project

This MRes project aimed to investigate the growth response of five human GCCLs, established from patients at different stages of the disease, to treatment with various types of HER inhibitors targeting one or more members of the HER family, other heterologous growth factor receptors (e.g., IGF-IR, c-MET, PDGFR, FGFR), CDK, and STAT3 compared to treatment with cytotoxic agents. The effect of treatment with such agents on tumour cell migration was also investigated. Furthermore, the cell surface expression of various biomarkers was determined by flow cytometry and whether there were an association between the expression level of growth factor receptors and the response to such treatment.

3. Methodology

3.1. Cancer cell lines and other materials used in this study

Five human GCCLs, AGS, FU97, HGC27, MKN1, and MKN74 were used. Three of them (MKN-74, MKN1 and FU97) were purchased from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). AGS and HGC27 were purchased from Public Health England (Table 3). Two control cancer cell lines, HN5 (EGFR overexpressing) and SKOV3 (HER2 overexpressing) were provided by Professor Modjathedi. A summary of drugs used in this study, their target antigens and suppliers are summarised in Table 4.

Table 3. Characteristics of each one of the five adherent gastric cancer cell lines used in this project.

GC cell line	Type	Source
AGS	Human Caucasian gastric adenocarcinoma. 54-year-old Caucasian female who had received no prior therapy	Primary (stomach)
FU97	Human gastric adenocarcinoma with alpha-fetoprotein production resected from a female before operation. Lymph node metastasis and pancreatic metastasis observed	Primary (stomach)
HGC-27	Human gastric carcinoma. Mucin producing. Lymph node metastasis	Metastatic-site (Lymph node)
MKN1	Adenosquamous carcinoma. 72-year-old male	Primary (Stomach)
MKN74	Stomach adenocarcinoma. metastasised to liver	Primary (stomach)

Table 4. The name and specificity of all drugs used in this project.

Drug name	Mode of action	Source
Erlotinib	Reversible EGFR inhibitor	Selleckham, USA
Lapatinib	Dual reversible HER2 and EGFR inhibitor	Selleckham, USA
HKI357	Dual irreversible EGFR and HER2 inhibitor	Selleckham, USA
TAK165	Irreversible HER2 inhibitor	Selleckham, USA
Neratinib	Irreversible EGFR and HER2 inhibitor	Selleckham, USA
Afatinib	EGFR, HER2, HER3 and HER4 inhibitor	Selleckham, USA
Palbociclib	CDK4/6 inhibitor	Selleckham, USA
Dinaciclib	CDK 1,2,5 and 9 inhibitor	Selleckham, USA
AT7519	CDK 1,2,4,6, and 9 inhibitor	Selleckham, USA
NVP-AEW742	IGFIR inhibitor	Selleckham, USA
Dasatinib	AB1, Src, ckit inhibitor	Selleckham, USA
Crenolanib	PDGFR a/b and FLT3 inhibitor	Selleckham, USA
Stattic	STAT3 inhibitor	Selleckham, USA
Brigatinib	ALK and ROS1 inhibitor	Selleckham, USA
Trametinib	MEK 1/2 inhibitor	Selleckham, USA
Crizotinib	CMET and ALK and ROS1 inhibitor	Selleckham, USA
Paclitaxel	Cytotoxic Antimicrotubule agent	Selleckham, USA
Docetaxel	Cytotoxic Antimicrotubule agent	Selleckham, USA

3.2. Cell culture

All of the cancer cell lines were cultured and grown in 75 cm^2 flasks at 37°C in a humidified atmosphere (5% CO_2). MKN74 and MKN1 were grown in Roswell Park Memorial Institute (RPMI) (Sigma-Aldrich; Merck KGaA), AGS in Ham's F12 (Sigma-Aldrich; Merck KGaA), HGC-27 in Minimum Essential Medium (MEM) (Sigma-Aldrich; Merck KGaA), FU97 in Dulbecco's Modified Eagle medium (DMEM) (Merck) and all were supplemented with 10% foetal bovine serum (FBS) (Sigma-Aldrich; Merck KGaA) and antibiotics (penicillin, streptomycin, and neomycin (all 50 µg/mL)) (Sigma-Aldrich; Merck KGaA). When cell lines reached about 90% confluency, tumour cell monolayers were washed with phosphate-buffered saline (PBS) (Sigma-Aldrich; Merck KGaA) and treated with 1mL of 0.005% trypsin-EDTA (Sigma-Aldrich; Merck KGaA) at 37°C until the cells became detached. The cell suspension was then transferred into a 15 mL centrifuge tube containing 1mL of FBS in order to deactivate the trypsin and centrifuged at 1200 RPM (270 X g) for 4 minutes. After the supernatant was discarded and the pellet was resuspended in 5 mL of fresh medium. The cells then were seeded into new T-75 flasks containing 25 mL of fresh medium and left in the incubator for subsequent cell culture.

3.3. Cryopreservation and thawing of cell lines

After routine trypsinisation, cells were suspended in a prepared freezing solution of 7% Dimethyl sulfoxide (DMSO) (Sigma Aldrich) in FBS and aliquoted in cryotubes in a volume of 1 ml. The cryotubes were then transferred to -80°C for 24 hours before being stored in liquid nitrogen tanks for long-term storage at -180 C. When required, frozen cryovials containing the cells were removed and thawed in the water bath (37°C). The cells were then suspended in 10mL of fresh medium, centrifuged at 1200rpm (270x g) for 4 minutes. After the supernatant was discarded and the pellet was resuspended in 15mL of fresh 10% FBS medium and seeded to cell culture flasks to be incubated at 37°C (humidified, 5% CO_2).

3.4. Cell counting

After routine trypsinisation, the cells were counted using a haemocytometer. The 4 squares on the edges and one in the middle of the 25 squares layout were used to count the live cells and then multiplied by 5 and 10^4 to determine the number of cells in 1mL.

3.5. Flow Cytometry

The surface expression of 7 biomarkers was assessed by flow cytometry analysis, as described previously (Puvanenthiran et al., 2016). The primary mouse antibodies against EGFR (HM43.16B) and HER-2 (HM50.67A) were in-house antibodies and the other primary mouse monoclonal antibodies against HER-3 (MAB3481), HER-4 (MAB11311), IGF-IR (MAB391), ALK (MAB77491), and HGF R/c-MET (MAB3583) were purchased from R&D Systems (Oxford, UK) and used as described previously (Puvanenthiran et al., 2016). Briefly, following routine trypsinisation, cells were counted and about 1 million cells were added into 1.5 ml Eppendorf tubes. The tubes containing tumour cells were then centrifuged at 1000rpm for 3 minutes. The supernatant was discarded and the pellet was resuspended in 1ml of cold PBS and centrifuged again. The supernatant was discarded and the cell pellet was resuspended in 1ml of cold PBS and 10 µg /ml of primary antibody or negative control medium and incubated for 1 hour on the rotator at 4°C. Next, the tubes were centrifuged and washed three times before adding the secondary antibody (Rabbit F(ab')2 anti Mouse IgG antibody, STAR9B) in 1 in 200 dilutions. The tubes were again placed on the rotator and covered with a foil due to the light sensitivity of STAR9B for another 1 hour. The cells were then washed three times and seeded into non-tissue culture round-bottom 96-well plate in the volume of 200µl. The plate was then read using Guava to obtain the readings. Both primary and secondary antibodies were guava flow cytometry verified.

3.6. Cell growth assay

Growth response studies were performed on each of the cell lines to analyse the inhibitory effect of various agents on the growth of GCCLs and to determine the IC50 values for each of them using Sulforhodamine B (SRB; Sigma Aldrich) colourimetric assay, as described previously (Ioannou et al., 2011; Khan et al., 2020). Briefly, GC cell lines were seeded into 96-well plates at a density of 5000 cells per well in 100µl of 2% FBS growth medium. The plates were then transferred into an incubator for a four-hour incubation period at 37°C (5% CO_2). After 4 hours, “time zero” plate containing the untreated cells was fixed with 10% trichloroacetic acid for 1h at room temperature and then washed three times and left to air dry for 24 hours. In regards to other plates, 100 µl of the double diluted drug-medium suspension of 24 agents including one controls (DMSO and PBS) were added into the cell plates in triplicates and incubated for 5-12 days (depending on the cell line) until the control wells which do not contain treatment became confluent. The list of drugs and their targets are summarised in table 3.

The plates were then fixed as described before and stained with 0.04% (w/v) SRB in 1% acetic acid for 1 hour, washed with 1% acetic acid and left to air dry for 24 hours. The dried stained plates were then solubilised with 100 µl /well of 10mM Tris-Base and read by Epoch plate reader to obtain the absorbance at 565 nm. IC50 values were then calculated using Gen5 software with the formula below:

Percentage Growth = (B-A) ÷ (C-A) ×100

A= Absorbance (565nm) before treatment (i.e. Time Zero)

B= Absorbance (565nm) after treatment with drugs

C= Absorbance (565 nm) of untreated (medium-only) cells

The morphology of cells following treatment were examined once they had become confluent prior to fixation process. The morphology pictures of the cells were taken with PixeLINK uScope software and are presented in figure 13.

3.7. Scratch wound healing assay

Scratch wound healing assay was used to assess the effect of highly effective agents, which were selected based on their IC50 values in growth inhibition studies, on the migration of the migratory GCCLs, as described previously (Khan et al., 2020). Succinctly, about 1×10^5 cells/100 µl of 10% FBS medium per well were seeded in a corning CELLBIND™ 96-well clear flat bottom sterile plate (Sigma-Aldrich; Merck KGaA). After incubating for 24 hrs at 37°C, wound maker was used to create the wounds on the wells. Once the wounds were created, medium from each of the wells was aspirated and washed with PBS. The cells were then treated with 200 µl of 10% FBS medium containing pre-specified drugs or medium only as a positive control. The plate was then incubated in the IncuCyte Zoom® instrument at 37°C for up to 72 h during which the cells were analysed every 3 hrs using Incucyte Zoom® software (Essen Bioscience, version 2018A).

3.8. Statistical Analysis

SPSS software was used to carry two statistical analysis. Linear regression correlation was performed to understand if there was a significant relationship between the expression level of the biomarkers and growth inhibitory effects of the various agents. A *p-value* of <0.05 was considered to be statistically significant, and the R2 value closer to 1 showed the reliability of the results.

Paired sample T-test was used to assess the significance of the migration results compared to the control; A *p-value* of <0.05 was considered to be statistically significant.

4. Results

4.1. Expression of all HER family members, IGF-IR, C-MET and ALK-7 in GC cell lines

The cell surface expression levels of 7 growth factor receptors were determined in a panel of five human GCCLs by flow cytometry as described previously. The HER2 overexpressing ovarian cancer cell line SKOV3 and the head and neck EGFR overexpressing cancer cell line HN5 were used as positive controls for HER2 and EGFR respectively. The results from each analysis are summarised in the form of MFI values in Table 5 and histograms of various biomarkers are presented in Figure 9.

As shown in the summary table 5, all cell lines were positive for EGFR, HER2 and IGF-IR. However, the expression levels varied across different cell lines. For example, AGS exhibited the highest level of EGFR expression (MFI=469), which was even higher than the EGFR expression in the positive control HN5 (MFI=368). AGS also showed the highest levels of HER2 and ALK-7 expression compared to all other four cell lines with the MFI value of 66 and 54.48, respectively.

In contrast, HGC-27 had the lowest EGFR expression (MFI=32.19) and a similar expression of HER2 (MFI= 31.10). HGC-27 cells were negative for HER3, C-MET and ALK-7, but had very low levels of HER4 (MFI=5.1) and IGF-IR (MFI=9.3).

Both MKN1 and MKN74 GCCLs had overexpression of the EGFR and intermediate level of HER2 (Table 5). However, the expression levels of both HER3, HER4 were low in both cell lines. The highest level of IGF-IR expression was present in MKN-74 followed by and MKN-1 with MFI-values of 36 and 15, respectively. The second highest expression of ALK-7 was present in MKN-74 with MFI value of 27. Finally, FU97 were found to have the highest level HER3 and HER4 with MFI values of 42 and 13, respectively (Table 5).

Table 5. The cell surface expressions of the seven biomarkers in all five cell lines determined by flow cytometry using the Guava machine. Data are provided in the form of MFI values.

Mean Fluorescence Intensity (MFI)								
Cell lines	Control	EGFR	HER2	HER3	HER4	IGF-IR	C-MET	ALK-7
AGS	4.18	**469**	**66**	22.81	5.24	11.70	**21.64**	**54.48**
FU-97	10.10	43.49	25.77	**41.77**	**12.62**	12.74	14.11	12.71
HGC-27	3.4	32.19	31.10	3.1	5.1	9.3	3.9	3.3
MKN-1	3.76	153.9	17.7	6.8	6.02	15.3	6.9	6.6
MKN-74	4.2	242.4	51.14	9.9	10.8	**35.6**	6.40	27.31
HN5*	N/A	368	N/A	N/A	N/A	N/A	N/A	N/A
SKOV3**	N/A	N/A	386	N/A	N/A	N/A	N/A	N/A

*EGFR positive control ** HER2 positive control. EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; IGF-IR, insulin-like growth factor 1 receptor; ALK-7, Anaplastic lymphoma kinase-7; C-MET, C- mesenchymal epithelial transition factor; N/A, Not available. Negative control cell lines were only treated with secondary antibody. These experiments were run once for each cell line.

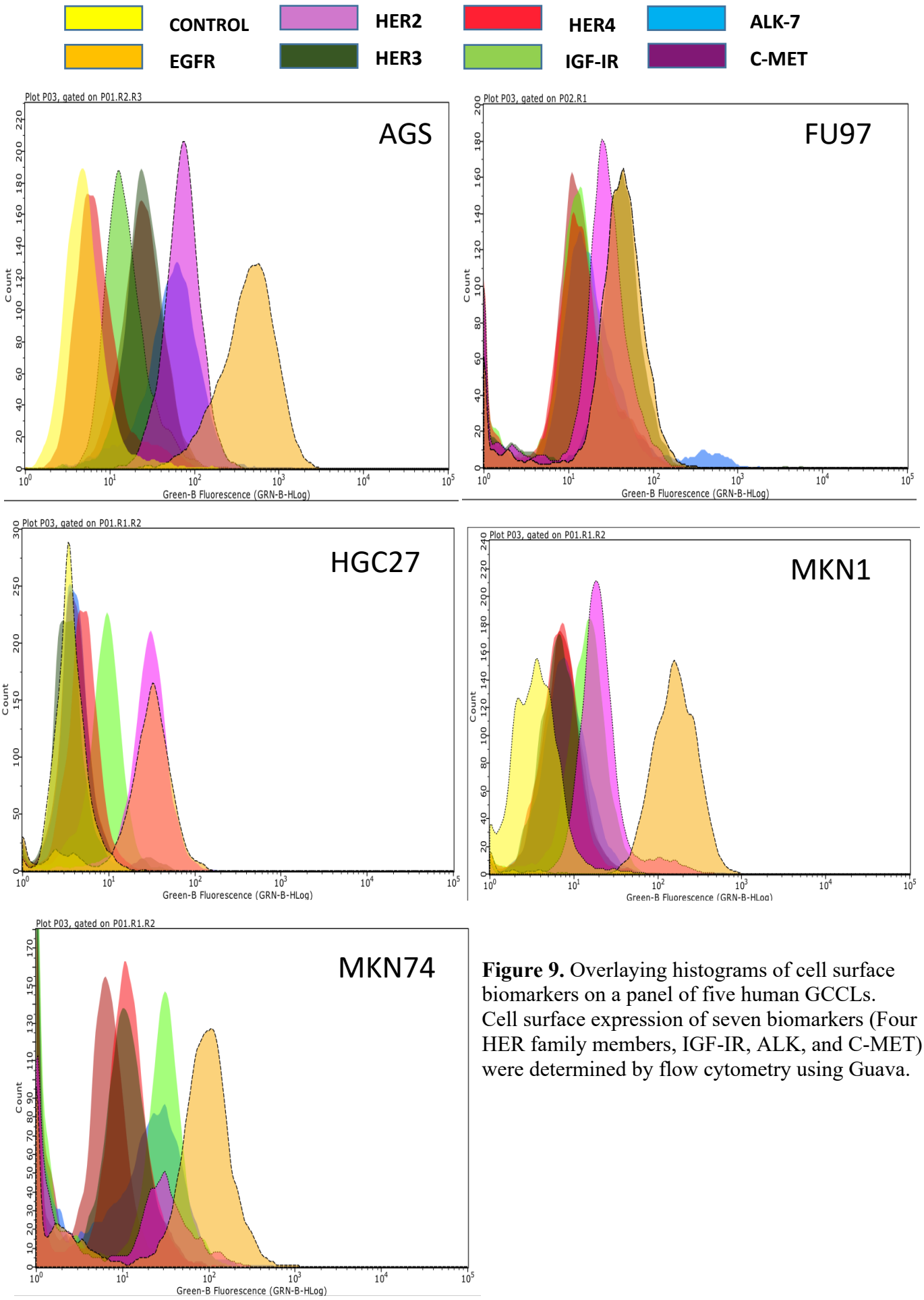


Figure 9. Overlaying histograms of cell surface biomarkers on a panel of five human GCCLs. Cell surface expression of seven biomarkers (Four HER family members, IGF-IR, ALK, and C-MET) were determined by flow cytometry using Guava.

4.2. Growth response of human GC cell lines to treatment with HER TKIs

Next, the effect of various types of HER inhibitors on the growth in vitro of the human HGCCLs was tested (Table 6 and Figure 10). Overall, as shown on table 6, all human GCCLs were highly sensitive to treatment with the irreversible pan HER family TKIs (i.e., Neratinib and afatinib) and the irreversible bispecific EGFR/HER-2 TKI HK1357 compared to the irreversible HER2 specific TKI, TAK165, or reversible EGFR specific TKI erlotinib (i.e., some IC50 values >10µM).

MKN-1 was the most sensitive cell line to treatment with the HER inhibitors as the IC50 values of all 6 HER TKIs were at their lowest in this cancer cell line compared to other human GCCLs. Generally, the reversible EGFR specific TKI erlotinib and the irreversible HER2 specific TAK165 were the least effective drugs having IC50 values above 10µM with all cell lines except MKN-1. However, even in MKN-1 cell line, the inhibitory effects of these two drugs were very low; IC50 values were 2.17 and 4.03 µM, respectively. The effect of doubling dilution of various types of HER inhibitors on growth of human GCCLs are presented in Figure 10.

4.3. Growth response of human GCCLs to treatment with inhibitors of IGF-IR, C-MET/ALK/ROS1, ALK/ROS1, BCR-AB1/Src/c-Kit/PDGFR/FLT3 and PDGFR/FLIT3 inhibitor.

Next, the effect of other targeting agents on the *in vitro* growth of the human GCCLs was investigated (Table 7, Figure 11). Of the 5 human GCCLs, MKN-74 was the most sensitive cell line to the IGF-IR inhibitor NVP-AEW742 with an IC50 value of 2.31µM, followed by MKN1 with an IC50 value of 4.9µM (Table 7). This was followed by the higher sensitivity of MKN-74 and HGC-27 among others to the treatment with the C-MET/ALK/ROS1 inhibitor, crizotinib, with the IC50 values of 1.21µM and 1.58 µM, respectively. In contrast, of all five human GCCLs studied, only HGC27 was not sensitive to treatment with the ALK/ROS1 inhibitor brigatinib (i.e., IC50 value>10µM). The growth of all other four GCCLs was inhibited by brigatinib with IC50 values which ranged from 0.96µM to 8.67µM respectively (Table 7).

All human GCCLs were also highly sensitive to the treatment with the BCR-AB1/Src/c-Kit/PDGFR/FLT3 inhibitor dasatinib (Table 7). Of these FU97 and MKN74 cell lines were the most sensitive cell lines to treatment with dasatinib with IC50 values of 0.12 and 0.17 uM, respectively. The growth of all human GCCLs was also inhibited by the PDGFR/FLIT3 inhibitor crenolanib 1.6 to 5.8µM (Table 7).

4.4. Growth response of human GCCLs to treatment with CDK inhibitors, MEK inhibitor, STAT3 inhibitor and cytotoxic drugs.

Out of three CDK inhibitors, CDK 1/2/5/9 inhibitor, dinaciclib, exhibited the strongest inhibitory effect on all five cell lines with IC50 values ranging between 4nM and 76 nM (Table 7, Figure 12). The CDK 1/2/4/6/9 inhibitor, AT7519, was the second most effective CDK inhibitor on all cell lines, apart from FU97, and inhibited their growth at IC50 values below 0.82µM. The most sensitive GCCL to treatment with the CDK 4/6 inhibitor palbociclib was FU97 (IC50=72 nM). It also inhibited the growth of the remaining cancer cell lines with IC50 values which ranged from 3.66 to 8.13µM (Table 7, Figure 11).

STAT3 inhibitor, stattic, exhibited its most potent inhibitory effect on FU97 cell line (IC50=0.49 µM). In contrast to the other GCCLs, HGC27 cell line was found to be resistant to treatment with stattic (i.e., IC50 value >10µM) (Table 7, Figure 11).

The most sensitive GCCL to treatment with the MEK1/2 inhibitor trametinib was AGS ((IC50=0.84 uM). In contrast, treatment at the maximum concentration of 10µM used in this study did not have any effect on the *in vitro* growth of MKN1 cells (Table 7, Figure 11).

The effect of the most commonly used chemotherapeutic agents paclitaxel and docetaxel on the *in vitro* growth and morphology of these five human GCCLs are presented in figure 12 and 14 and their IC50 values are summarised in Table 7. As the results show, both drugs were effective and inhibited the *in vitro* growth of all human GCCLs with IC50 values ranging from 2nM to 8nM (Table 7).

As an example, the morphology pictures of AGS cell line following treatment with each agent are presented in figure 13.

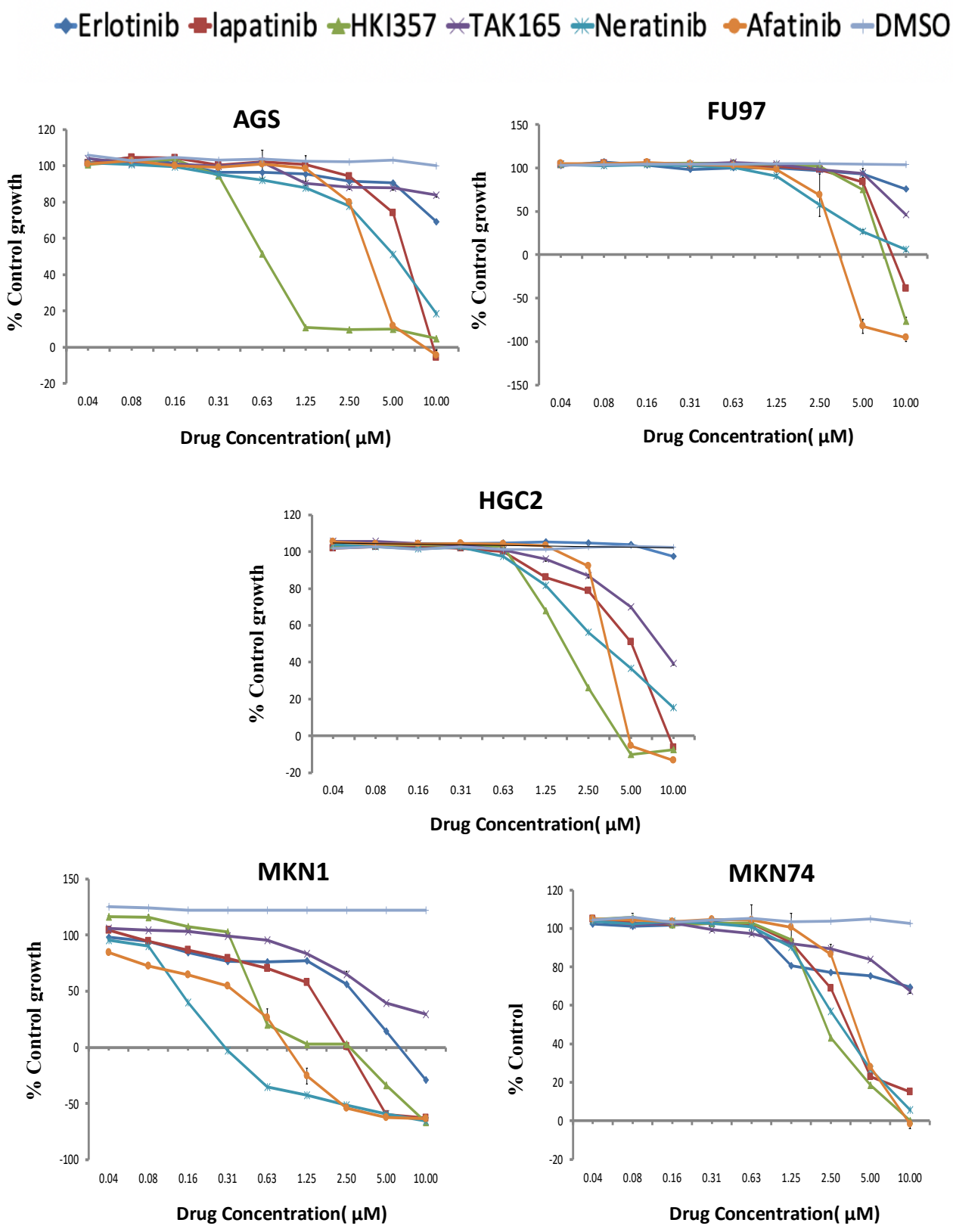


Figure 10. The growth response of all five GC cell lines to treatment with doubling dilutions of HER inhibitors. The average duration of treatment ranged from 5 to 12 days depending on the cell line's growth rate. Each value is the mean + standard deviation of triplicate samples. These experiments were repeated for three times.

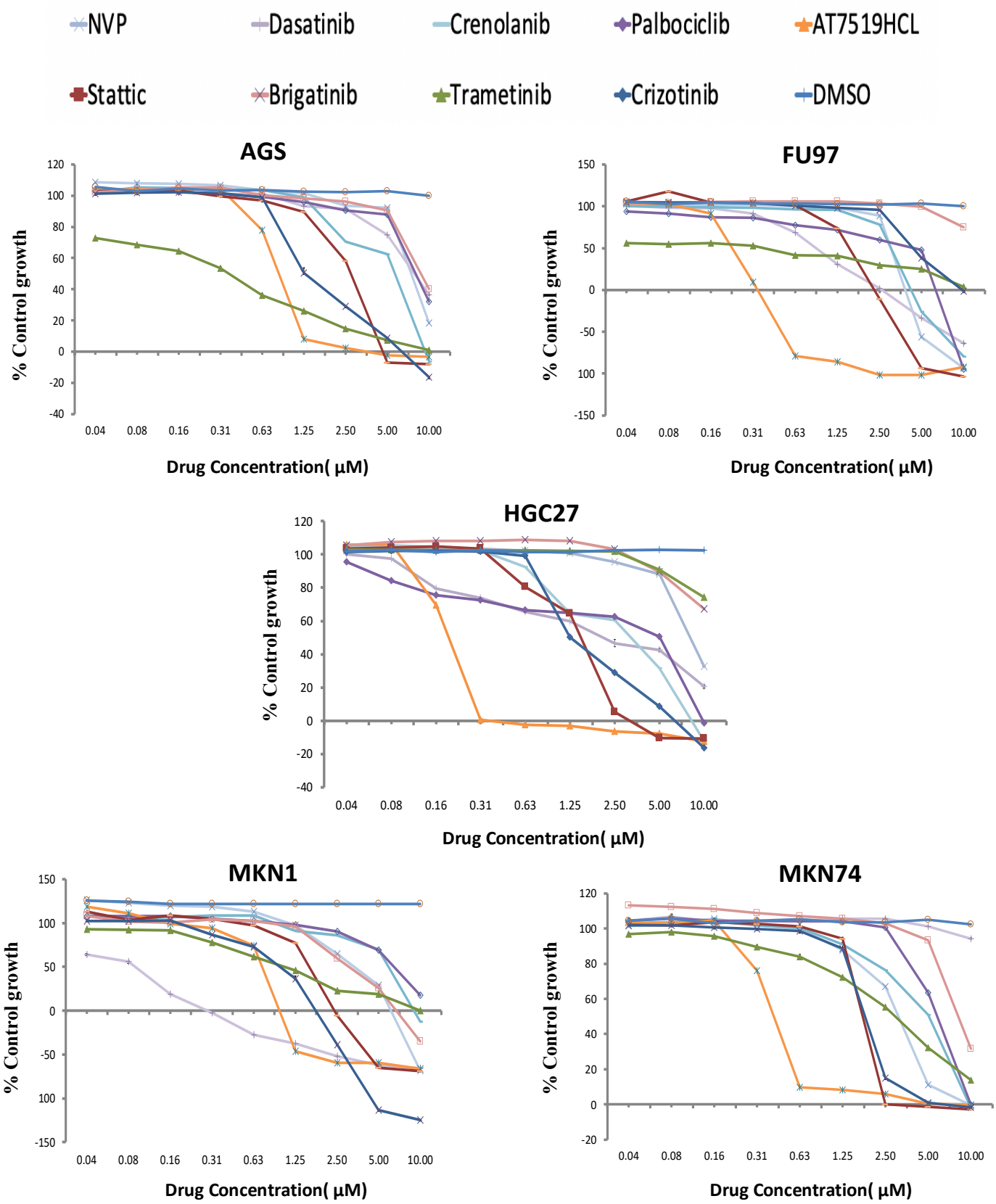


Figure 11. The growth response of all five GC cell lines to treatment with doubling dilutions of CDK, IGF-IR, C-MET, PDGFR, ALK7, and STAT3 inhibitors. The average duration of treatment ranged from 5 to 12 days depending on the cell line's growth rate. Each value is the mean + standard deviation of triplicate samples. These experiments were repeated for three times.

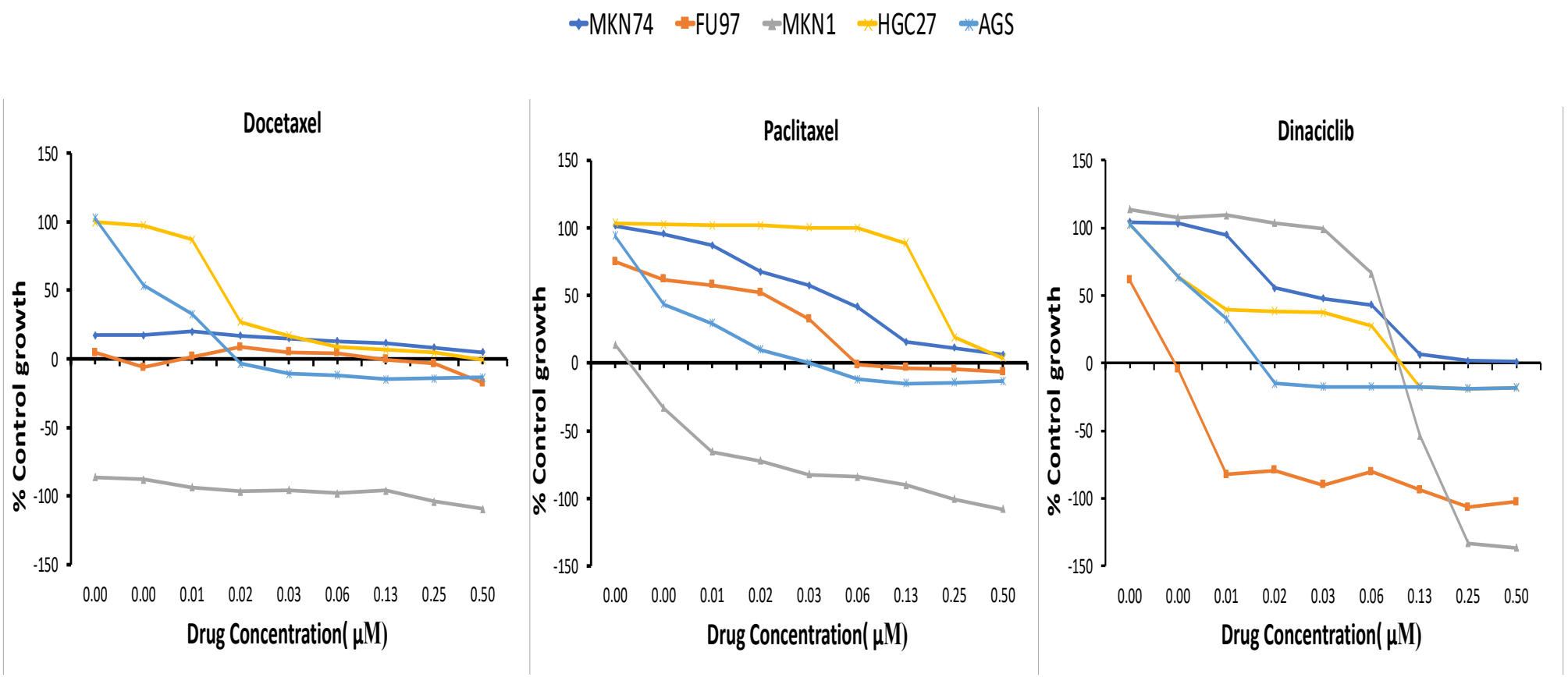


Figure 12. The growth response of all five GC cell lines to treatment with doubling dilutions of CDK inhibitor dinaciclib and two cytotoxic drugs docetaxel and paclitaxel. These drugs were the most effective agents among all the tested drugs. The growth inhibition assay was repeated with reduced drug concentrations in order to obtain the IC50 values for these drugs. The average duration of treatment ranged from 5 to 12 days depending on the cell line's growth rate. Each value is the mean + standard deviation of triplicate samples. These experiments were repeated for three times.

Table 6. The effects of various receptor tyrosine kinases on the growth of HGCCLs presented in the form of mean IC50 values and their standard deviations. The average duration of treatment ranged from 5 to 12 days depending on the cell line's growth rate. All the IC50 values are calculated by the mean ± standard deviation of triplicate samples of three sets of growth inhibition assay.

	IC_{50} Values (μM) + standard deviation										
Cell lines/Drug names	Erlotinib (reversible EGFR inhibitor)	Lapatinib (reversible EGFR and HER2 inhibitor)	HKI357 (irreversible EGFR and HER2 inhibitor)	TAK165 (irreversible HER2 inhibitor)	Neratinib (irreversible pan EGFR and HER2)	Afatinib (irreversible pan EGFR, HER2 and HER4 inhibitor)	NVP-AEW742 (IGF-IR inhibitor)	Dasatinib (BCR-Ab1,Src, Ckit, PDGFR inhibitor)	Crenolanib (PDGFR a/b and FLT3 inhibitor)	Brigatinib (ALK and ROS1 inhibitor)	Crizotinib (ALK, C-MET, and ROS1 inhibitor)
AGS	>10.00	6.36±0.45	0.65±0.44	>10.00	5.30±0.28	3.63±0.58	8.16±0.22	8.33±0.49	5.77±0.08	5.14±0.28	3.57±0.45
FU97	>10.00	7.26±0.36	6.29±0.45	>10.00	2.43±0.12	3.91±0.22	>10.00	0.12±0.74	1.98±0.12	0.96+0.12	3.49±0.12
HGC27	>10.00	6.47±0.11	1.62±0.74	>10.00	5.50±0.007	3.13±0.36	>10.00	3.37±0.007	1.62±0.74	>10.00	1.58±0.007
MKN1	2.17±1.30	1.37±0.51	0.59±0.13	4.03±0.115	0.17±0.24	0.38±0.45	4.92±0.45	6.47±0.33	4.63±0.007	3.7±0.11	1.21±0.12
MKN74	>10.00	2.35±0.53	1.52±0.33	>10.00	2.50±0.23	3.19±0.16	2.31±0.39	0.17±0.33	5.35±0.22	8.67±0.20	2.21±0.49

Table 7. The growth inhibition effects of three CDK inhibitors, two cytotoxic drugs, and one STAT3 inhibitor were examined and expressed as IC50 values and their standard deviations. The average duration of treatment ranged from 5 to 12 days depending on the cell line's growth rate. All the IC50 values are calculated by the mean ± standard deviation of triplicate samples of three sets of growth inhibition assay.

	IC_{50} Values (µM)) + standard deviation						
Cell lines/Drug names	Palbociclib (CDK4/6 inhibitor)	Dinaciclib (CDK 1,2,5 and9 inhibitor)	AT7519 (CDK 1,2,4,6, and 9 inhibitor)	Stattic (STAT3 inhibitor)	Trametinib (MEK 1/2 inhibitor)	Paclitaxel (Antimicrotubule agent)	Docetaxel (Antimicrotubule agent)
AGS	8.13±±0.081	0.005±0.059	0.82±0.012	2.63±0.17	0.84±0.035	0.004±0.55	0.003±0.16
FU97	0.07±0.029	0.014±0.081	3.32±0.19	0.49±0.18	4.78±0.25	0.005±0.15	0.006±0.05
HGC27	3.66±0.02	0.004±0.05	0.21±0.033	>10.00	5.49±0.41	0.004±0.46	0.008±0.04
MKN1	7.66±0.04	0.076±0.043	0.63±0.08	1.84±0.083	>10.00	0.03±0.26	0.002±0.17
MKN74	5.18±0.17	0.05±0.06	0.40±0.16	1.54±0.05	1.04±0.54	0.05±0.05	0.005±0.12

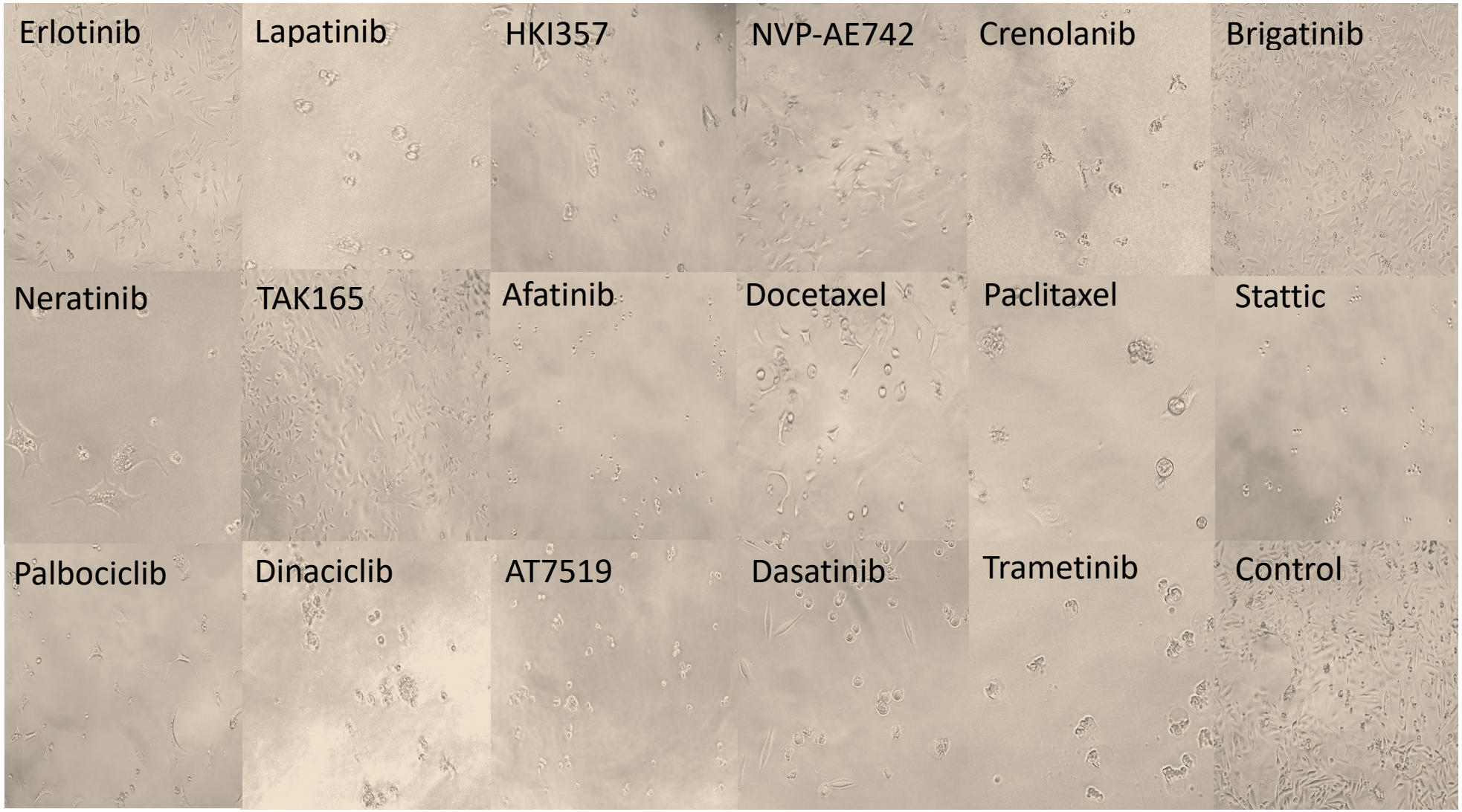


Figure 13. Treatment of AGS cell line with doubling dilution of dinaciclib for 7 days and its effect on the cell growth and morphology. The picture from control well shows the cells in Ham's F12 medium only. All the pictures of 10 various drugs with the highest concentration (μM) were taken on day 7 when the control became confluent.

4.5. Analysis of the association between HER family and other biomarkers' expression levels and the response to various classes of agents.

The linear regression analysis was performed to identify any significant correlations between the expression levels of the biomarkers and the IC_{50} values obtained from the growth inhibition analysis of various therapeutic agents in 2% FBS (for AGS, HGC27, MKN1, MKN74) and 5% FBS (for FU97).

As shown in table 8, there was no significant correlation between IC50 values of any of the HER inhibitors and the HER family members' expression levels. However, there was a significant correlation found between the EGFR expression and the response to crenolanib. Also, there were significant correlations found between IGF-IR expression and the response to NVP-AEW742, and two cytotoxic drugs (paclitaxel and docetaxel, Table 8).

Table 8. Linear regression analysis was performed using SPSS software, to identify any correlations between the MFI values of the biomarkers and the IC50 values of the tested drugs for each cell line. The expression of the cell surface marker was used as the independent variable, while the IC50 value of the agent was the dependent variable. The R^2 and P-values are illustrated in the table below; an R^2 value closer to 1 demonstrated the reliability of the results and the p-value less than 0.05 (<0.05) was considered statistically significant (as demonstrated in bold by asterisks.

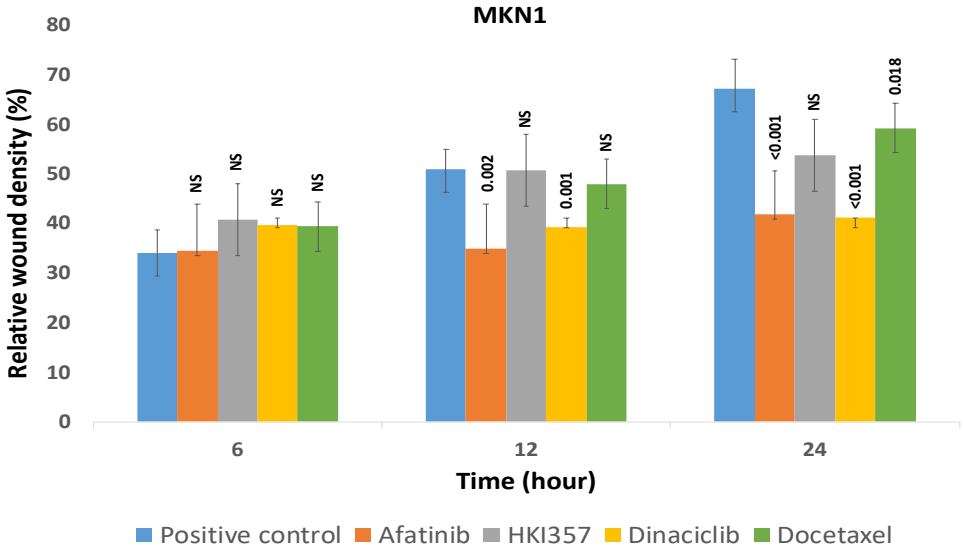
Drugs/ cell surface markers	EGFR R^2 (p-value)	HER2 R^2 (p-value)	IGF-IR R^2 (p-value)	ALK-7 R^2 (p-value)	C-MET R^2 (p-value)
Erlotinib	0.011 (0.870)	0.274 (0.366)	0.001 (0.970)	0.050 (0.717)	0.125 (0.560)
Lapatinib	0.011 (0.864)	0.013 (0.855)	0.480 (0.194)	0.094 (0.615)	0.071 (0.664)
HKI357	0.306 (0.334)	0.216 (0.430)	0.136 (0.542)	0.115 (0.577)	0.085 (0.634)
TAK165	0.011 (0.870)	0.274 (0.366)	0.001 (0.970)	0.050 (0.717)	0.125 (0.560)
Neratinib	0.066 (0.676)	0.358 (0.287)	0.073 (0.660)	0.238 (0.404)	0.008 (0.884)
Afatinib	0.013 (0.853)	0.217 (0.429)	0.011 (0.868)	0.085 (0.635)	0.098 (0.608)
NVP-AEW742	0.101 (0.602)	0.045 (0.733)	**0.835 (0.030)***	0.024 (0.804)	0.407 (0.247)
Dasatinib	0.365 (0.281)	0.097 (0.611)	0.109 (0.587)	0.492 (0.187)	0.003 (0.935)
Crenolanib	**0.766 (0.049)***	0.167 (0.495)	0.333 (0.308)	0.328 (0.313)	0.624 (0.112)
Brigatinib	0.002 (0.943)	0.426 (0.232)	0.225 (0.419)	0.015 (0.843)	0.053 (0.708)
Crizotinib	0.170 (0.490)	0.188 (0.465)	0.072 (0.663)	0.371 (0.275)	0.131 (0.550)
Palbociclib	0.548 (0.152)	0.244 (0.398)	0.078 (0.650)	0.302 (0.337)	0.139 (0.536)
Dinaciclib	0.002 (0.941)	0.120 (0.568)	0.282 (0.357)	0.161 (0.503)	0.009 (0.878)
AT7519	0.104 (0.596)	0.164 (0.499)	0.221 (0.425)	0.008 (0.889)	0.058 (0.695)
Stattic	0.095 (0.614)	0.000 (0.999)	0.065 (0.678)	0.022 (0.812)	0.195 (0.457)
Trametinib	0.348 (0.296)	0.742 (0.061)	0.136 (0.541)	0.275 (0.364)	0.639 (0.104)
Paclitaxel	0.012 (0.862)	0.008 (0.885)	**0.893 (0.015)***	0.133 (0.546)	0.329 (0.312)
Docetaxel	0.008 (0.889)	0.005 (0.908)	**0.886 (0.017)***	0.148 (0.523)	0.312 (0.328)

4.6. Effect of selected agents on the migration of gastric cancer cell lines

The results from the effect of pre-selected agents on the migration of three GCCLs was established using the scratch wound healing assay as described previously in the methodology section. The results obtained from three time points 6, 12 and 24 h are demonstrated below in figures 14, 15, and 16 and the data obtained from the statistical analysis can be found in the supplementary data (Table S2).

The results from MKN1 cell line demonstrated a significant correlation between the relative wound density and two drugs, afatinib and dinaciclib, as early as 12 hours. These results were consistent for the HCG27 cell line which also showed significant correlations with afatinib, AT7519, dinaciclib and docetaxel after 12 hrs which was maintained until 24hrs. In regards to the AGS, only AT7519 demonstrated significant results at 12 hrs and maintained until 24 hrs.

A



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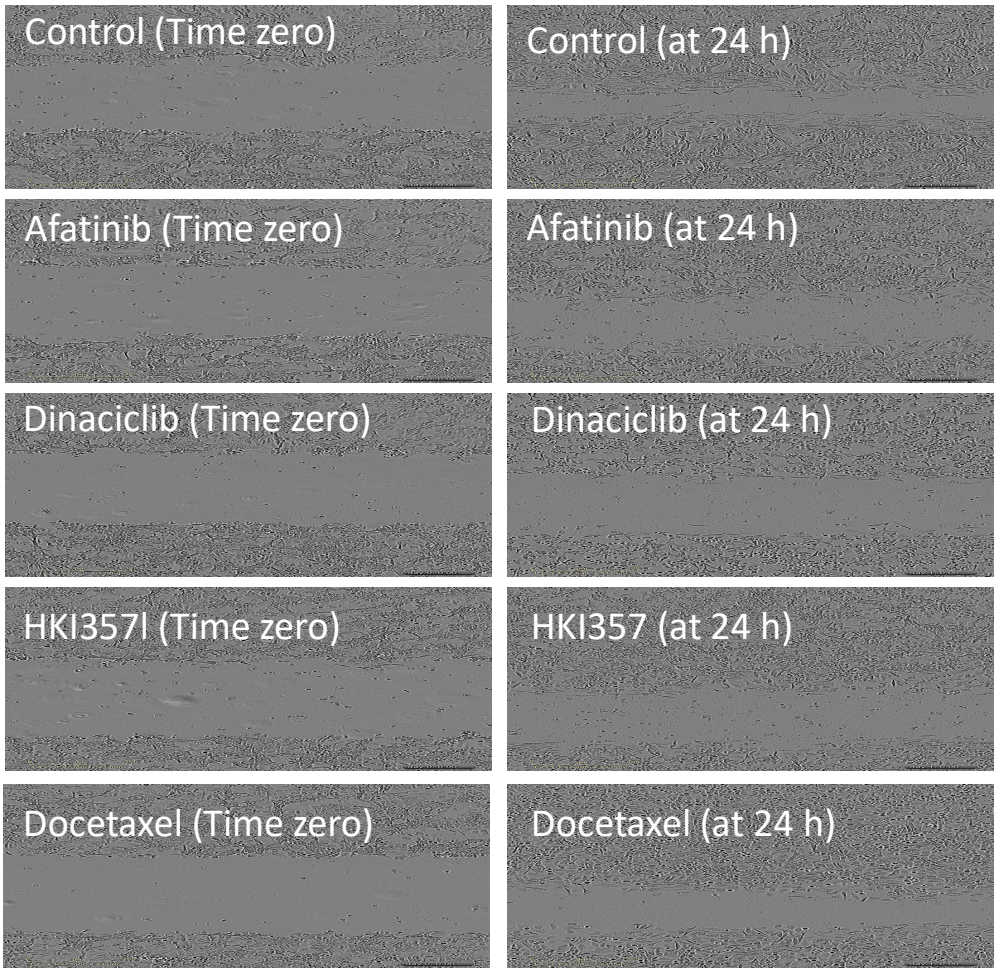

Figure 14. (A) Effect of selected drugs on the migration of MKN1 cell line at different time intervals. (B) Effect of selected agents on the migration of MKN1 at 24h post treatment using scratch wound healing experiment. Paired sample T-test (95% CI) was used to assess the significancy. P<0.05 was considered significant. NS=not significant. This experiment was performed once but in triplicates.

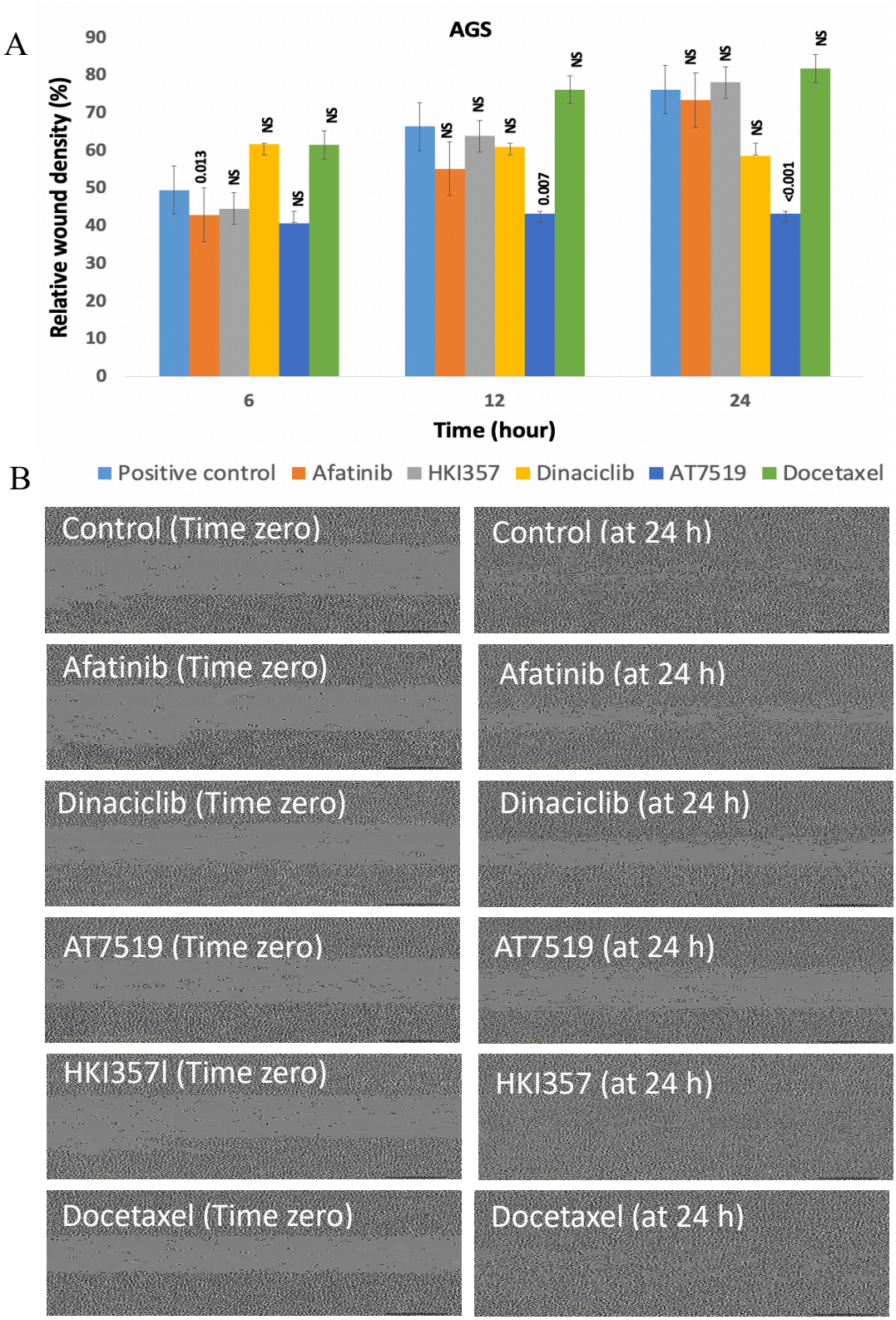

Figure 15. (A) Effect of selected drugs on the migration of AGS at different time intervals. (B) Effect of selected agents on the migration of AGS at 24h post treatment using scratch wound healing experiment. Paired sample T-test (95% CI) was used to assess the significancy. P<0.05 was considered significant. NS=not significant. This experiment was performed once but in triplicates.

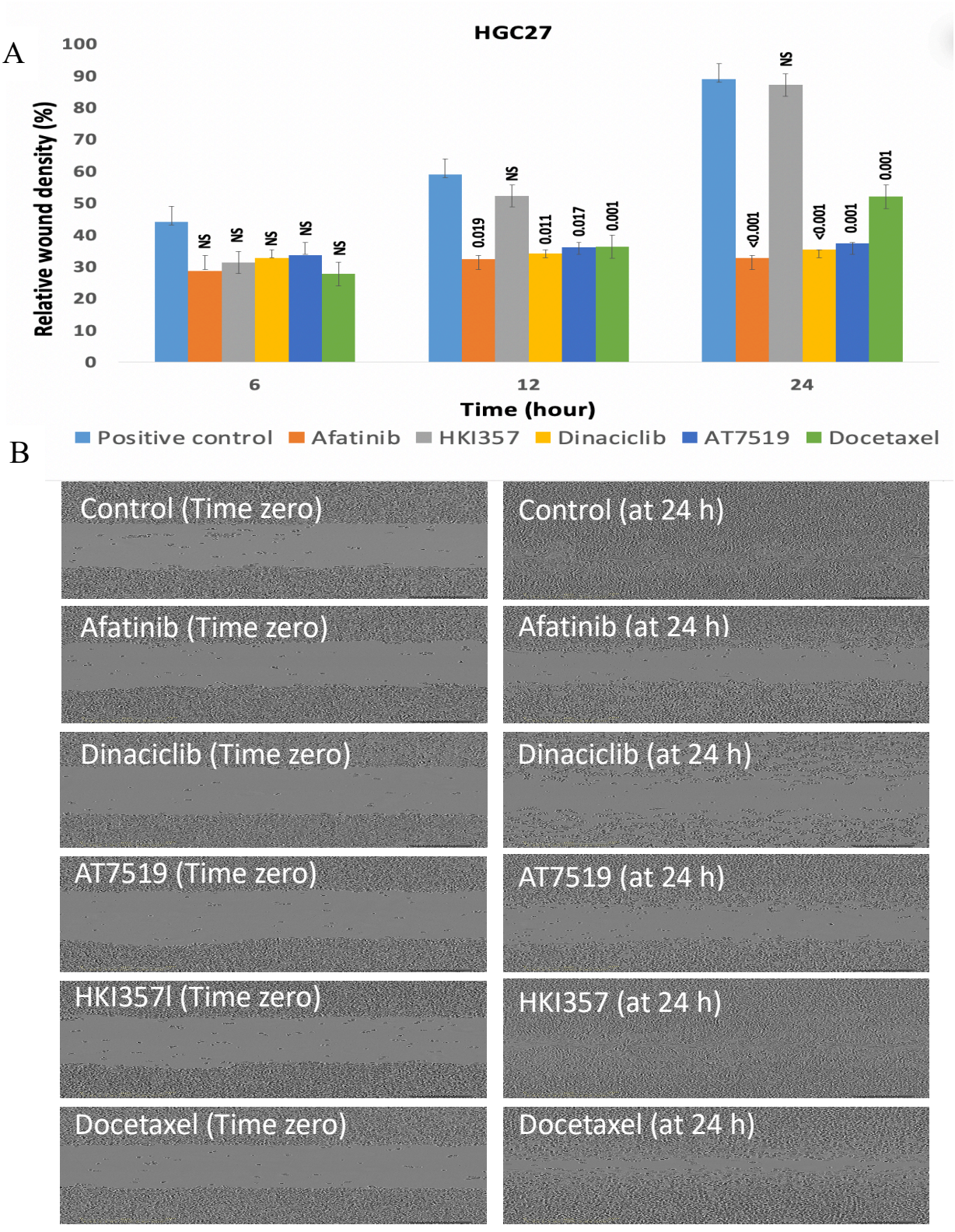

Figure 16. (A) Effect of selected drugs on the migration HCG27 at different time intervals. . (B) Effect of selected agents on the migration of HCG27 at 24h post treatment using scratch wound healing experiment. Paired sample T-test (95% CI) was used to assess the significancy. P<0.05 was considered significant. NS=not significant. This experiment was performed once but in triplicates.

5. Discussion

In the past few decades, advances in the screening and the early detection of human cancers together with development of more effective and less toxic therapeutic agents have led to significant improvements in the survival of patients with various types of cancers. However, there is still an unmet medical need in treating patients with GC. Patients at the early stages of GC normally show no or minimal symptoms, which leads to the poor prognosis (Dicken et al., 2005). This is because, most of these patients, when diagnosed, have already passed the early stage (T1N0), and reached the advanced stage which minimises the efficacy of chemotherapy leading to lower survival rates (Shah, 2015). Also, for patients with advanced-stage GC, there is a high chance of tumour recurrence post-surgery; thus, adjuvant chemotherapies have been employed to improve this issue (Hayashi et al., 2008).

Interestingly, in countries with a high prevalence of GC, prognosis rates tend to be higher. For example, in Japan, where there is an annual mass examination on high-risk patients, the five-year survival rate reaches 90%. Therefore, this demonstrates the importance of earlier diagnosis and consecutive early tumour resection. Treatment strategies also depend on several factors, including the tumour type, location, stage and patient performance status. In earlier stages, tumour resection exhibits satisfactory results; however, in later stages, chemotherapy with or without radiation therapies are involved, but all patients may not benefit from such therapeutic approaches (Sitarz, 2018).

Despite the continued research on the field of targeted therapies for human cancers, there are only three approved targeted therapies for stomach cancer; 1) the humanised anti-HER2 mAb trastuzumab, 2) anti-HER2 drug antibody conjugate trastuzumab deruxtecan (only in the US) and 3) the humanised mAB ramucirumab, which is an anti-angiogenesis therapy (Shitara et al., 2020, American Cancer society, 2020). However, the duration of response can be short in many of the patienst receiving trastuzumab (Mitani and Kawakami 2020). The newly approved novel trastuzumab deruxtecan is approved for these locally advanced or metastasised patients who have progressed after a prior trastuzumab based regimen. It is anticipated that this revolutionary novel drug will reduce the risk of death significantly in these patients in real life settings according to its promising results in DESTINY-Gastric-1 trial (41% reduction in the risk of death; p=0.0097) (Shitara et al., 2020).

However, not all patients are HER2 positive and at the advanced stage to be eligible for this drug. Therefore, in addition to its early diagnosis, it is essential to discover novel therapeutic targets and biomarkers responsible for the poor response to current therapies in order to improve the OS rates in patients with different stages of GC and molecular subtypes. It is also very important to develop more effective and less toxic therapeutic approaches.

One of the major contributors of the poor response to cancer therapy is the heterogeneous nature of human cancer including intra-tumour and inter-tumour heterogeneity (Dagogo-Jack and Shaw, 2018). As a result, a major aim of this project is to investigate the effect of various targeted agents on proliferation and migration of tumour cells, the two hallmarks of human cancer, and whether there are any associations between the expression levels of various biomarkers and the response to the treatment.

5.1. Of the CDK inhibitors, dinacicilib was most effective in the growth inhibition of gastric cancer cells

Out of all drugs tested in our study, the CDK inhibitor dinaciclib showed the most potent inhibitory effect in all five GC cell lines. AT7519 was the second most effective CDKI and palbociclib the least effective drug (Table 7). The three CDK inhibitors employed in this study target different CDKs with those which inhibit more CDKs exhibiting stronger inhibitory effects (Table 7). This can be explained further when we compare palbociclib (CDK 4/6 inhibitor) and AT7519 (CDK1, CDK2, CDK4, CDK6 and CDK9 inhibitor). Although CDK4/6 inhibition is of interest as a cancer therapeutic agent, in our study, palbociclib was not as strong as AT7519, which not only targets CDK4/6 but also targets CDK1, CDK2 and CDK9. Interestingly, these extra targets by AT7519 are also found in dinaciclib's mode of action. Dinaciclib, which showed the strongest inhibitory effect on five GCCLs targets all these three CDKs (CDK1, CDK2 and CDK9) as well as CDK5. This may explain the stronger inhibitory effect of dinaciclib compared to AT7519 by the ability of the former to co-target CDK5. In one study, Takano et al. found over-expression of Cyclins E and D and CDK4 proteins in a high percentage of 260 GC cases (Takano et al., 2000). It will, therefore, be interesting to study the expression levels of these CDK proteins in patients with GC and find any possible correlations between their expression levels and the response to various types of the CDK inhibitors in patients with GC.

5.2. The irreversible Pan HER family inhibitors are more effective than the dual and monospecific type of HER inhibitors in inhibiting the growth of gastric cancer cells

Of various types of HER inhibitors studied to date, the irreversible dual and pan-HER inhibitors (HKI-357, neratinib, and afatinib) were shown to be more effective than the reversible dual inhibitor lapatinib and erlotinib and TAK165 were found to be the least effective drugs. As far as reversibility is concerned, it was also noticed that irrespective of the reversibility, dual inhibitors targeting both EGFR and HER2 were more effective than EGFR or HER-2 specific drugs (Table 6). For example, in terms of reversible drugs, dual EGFR and HER2 inhibitor, lapatinib, exhibited stronger inhibitory effect than the EGFR specific erlotinib. When comparing irreversible drugs, the dual EGFR and HER2 inhibitor HKI357 was more effective than the HER2 specific TAK165 in inhibiting the *in vitro* growth of human GCCLs (Table 6, Figure 10). A major contributing factor could be that in addition to HER2 and EGFR positivity, some of the cancer cell lines have also expressed low levels of HER3 and HER4. As a result, the pan-HER family blockers can also be effective in inhibiting cell signalling via all these receptors.

Of all the 18 drugs employed in this study, only the MEK1/2 inhibitor trametinib did not have an inhibitory effect on MKN-1 cell line. Interestingly, among all the other four cell lines, MKN-1 was the most sensitive cell line to treatment with all six HER TKIs, especially, to neratinib, afatinib and HKI357 (Table 6). Further studies involving a larger panel of human GCCLs will be needed and should confirm whether the high response rate of MKN-1 cancer cell line could be due its clinicopathological feature being adenosquamous carcinoma, unlike other GCCLs used in this study, which were adenocarcinoma. Although, there is limited information in regards to the clinical consequences of these two pathological types of GC, adenosquamous GC is considered as a rare type comprising less than 2% of GCs (Akce M, et al. 2019). In one multivariate analysis of 61,215 patients between 2004 and 2013, adenosquamous cell histology was associated with worse survival versus adenocarcinoma (HR =1.52; 95% CI, 1.35–1.73, $P<0.001$).

Although there was no significant correlation found between the expression levels of the seven growth factor receptors investigated and the IC50 values of the HER inhibitors, it is still interesting to notice the importance of reversibility of the drug and its ability to inhibit multiple

receptors. For example, while EGFR was overexpressed in most of the cell lines and its expression was higher than HER2 in all cell lines, the reversible EGFR specific erlotinib was not effective in inhibiting the growth of these GCCLs in low doses compared to other pan-HER inhibitors (Table 6).

5.3. A significant association was only found between the IGF-IR expression and the response to treatment with the NVP-AEW742 (IGF-IR inhibitor), and paclitaxel and docetaxel, and the EGFR expression and response to treatment with the PDGFRα/β TKI crenolanib

In regards to the expression levels of HER family members, it is important to point out the consistent results observed in this study in regards to the high EGFR expression in most of the GC cases, as was also reported by other studies (Dulak et al., 2012, Kim et al. 2008, Chen et al. 2013 and Kandel et al. 2014). Despite the high overexpression levels of EGFR in most of the GC cases, there is still no established data on the prognostic significance of this RTK and its validation as a therapeutic target in patients with GC. The results from the linear regression demonstrated a significantly positive correlation between EGFR expression and the response to treatment with the PDGFR and FLT3 inhibitor, crenolanib. It will be interesting to determine the expression level of EGFR in future clinical trials and its potential as a biomarker of the response to therapy with the PDGFR/FLT3 TKI in patients with GC.

In regards to ALK-7, the highest expression levels of this growth factor receptor were found in AGS and MKN-74 with MFI-values of 54.5 and 27.3, respectively (Table 5). AGS also had the highest level of c-MET with an MFI value of 21.6. However, AGS did not show higher sensitivity to either brigatinib (ALK and ROS1 inhibitor) and crizotinib (C-MET, ALK and ROS1) when compared to MKN74. Major contributing factors could that be that, in addition to ALK-7 and C-MET overexpression, AGS cells also overexpressed both EGFR and HER2. These in turn could have been responsible for the inadequate response to treatment with the ALK inhibitors. Future studies using a combination of the HER family and ALK/C-Met inhibitors would be needed to determine whether such treatments result in the synergistic growth inhibition of such GCCLs. Moreover, the results of Western blot analysis should unravel the impact of such treatments on the down-stream cell signalling molecules.

Of the other TKIs employed in this study, the IGF-IR inhibitor NVP-AEW742 was most effective at inhibiting the growth of MKN-74 (IC50=2.31 μM), which was also found to have

the highest level of the IGF-IR expression (MFI=35.6) among the other cell lines (Table 6 & 7). The linear regression analysis showed a positive correlation between this biomarker and the NVP-AEW742 (Table 8). According to a study by Liu et al., the expression or activation of C-MET may lead to resistance to treatments with IGF-IR inhibitors in GC, and the addition of crizotinib as a C-MET inhibitor shows satisfactory results in GC cases exhibiting positive C-MET expression (Liu et al., 2014). Therefore, in addition to the expression of all members of the HER family, it will also be interesting to determine the relative expression, prognostic significance and predictive values of the IGF-IR, c-MET and ALK in patients with GC. In particular, in this study, for the first time, a positive correlation was also found between the IGF-IR expression and the response to treatment with the cytotoxic drugs docetaxel and paclitaxel (Table 8). It might, therefore, be useful to investigate the predictive value of IGF-IR determination in the selection of GC patients who would benefit from such therapeutic agents.

Trametinib, which is a MEK1/2 inhibitor, was more effective in inhibiting AGS and MKN74 cell lines and also inhibited the other cell lines in higher doses. According to a study by Mizukami et al., when trametinib was used as monotherapy on a MEK1-mutated GC cell line, resistance to this drug was developed. They found that inhibition of the MEK pathway by trametinib caused phosphorylation of EGFR and HER2 and this, in turn, reactivated the ERK1/2 pathway and led to resistance to this agent. In order to reverse the activation of this pathway, treatment with a combination of the dual EGFR/HER2 reversible and trametinib resulted in synergistic growth inhibition of this cell line and induction of apoptosis (Mizukami et al., 2015), highlighting the need for further investigation using a combination of the HER inhibitors in combination with the MEL1/2 inhibitors and other targeted agents.

5.4. CDK inhibitors, dinaciclib and AT7519 and Pan- HER inhibitor, Afatinib, were the most effective agents in inhibiting the migration of gastric cancer cell lines

Metastasis in cancer leads to significantly high mortality rates (>90%) and therefore is considered as the hallmark of cancer. Cell migration has been classified as the key for cancer progression and metastasis and therefore it is important to identify the therapeutic agents which inhibit the migration of these cells and consequently inhibit the metastasis. In normal cells, cell motility and migration are less active, but there are certain cases where these are essential such as embryological development, immune defense etc. For example, the rates of cell migration

increase in healthy cells for wound healing purposes and therefore these processes possess important roles in cell physiology (Jiang et al., 2015).

Wound healing assay is an important technique to extrapolate the migration of cancer cells and the effect of therapeutic agents on the migration of these cells. There were five pre-selected agents in total used in the scratch wound healing assay following their effects observed from the growth inhibition assay. All five agents (Afatinib, dinaciclib, HKI357, AT7519 and docetaxel) were tested on AGS and HGC27 cell lines while only four (Afatinib, dinaciclib, HKI357, and docetaxel) were tested on MKN1.

Consistent results with growth inhibition were observed for CDK inhibitors (Dinaciclib and AT7519) and the pan-HER inhibitor afatinib. These drugs which had demonstrated very low IC50 values in the growth inhibition assay succeeded to induce a statistically significant inhibition on the migration of the tumour cells. While AT7519 was not tested on MKN1, it is challenging to conclude its effect on this cell line. However, as dinaciclib which is also a CDK inhibitor demonstrated significant results from as early as 12 hrs of treatment, it can be anticipated that AT7519 might also demonstrate similar results on this cell line.

As mentioned previously, it would be very helpful to assess the expression levels of these cell lines for different CDKs in order to further support the effectiveness of these agents. Interestingly, HKI357, an irreversible EGFR and HER2 inhibitor, which had demonstrated very low IC50 values in the EGFR overexpression AGS and MKN1 cell lines in the growth inhibition assay (Tables 4 & 5), failed to induce any significant effect on the migration of any of the three cell lines (Figures 14-16). Also, treatment with the cytotoxic drug docetaxel resulted in statistically significant inhibition of migration of both MKN1 and HGC27 cell lines but not AGS cells. These results suggest that while some agents can be highly effective at inhibiting the proliferation of human GCCLs, the same agents may not be as effective in inhibiting the migration of such tumours and warrants further investigation using a larger panel of human GCCLs.

Interestingly, to date, there has been no clinical trials conducted to investigate the effects of either dinaciclib or AT7519 in GC patients. And although our study is not sufficient enough to support and make claims on the efficacy of any agents used in this study, we believe that future studies employing larger panel of cells and consequently *in vivo* animal studies would lead to clear answers and interpretations around these results.

5.5. Concluding remarks and future work

In summary, in this study, the growth response of a panel of five human GCCLs to treatment with 18 agents including various types of the HER inhibitors, inhibitors of other RTKs, down-stream cell signalling molecules and CDKs were investigated. The results to date showed that: 1) the irreversible pan-HER family TKIs were more effective than the reversible EGFR specific erlotinib or the irreversible HER2 specific TKIs at inhibiting the growth *in vitro* of GCCLs, 2) the CDK1/2/59 inhibitor dinaciclib was the most effective CDK for inhibiting the growth of these GCCLs, and 3) all human GCCLs were most sensitive to treatment with the two cytotoxic drugs. However, the growth of GCCLs was also inhibited following the treatment with other agents by various amounts. At present, only a significant association was found between the IGF-IR expression and the response to the IGF-IR TKI NVP-AEW742 and the cytotoxic drugs, and between the EGFR expression and the response to the PDGFR/FLT3 TKI crenolanib.

However, in the study, the effect of these agents was investigated only on the growth of five human GCCLs (i.e., 4 derived from the primary tumours and 1 from lymph node metastasis) and the migration of three human GCCLs. Therefore, due to the heterogenous nature of human malignancy, including GC, these results support the need for further investigations on the therapeutic potential of such agents in GC and in particular:

1) The therapeutic potential of dinacicilib in combination with the pan HER family blocker such as afatinib and whether such treatments result in the synergistic inhibition of growth and migration and invasion (e.g. via Matrigel invasion assay) and induction of apoptosis (e.g. by flow cytometry based measurement of DNA content in sub-G1 or annexin V staining) of a larger panel of human GCCLs and ultimately in the ongoing clinical trials with various types of HER2 inhibitors (Tables 2 and Table 9) .
2) The impact of such treatments on the down-stream cell signalling molecules and potential mechanisms of resistance to such therapeutic agents
3) To examine relative expression of all HER family members, CDKs and IGF-IR in tumour specimens from patients with GC and to determine their prognostic significance and predictive values for the response to treatment with various agents including the recently approved HER-2 inhibitor Enhertu for the treatment of patients with previously treated HER2-positive advanced GC (AstraZeneca press release, 2021)

Table 9. Active (recruiting or not recruiting) clinical trials of trastuzumab or trastuzumab based regimens in monotherapy or combination therapy of patients with GC or GEJC. The results are updated as of 28/02/2021 and derived from clinicaltrials.gov website.

	NCT Number	Title	Conditions	Interventions	Characteristics
1	NCT04168931	Trastuzumab to Patients With Advanced Gastric Cancer With HER2 Positive Expression in CTC	·Gastric Cancer Stage IV	·Drug: Trastuzumab	Phase: Phase 2
2	NCT04661150	A Study of Atezolizumab and Trastuzumab in Combination With Capecitabine and Oxaliplatin in Patients With HER2 Positive Locally Advanced Resectable Gastric Cancer of Adenocarcinoma of Gastroesophageal Junction	·Gastric Cancer ·Gastroesophageal Junction Adenocarcinoma	·Drug: Atezolizumab ·Drug: Trastuzumab ·Drug: Capecitabine ·Drug: Oxaliplatin	Phase: Phase2
3	NCT04704934	Trastuzumab Deruxtecan for Subjects With HER2-Positive Gastric Cancer or Gastro-Esophageal Junction Adenocarcinoma After Progression on or After a Trastuzumab-Containing Regimen (DESTINY- Gastric04)	·Gastric Cancer, Adenocarcinoma ·Gastroesophageal Junction Adenocarcinoma	·Drug: Trastuzumab deruxtecan ·Drug: Ramucirumab ·Drug: Paclitaxel	Phase: Phase 3
4	NCT04379596	Ph1b/2 Study of the Safety and Efficacy of T-DXd Combinations in Advanced HER2+ Gastric Cancer (DESTINY-Gastric03)	·Gastric Cancer	·Drug: Fluorouracil (5-FU) ·Drug: Capecitabine ·Biological: Durvalumab ·Drug: Oxaliplatin ·Biological: Trastuzumab ·Drug: Trastuzumab deruxtecan ·Drug: Cisplatin	Phase: Phase 2
5	NCT03950271	SHR-1210 Combined With Trastuzumab , Oxaliplatin and Capecitabine for Neoadjuvant Therapy of Gastric Adenocarcinoma/Gastroesophageal Junction Adenocarcinoma	·Gastric Cancer	·Drug: SHR-1210 Combined With Trastuzumab , Oxaliplatin and Capecitabine	Phase: Phase 2

6	NCT04181333	Safety and Efficacy of Trastuzumab BS	·Gastric Cancer		
7	NCT03588533	Herzuma-capecitabine/Cisplatin for Gastric Cancer	·HER-2 Positive Gastric Cancer ·Metastatic Cancer	·Drug: Trastuzumab ·Drug: Capecitabine ·Drug: Cisplatin	Phase: Phase 2
8	NCT02205047	Neoadjuvant Study Using Trastuzumab or Trastuzumab With Pertuzumab in Gastric or Gastroesophageal Junction Adenocarcinoma	·Malignant Neoplasm of Stomach ·Malignant Neoplasm of Cardio- esophageal Junction of Stomach ·Epidermal Growth Factor Receptor (EGFR) Protein Overexpression	·Drug: Cisplatin ·Drug: 5-fluorouracil or Capecitabine ·Drug: Trastuzumab ·Drug: Pertuzumab ·Procedure: gastrectomy	Phase: Phase 2
9	NCT03615326	Pembrolizumab/Placebo Plus Trastuzumab Plus Chemotherapy in Human Epidermal Growth Factor Receptor 2 Positive (HER2+) Advanced Gastric or Gastroesophageal Junction (GEJ) Adenocarcinoma (MK-3475-811/KEYNOTE-811)	·Gastric Neoplasms ·Gastroesophageal Junction Adenocarcinoma	·Biological: Pembrolizumab ·Biological: Placebo ·Drug: Cisplatin ·Drug: 5-FU ·Drug: Oxaliplatin ·Drug: Capecitabine ·Drug: S-1 ·Biological: Trastuzumab	Phase: Phase 3
10	NCT04520295	ctDNA Screening in Advanced HER2 Positive Gastric Cancer	·HER2-positive Gastric Cancer	·Genetic: ctDNA screening	

	NCT Number	Title	Conditions	Interventions	Characteristics
11	NCT04082364	Combination Margetuximab, INCMGA00012, MGD013, and Chemotherapy Phase 2/3 Trial in HER2+ Gastric/ GEJ Cancer (MAHOGANY)	·Gastric Cancer ·Gastroesophageal Junction Cancer ·HER2-positive Gastric Cancer	·Combination Product: margetuximab plus INCMGA00012 ·Combination Product: Margetuximab plus INCMGA00012 plus chemo ·Combination Product: Margetuximab plus MGD013 plus chemo ·Combination Product: Margetuximab plus chemo ·Combination Product: Trastuzumab plus chemo	Phase: ·Phase 2 ·Phase 3
12	NCT04510285	Study of Pembrolizumab Plus Trastuzumab or Trastuzumab Alone After Surgery in Patients With Esophagogastric Tumors	·Esophagogastric Tumors ·Gastric Cancer ·Gastric Tumor ·Esophageal Cancer ·Esophageal Neoplasms ·Esophageal Tumor ·GastroEsophageal Cancer ·Gastroesophageal Junction Adenocarcinoma ·Gastroesophageal Junction Tumor ·Gastroesophageal Junction Cancer	·Drug: Trastuzumab ·Drug: Pembrolizumab	Phase: Phase 2
13	NCT04309578	A Study of Trastuzumab in Combination With Capecitabine and Cisplatin in Patients With Tissue HER2- But Serum HER2+ AGC	·Gastric or Gastroesophageal Junction(GEJ) Adenocarcinoma	·Drug: Trastuzumab, Capecitabine and Cisplatin	Phase: Phase 2

14	NCT04281576	Effect of Tumor Treating Fields (TTFields, 150 kHz) Concomitant With Chemotherapy as First Line Treatment of Unresectable Gastroesophageal Junction or Gastric Adenocarcinoma	·Gastric Cancer ·GastroEsophageal Cancer	·Device: NovoTTF-100L(P) ·Drug: Oxaliplatin ·Drug: Capecitabine ·Drug: Trastuzumab	Phase: Not Applicable
15	NCT04714190	A Study of RC48-ADC in Local Advanced or Metastatic Gastric Cancer With the HER2-Overexpression	·Gastric Cancer ·HER2 Overexpressing Gastric Carcinoma	·Drug: RC48-ADC ·Drug: Paclitaxel injection ·Drug: Irinotecan Hydrochloride Injection	Phase: Phase 3
16	NCT02578368	Chemotherapy Alone vs. Chemotherapy + Surgical Resection in Patients With Limited-metastatic Adenocarcinoma of the Stomach or Esophagogastric Junction	·Gastric Cancer	·Drug: 5-Fluorouracil ·Drug: Leucovorin ·Drug: Oxaliplatin ·Drug: Docetaxel ·Drug: Trastuzumab ·Drug: sodium folinate ·Procedure: Surgery	Phase: Phase 3
17	NCT03694977	Biomarker Study of PDR001 in Combination With MCS110 in Gastric Cancer	·Gastric Cancer	·Drug: MCS110/PDR001 combination	Phase: Phase 2
18	NCT04086888	Real World Study of Immune Checkpoint Inhibitors for Advanced Gastric Cancer	·Gastric Cancer		

	NCT Number	Title	Conditions	Interventions	Characteristics
19	NCT04464967	Safety and Preliminary Efficacy of SNK01 in Combination With Trastuzumab or Cetuximab in Subjects With Advanced HER2 or EGFR Cancers	·Advanced Solid Tumor ·Metastatic Cancer ·HER2-positive Breast Cancer ·HER2-positive Gastric Cancer ·HER-2 Protein Overexpression ·Esophageal Cancer ·Ovarian Cancer ·Endometrium Cancer ·Bladder Cancer ·Pancreatic Cancer ·and 7 more	·Biological: SNK01 ·Drug: Trastuzumab ·Drug: Cetuximab	Phase: ·Phase 1 ·Phase 2
20	NCT03253107	Predicting Biomarker of Gastric Cancer Chemotherapy Response	·Gastric Cancer ·Chemotherapy Effect ·Predictive Cancer Model	·Drug: Chemotherapy	
21	NCT02678182	Planning Treatment for Oesophago-gastric Cancer: a Maintenance Therapy Trial	·Adenocarcinoma of the Oesophagus ·Adenocarcinoma of the Gastro-oesophageal Junction ·Adenocarcinoma of the Stomach	·Drug: Capecitabine ·Drug: MEDI4736 ·Drug: Trastuzumab ·Drug: Rucaparib ·Drug: Ramucirumab	Phase: Phase 2

22	NCT02393248	Open-Label, Dose-Escalation Study of Pemigatinib in Subjects With Advanced Malignancies - (FIGHT-101)	·Lung Cancer ·Solid Tumor ·Gastric Cancer ·Urothelial Cancer ·Endometrial Cancer ·Multiple Myeloma ·Myeloproliferative Neoplasms ·Breast Cancer ·Cholangiocarcinoma ·UC ·MPN	·Drug: Pemigatinib ·Drug: Gemcitabine + Cisplatin ·Drug: Pembrolizumab ·Drug: Docetaxel ·Drug: Trastuzumab ·Drug: INCMGA00012	Phase: ·Phase 1 ·Phase 2
23	NCT02465060	Targeted Therapy Directed by Genetic Testing in Treating Patients With Advanced Refractory Solid Tumors, Lymphomas, or Multiple Myeloma (The MATCH Screening Trial)	·Advanced Malignant Solid Neoplasm ·Bladder Carcinoma ·Breast Carcinoma ·Cervical Carcinoma ·Colon Carcinoma ·Colorectal Carcinoma ·Endometrial Carcinoma ·Esophageal Carcinoma ·Gastric Carcinoma ·Glioma ·and 40 more	·Drug: Adavosertib ·Drug: Afatinib ·Drug: Afatinib Dimaleate ·Drug: Binimetinib ·Drug: Capivasertib ·Drug: Copanlisib ·Drug: Copanlisib Hydrochloride ·Drug: Crizotinib ·Other: Cytology Specimen Collection Procedure ·Drug: Dabrafenib ·and 23 more	Phase: Phase 2

	NCT Number	Title	Conditions	Interventions	Characteristics
24	NCT03219268	A Study of MGD013 in Patients With Unresectable or Metastatic Neoplasms	·Advanced Solid Tumors ·Hematologic Neoplasms ·Gastric Cancer ·Ovarian Cancer ·GastroEsophageal Cancer ·HER2-positive Breast Cancer ·HER2-positive Gastric Cancer ·DLBCL	·Biological: MGD013 ·Biological: MGD013 in combination with margetuximab	Phase: Phase 1
25	NCT04278144	A First-in-human Study Using BDC-1001 in Advanced HER2-Expressing Solid Tumors	·Neoplasm, Metastatic ·Neoplasm, Breast ·Neoplasm, Gastric	·Drug: BDC-1001 ·Drug: Pembrolizumab	Phase: ·Phase 1 ·Phase 2
26	NCT03966118	Avelumab + Paclitaxel/ Ramucirumab (RAP) as Second Line Treatment in Gastro-esophageal Adenocarcinoma	·Gastroesophageal Junction Adenocarcinoma ·Adenocarcinoma of the Stomach	·Drug: Avelumab ·Drug: Ramucirumab ·Drug: Paclitaxel	Phase: Phase 2
27	NCT04246671	Intravenous TAEK-VAC-HerBy Vaccine Alone and in Combination Treatment in HER2 Cancer Patients	·Breast Cancer ·Gastric Cancer ·Chordoma ·Lung Cancer ·Ovarian Cancer ·Prostate Cancer ·Colorectal Cancer ·Pancreatic Cancer ·Hepatocellular Cancer ·Merkel Cell Carcinoma ·Small-cell Lung Cancer	·Biological: TAEK-VAC-HerBy	Phase: ·Phase 1 ·Phase 2

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