Chapter 1
Introduction

CHAPTER 1 INTRODUCTION

1.1. GENERAL

Cancer represents a group of diseases which involves uncontrolled cell proliferation, abnormal cell growth, ability to destroy and migration to different organ systems of the body. (Hanahan and Weinberg 2011). It is the second major health problem around the globe. According to cancer statistics 2021, in the United States of America roughly 608,570 cancer related deaths with 1,898,160 new cases are expected (Siegel, Miller et al. 2021). In general, about 100 different types of cancer affect human being. The majority of the cancers develop due to alteration in the genetic makeup. Some other factors like lifestyle, diet, obesity, behavioral factors etc., also contribute to the development of cancer (Blackadar 2016). Cancer is categorized into many types based on its origin. These includes carcinoma, sarcoma, lymphoma, leukemia and blastoma. Among all, cancer that is derived from epithelial cells *i.e.*, carcinoma, is most common type of cancer which further includes, lung cancer, breast cancer, prostate cancer, colon cancer, pancreatic cancer etc (Yuan, Norgard et al. 2019; Bade and Cruz 2020). Based on types of cancer, many treatment approaches are available for the mitigation and treatment of cancer including surgery, radiation therapy, chemotherapy, targeted therapy, hormone therapy and immunotherapy (A Baudino 2015). Till date, number of chemotherapeutic agents are developed for the treatment, mitigation and management of cancer. Despite all these efforts, there are still many pitfalls in cancer chemotherapy (Marzo and Naval 2013). One of the major challenges is development of resistance against approved chemotherapeutic agents. The reasons behind development of resistance against anti-cancer drugs vary according to the types of cancer, from acquired mutations to cross talk among signaling pathways, to inactivation of active drug form, to altered cell cycle regulation and check points, etc., all have been reported to result in development of resistance (**Figure 1.1.**) (Housman, Byler et al. 2014). These complications led to discovery of new approaches in the field of anti-cancer drug discovery like targeted therapy. In targeted therapy, focused efforts are made to inhibit dysregulated cellular targets specific to tumor cells (Lee, Tan et al. 2018). Mainly members of overexpressed signaling pathways are targeted in

this approach. Targeted therapy may exert it's anticancer action *via* multiple mechanisms like inhibition of cell proliferation, apoptosis induction, suppression of metastasis and reversal of multidrug resistance (Ke and Shen 2017). Many targets like HER2, EGFR, PI3K, ALK, ERK1/2, CDKs, HER2, etc are overexpressed in different types of cancer and are continuously explored by the researchers in concerted efforts to develop targeted therapy, overcoming the problem of resistance and developing specific treatment for cancer.

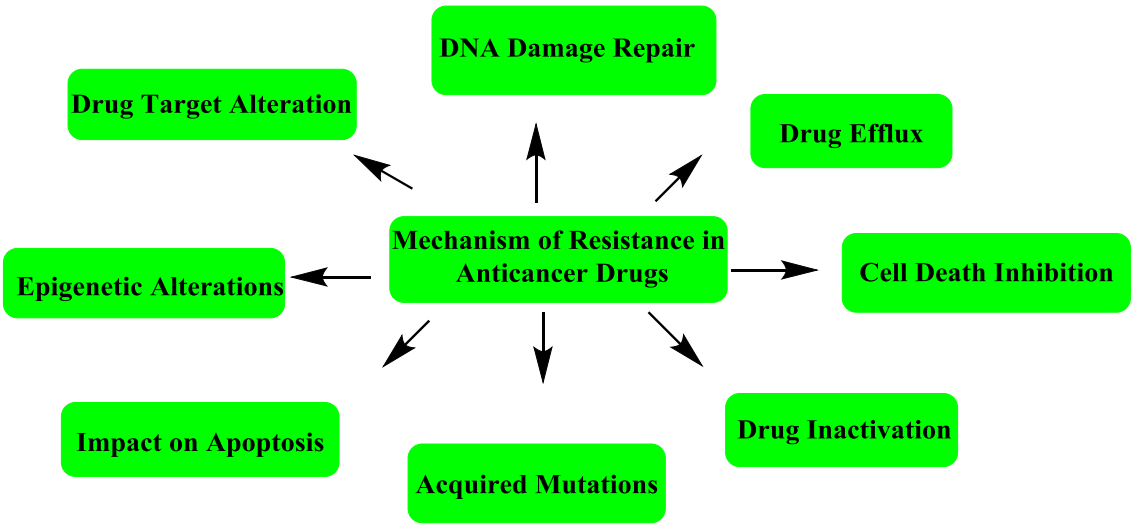

Figure 1.1. Mechanisms of anticancer drug resistance.

1.2. BREAST CANCER

Amongst all types of cancer, one very often observed in women is breast cancer (Akram, Iqbal et al. 2017). Similarly, it is also one of main reasons of mortality in women. Also, it could be surprising for many but 1 out of every 100 breast cancer diagnosis in US is in a man (Elimimian, Elson et al. 2021). Breast cancer is known to be the second most common cause of death among women (Garcia-Estevez and Moreno-Bueno 2019). According to WHO data, breast cancer cases rise every year throughout the world. On the basis of overexpression of molecular targets, breast cancer is classified into many sub-types such as ER/PR+ (showing overexpression of

estrogen and progesterone receptors; 70% of all breast cancers cases) and HER2+ (showing overexpression of human epidermal growth factor receptor 2; 20% of breast cancers cases) (Dai, Xiang et al. 2016). A critical subtype of breast cancer is TNBC (Triple-Negative Breast Cancer), which is among most aggressive and lethal form of breast cancer because of the absence of molecular targets such as HER2, ER and PR (Pal, Childs et al. 2011). TNBC is observed in around 15% of the breast cancers cases and usually harbors signature of a BLBC (Basal-like Breast Cancer) gene expression (Giltnane and Balko 2014). As TNBC lacks overexpression of ER, PR and HER2, it is resistant towards hormonal and targeted therapies. Therefore, targeted therapy such as anti-HER2 therapy do not benefit TNBC patients clinically in comparison to chemotherapy (Denkert, Liedtke et al. 2017). Hence, there is a requirement of developing new potent therapeutic agents with specific mechanism of action, which can overcome the existing challenges in the management of TNBC. Key signaling pathways such as MAPK (Mitogen-Activated Protein Kinase) pathway have been reported to be overexpressed in the intracellular signaling process of TNBC (Eralp, Derin et al. 2008; Peng, He et al. 2019). Several reports indicate that prevalent transcriptional signatures of activated MAPK pathway are observed in TNBC and BLBC in comparison to other isoforms of breast cancer (Hoeflich, O'Brien et al. 2009; Pratilas, Taylor et al. 2009; Balko, Cook et al. 2012). Presence of such signatures suggests that the signaling output of the MAPK pathway constitutes a major component of oncogenic activity and further corroborates immunohistochemistry associations of MAPK activity in TNBC (Jiang, Wang et al. 2020).

1.3. MAPK PATHWAY

MAPK signaling pathway is one of the important cellular signaling pathways, essential for the functioning of cells (Garrington and Johnson 1999). It comprises of several signaling protein which participate in downstream signaling to the nucleus, resulting in the eventual outcome. Briefly, the pathway initiates from the receptor linked kinases (EGFR, c-Kit, etc) whereby the binding of extracellular ligands result

in their activation followed by recruitment of Sos (guanine exchange factor) to the small RAS GTPase (a GTP to GDP converter), which binds to RAS, the first member of the MAPK signaling pathway, resulting in a conformational change in RAS that allows it to interact with its downstream effectors. Upon activation of RAS, the downstream effector RAF, a serine/ threonine kinase, is translocated to the plasma membrane, where it activates *via* phosphorylation and de-phosphorylation. The next member of the pathway, MEK, is phosphorylated and activated *via* Raf, followed by activation of ERK, resulting in altered gene expression and protein activity (Seger and Krebs 1995). Activated ERKs dimerize and translocate to the nucleus; downstream ERK1/2 proteins include transcription factors, such as c-Fos and Elk-1, while cytoplasmic substrates include Rsk (**Figure 1.2.**). In cancer, MAPK pathway is abnormally activated due to the over activation of the growth factor receptors and mutations in RAS and BRAF proteins (Schubbert, Shannon et al. 2007; Kim and Choi 2010). Approximately 33% and 8% of the human cancers are found to have overexpressed RAS and BRAF proteins, respectively. Targeting different members of MAPK pathway such as RAF and MEK by small heterocyclic molecules has been reported as an efficient strategy for the management of advanced melanomas that harbor the BRAF V600E mutation (Jang and Atkins 2014). Thus, successes of RAF and MEK inhibitors in melanoma treatment reinforce and validate the concept that some cancers can be 'addicted' to MAPK pathway activity (Lee, Rauch et al. 2020).

Surprisingly in breast cancer, activating mutations are observed infrequently in the different members of MAPK pathway (Giltnane and Balko 2014). Importantly, the low frequency of such activating mutations in different members of MAPK pathway indicate that this pathway may not be obligatory for the growth and survival of breast cancer and its normal epithelial cell precursors. On the other hand, sufficient literature reports validate a pivotal role of MAPK pathway in the survival and development of breast cancer (Normanno, Luca et al. 2006). Studies establishing elevated ERK1/2 phosphorylation, which is a primary output of aberrant RAS function, in tumor sites relative to primary breast metastasis also validate this role (Adeyinka, Nui et al.

2002). In general, the upregulated activity of RAS is considered to be actively involved in the development of TNBC/basal-like tumors. It also indicates that MAPK activity is abnormally stimulated in breast cancer, may be *via* other mechanisms such as overexpression of RTKs (Receptor Tyrosine Kinases) like EGFR and HER2. Another alternative mechanism of MAPK pathway activation include copy number alterations in canonical MAPK pathway constituents *i.e*., amplifications or gains of KRAS and BRAF (Craig and Petticrew 2013). Another mechanism involving loss of negative regulation have also been proposed for the activation of the MAPK pathway. Alterations including truncations and deletions in NF1 (Nissan, Pratilas et al. 2014), which accelerate the hydrolysis of GTP to GDP and thereby catalyzes the inactivation of RAS, are hypothesized to result in constitutive activity of RAS and the downstream MAPK pathway, a bit similar to PTEN mutations or deletion resulting in PI3K/Akt pathway activation. Loss of function of miRNAs which specifically target KRAS have been shown to be lost or deregulated in TNBC (Masliah-Planchon, Garinet et al. 2016). This may serve as an additional mechanism for aberrant MAPK pathway activity in TNBC. Following these findings, researchers are involved in the development of small-based molecules targeting MAPK signaling cascade for the management of cancer.

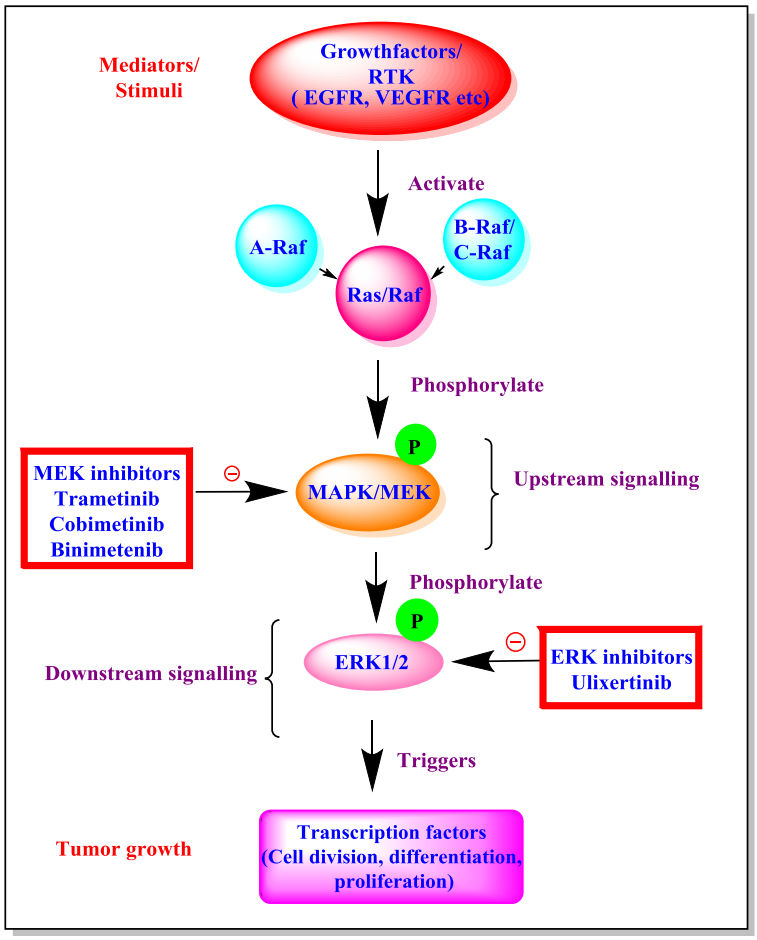

Figure 1.2. Targeting extracellular signal-regulated kinase (ERKs) upregulation in downstream pathway for the management of cancer.

1.4. TARGETING DIFFERENT MEMBERS OF MAPK PATHWAY

1.4.1. KRAS inhibitors

KRAS is one of the initially identified oncogenes but till today most research efforts to find its inhibitors have been unsuccessful. The main reasons behind such failure include picomolar level affinity of KRAS for GDP and GTP which hampers development of nucleotide binding competitors (Roskoski Jr 2021), and floppy surface of KRAS *i.e.*, absence of any deep and significant hydrophobic pockets on its

surface, limiting the possibility of identifying allosteric inhibitors (Bannoura, Uddin et al. 2021). However, recently a lot of small molecule inhibitors targeting KRAS have been reported for the management of cancer. One of the earliest direct inhibitors of KRAS was **SCH-53239**, reported in 1997 (Korzeniecki and Priefer 2021). Although it inhibited GDP to GTP conversion, non-selective binding to wild-type KRAS led to toxicity. In 2013, Kevan M. Shokat and group identified **Compound 6** which binds in the allosteric S-IIP in the GDP state of G12C mutant (Ostrem, Peters et al. 2013). This allosteric binding resulted in the blockade of interaction between KRAS and its effector proteins like RAF and SOS. This work opened up the approach of targeting allosteric site to attain specificity for the mutant KRAS G12C. In 2019, Amgen reported development of allosteric inhibitor, **AMG510**, *via* utilization of crystallographic information obtained from a lead compound to rationally perform structure-based designing followed by a custom library synthesis (Govindan, Fakih et al. 2019). In the same year, Mirati Therapeutics reported discovery of **MRTX849/Adagrasib**, another allosteric inhibitor which is now in clinical trials (**Figure 1.3.**). Briefly, they reported optimization of a previously identified covalent inhibitor, **Compound 4**, by altering substituents on its naphthyl side flank and pyrido-pyrimidine core leading to enhanced cellular potency and solubility (Fell, Fischer et al. 2020). In 2020, Johnson and Johnson and Wellspring Bioscience reported discovery of another KRAS inhibitor, **ARS-3248**, which has been now withdrawn from clinical trials (Goebel, Müller et al. 2020). Nowadays, two more inhibitors of KRAS are being evaluated in Phase I of clinical trials, *i.e*., **GDC-6036** developed by Roche and **D-1553**, developed by InventisBio. *Structural and pharmacophoric details of all these three (ARS-3248, GDC-6036 and D-1553) compounds are yet not publicly disclosed*.

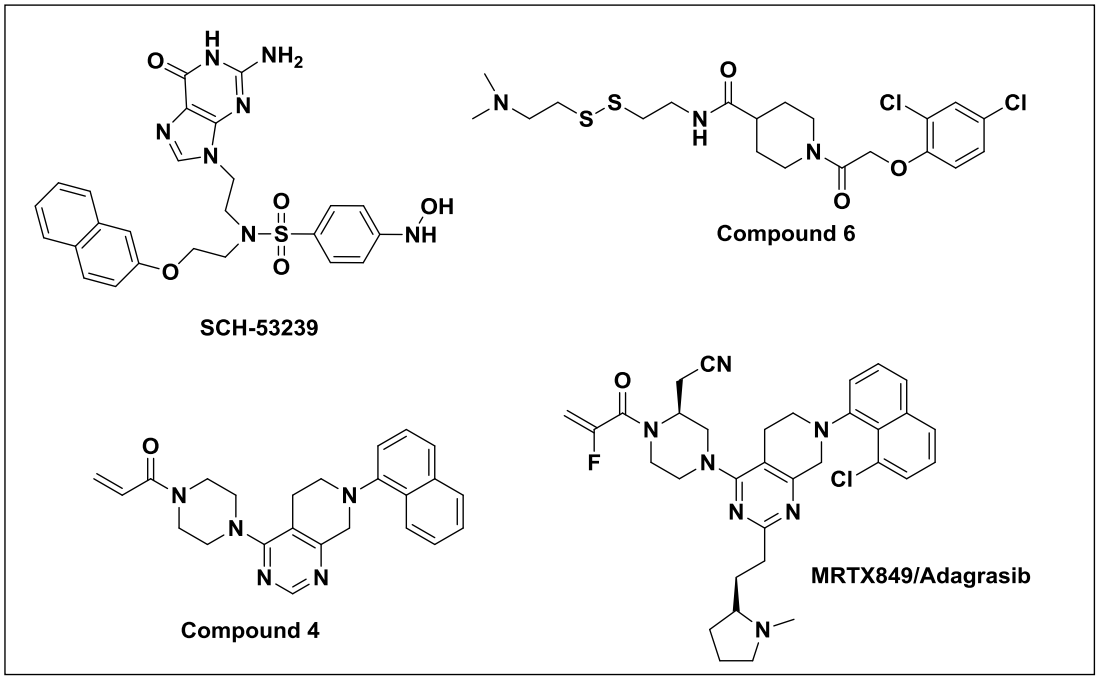

Figure 1.3. Some reported KRAS inhibitors.

1.4.2. Problems associated with KRAS inhibitors

First of all, it has been noted in pre-clinical models that absence of dependency on KRAS signaling could result in resistance but it may vary depending on the patients. Secondly, the mechanism of resistance offered by KRAS and how it encourages resistance to inhibition of MEK has still not been fully known but it has been somewhat associated with its ability to dimerize with mutant KRAS. Therefore, both wild-type RAS and mutant form of KRAS mediate resistance towards inhibition of MEK signaling. Irrespective of both of these observation, studies clearly indicate role of activated RTK and SHP2 are in acquired resistance towards KRAS inhibitors (Hyun and Shin 2021).

1.4.3. B-RAF inhibitors

Sorafenib, a multi-kinase inhibitor, was primarily developed as a first generation kinase inhibitor of RAF inhibitor for the management of melanoma but it performed poorly in improvement of survival rate as a single drug (Mangana, Levesque et al.

2012). As a multi-kinase inhibitor, sorafenib can inhibit VEGFR, c-kit, PDGFR, Flt-3, and FGFR1. The second generation of RAF inhibitor, **Vemurafenib** was approved by FDA in the year 2011 for the treatment of metastatic melanoma with BRAF V600E mutation. Mechanistically, it occupies the ATP pocket of mutant B-RAF monomer but can only inhibit MEK and ERK signaling if there is no activation of RAS (Sharma, Shah et al. 2012). It is also reported to show certain adverse effects such as ~30% patients developing squamous cell carcinoma, skin rash, etc. Another inhibitor of pan-RAF kinases is **ARQ736**, an ATP-competitive inhibitor (Martin-Liberal and Larkin 2014). Various mouse models and cancer cell lines like colon, melanoma and thyroid have demonstrated that this molecule effectively inhibits wild-type BRAF, mutant BRAF (V600E), and c-RAF. Another small molecule heterocycle, **Dabrafenib**, a selective inhibitor of B-Raf V600E mutant, is currently in clinical trials (Li, Feng et al. 2014). Similarly, **PLX3603** is also in phase I clinical trial for the management of advanced solid tumors (NCT01143753) (Martin-Liberal and Larkin 2014). **RAF265**, a dual inhibitor of mutant BRAF V600E and VEGFR2 with EC_{50} value of 0.14 μM and 0.19 μM, respectively is also reported to inhibit downstream ERK signaling in B-CPAP thyroid cancer cells. **GDC0879**, another kinase inhibitor has been reported to inhibit B-Raf V600E in 130 cancer cell lines and mouse models (**Figure 1.4.**).

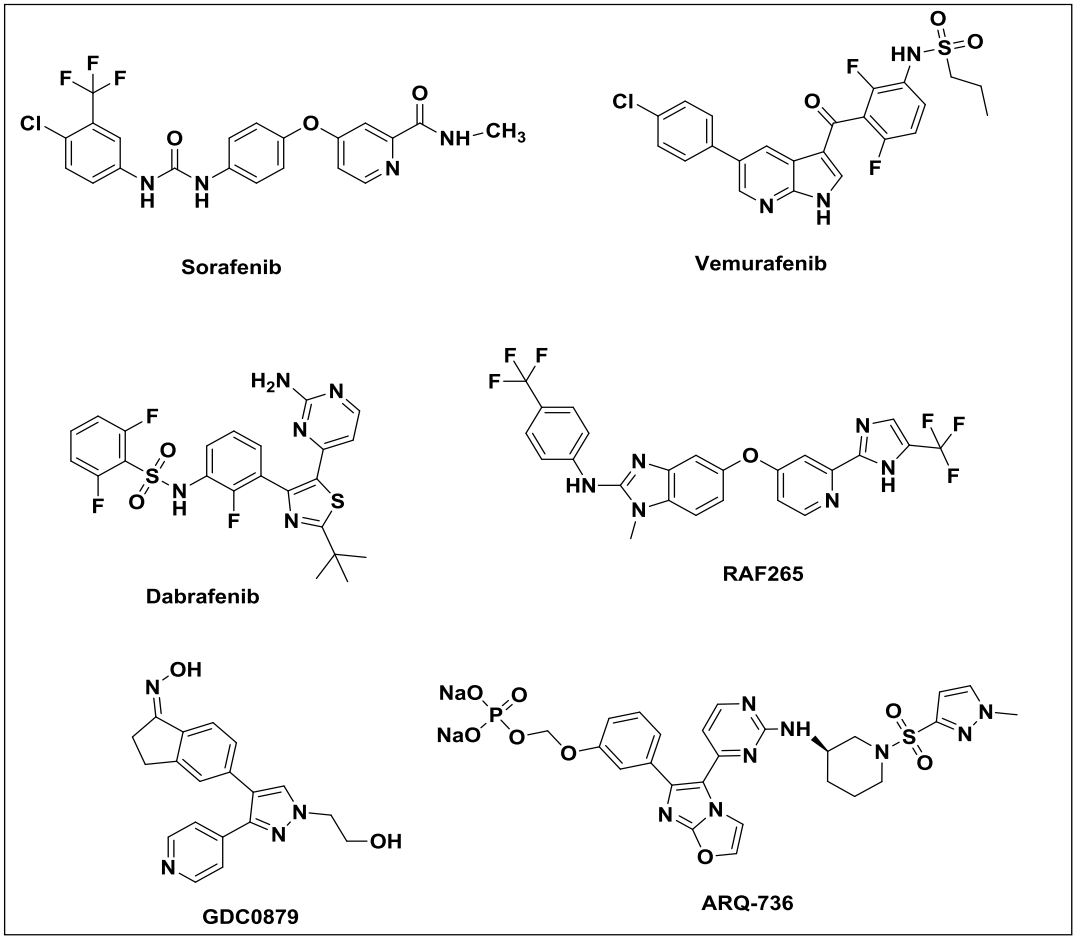

Figure 1.4. Some clinically reported B-RAF inhibitors.

1.4.4. Problems associated with BRAF inhibitors

Firstly, RAF inhibitors have been known to possess a peculiar ability whereby they inhibit ERK signaling in cells with mutant BRAF while they augment the same signaling

in cells with wild type BRAF. Secondly, BRAF inhibitors usually interact and inhibit only one protomer of the RAF complex *i.e.*, either the C-Raf/C-Raf homodimer or the C-Raf/B-Raf heterodimer, which permits the other member to be trans-activated and continue signaling. Also, it has been reported that RAF inhibitors fail to inhibit ERK signaling in cells expressing both BRAF V600E and mutant RAS. Therefore, it is suggested that RAS activation and RAF dimerization, both result in resistance against

BRAF inhibitors. Additionally, analogous signaling *via* other cellular proteins which can cross talk with different members of MAPK pathway have also been found responsible for resistance against BRAF inhibitors. One study disclosed that tumor cells have the capacity to revamp signaling properties to continue ERK activation *via* recruiting other active isoforms of RAF and thereby developing resistance against RAF inhibitors in cancer cells. Although BRAF mutations are extensively observed, irony is the fact that clinical benefits of BRAF inhibitors are restricted to metastatic melanomas with expression of mutant BRAF (Luebker and Koepsell 2019).

1.4.5. MEK inhibitors

PD098059 was the first MEK inhibitor reported in 1995 (Grimaldi, Simeone et al. 2014). There have been several MEK inhibitors in clinical trials. One of the most clinically efficient MEK inhibitor is **Trametinib** (**Figure 1.5.**). It is the only selective MEK1/2 inhibitor having proven clinical efficacy (Wright and McCormack 2013). Mechanistically, it is second generation allosteric inhibitor with potent nanomolar range inhibitory activity against purified MEK 1 and 2. Another selective second generation allosteric inhibitor of MEK1/2 is **Pimasertib** (Martinelli, Troiani et al. 2013). **Selumetinib** is also a nanomolar range selective second generation allosteric inhibitor of MEK 1/2 (Markham and Keam 2020). Xenograft models validated that mechanism of anti-cancer activity of Selumetinib involved inhibition of ERK1/2 phosphorylation. Another one belonging to the same class of allosteric inhibitor of MEK1/2 is **PD-0325901**, a synthetic derivative of **CI-1040**, with nanomolar range inhibitory activity (Barrett, Bridges et al. 2008).

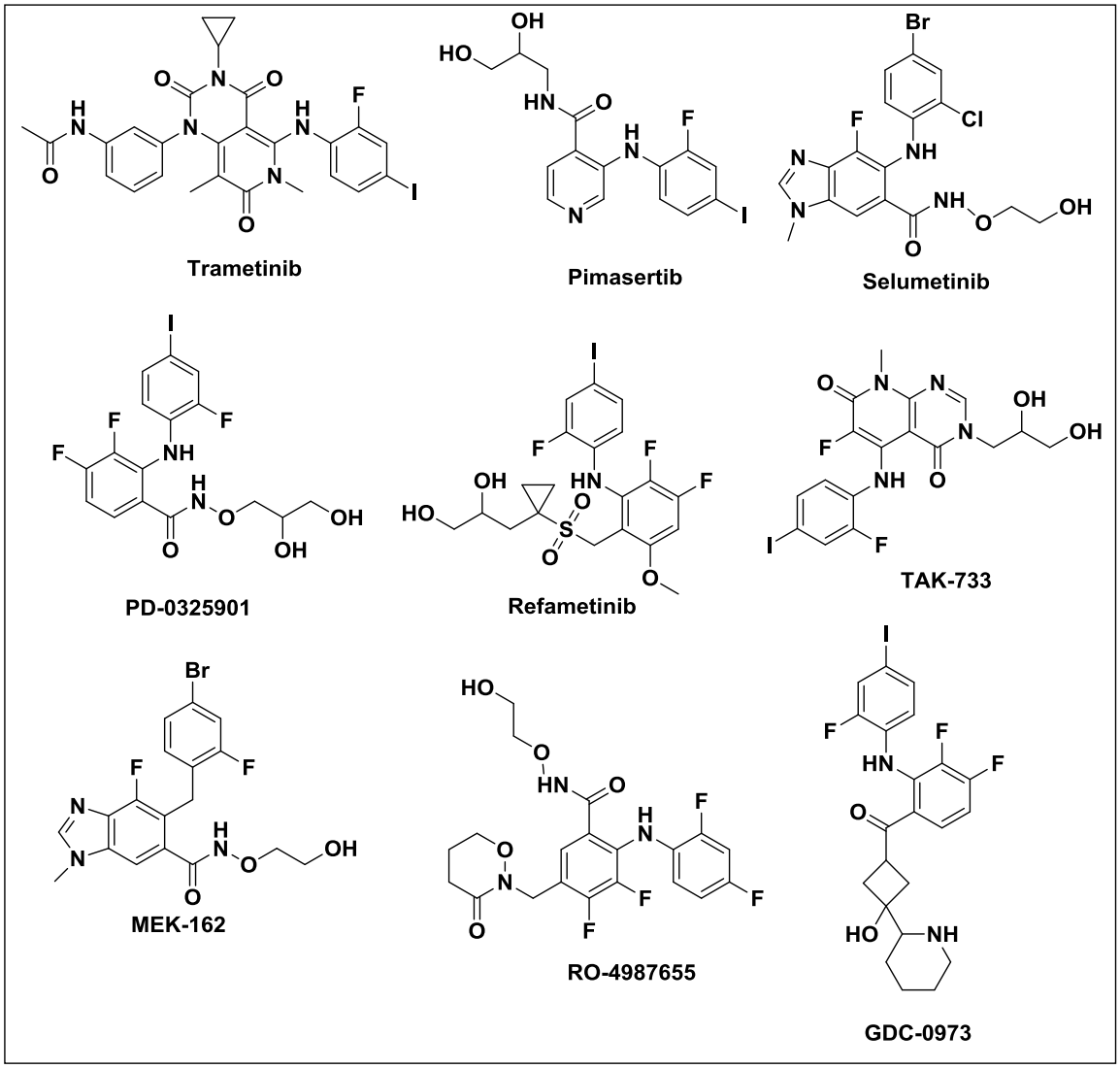

Figure 1.5. Some clinically reported MEK inhibitors.

Mechanistic analysis in melanoma and BRAF mutated papillary thyroid cancer (PTC) cell lines disclosed inhibition of ERK1/2 phosphorylation upon treatment with PD-0325901. Another selective allosteric inhibitor of MEK 1/2 is **Refametinib**, a derivative of cyclopropane-1-sulfonamide (Schmieder, Puehler et al. 2013). Other potent second generation nanomolar range MEK1/2 inhibitors include TAK733 and MEK162 (Dong, Dougan et al. 2011)(Bendell, Javle et al. 2017). **RO5126766** was reported as the first-in-class, highly potent, dual MEK/Raf inhibitor which binds

selectively to MEK 1/2. A panel of human cell lines study showed that RO5126766 exhibit its inhibitory activity through cell cycle arrest (Wada, Horinaka et al. 2014). **RO4987655** is another highly selective MEK inhibitor having unique 3-oxo-oxazinane moiety which promote metabolic stability of the compound (Leijen, Middleton et al. 2012). An orally bioavailable, small-molecule inhibitor of MEK 1 is **GDC-0973**, derived from methanone with potent anticancer activity against BRAF and K-RAS mutated cancer cell lines (Rice, Aay et al. 2012).

1.4.6. Problems associated with MEK inhibitors

The common mechanism of acquired resistance against MEK inhibitors in any type of cancer involve reactivation of the MAPK pathway, finally leading to activation of ERK. This could occur via multiple ways, most common being alterations or mutations in upstream signaling proteins like RAF, RAS, or MEK. In certain cases, upon treatment with MEK inhibitors, MEK develops mutations which result in its over-activation limiting efficacy of its inhibitors. Parallel signaling cascades activated by different RTKs which eventually leads to the activation of similar signaling molecules and cross-talk with members of MAPK pathway resulting the continued cellular growth and proliferation also results in resistance against MEK inhibitors. Additionally, as a mechanism of adaptive resistance, cancer cells upon successful inhibition of MAPK pathway, revert to other signaling pathways such as STAT, PI3K, and Hippo to continue growth (McCubrey, Steelman et al. 2011).

1.5. PUTATIVE WAY TO BYPASS RESISTANCE AGAINST MAPK PATHWAY

Amongst all the members of the MAPK pathway, the focus on ERK1/2 inhibitors has been very limited while a lot of clinical evaluation of MEK and RAF inhibitors has been studied (Liu, Yang et al. 2018). One commonly known reason is the fact that ERK lies downstream of MEK and if MEK is successfully inhibited then inhibiting ERK seemed redundant. However, with the disclosure of several resistance

mechanism against MEK inhibitors, inhibition of ERK does not seem so redundant after all. Nonetheless, there are several other known phenomenon which suggest that targeting ERK1/2 may provide a possible way to bypass resistance mechanisms against other members of the MAPK pathway (Inamdar, Madhunapantula et al. 2010). First of all, in spite of cancer heterogeneity, genomic instability and other variable factors, one commonality in multiple underlying mechanisms of resistance is the activation of upstream components *via* some compensatory mechanism and therefore continuation of MAPK signaling into nucleus. Several studies have revealed that in each case of parallel signaling, cross-talk signaling or reactivation dependent signaling, the eventual activation of ERK1/2, which then moves into nucleus, is essential and therefore, inhibiting ERK1/2, the eventual downstream kinase is beneficial in overcoming resistance. Secondly, in MAPK pathway ERK1/2 is positioned at a unique place. The signaling protein, RAF, upstream to it has only limited effectors besides MEK, which does not have any other substrate besides ERK1/2 and therefore ERK1/2 are the prime activators to execute the functional outcome of the MAPK pathways (Montagut and Settleman 2009), and accordingly ERK1/2 inhibitors are reported to overcome the mutated upstream signaling molecules induced activation of MAPK pathway (Liu, Yang et al. 2018). On top of that literature suggests that inhibition of ERK1/2 along with MEK and RAF is also considered useful in cases of acquired resistance (Hatzivassiliou, Liu et al. 2012). Additionally, other over-activated pathways in cancer also execute their functional outcome *via* cross-talk with ERK1/2, which enhance the clinical relevance of ERK inhibition in the management of cancer (Von Kriegsheim, Baiocchi et al. 2009). Supporting this hypothesis, studies show that treatment with selective ERK inhibitors reversed acquired resistance to MEK and BRAF inhibitors. Sullivan and colleagues reported the clinical evidences of ERK1/2 inhibitors overcoming BRAF mutations double acquired resistance against BRAF and MEK inhibitors in melanomas (Cohen and Sullivan 2019). This study for the first time indicated towards possible success of ERK1/2 inhibitors in patients with MAPK-dependent malignancies, where BRAF and

BRAF-MEK inhibitor therapy has failed.

Both ERK1 and ERK2 contain 85% of the same amino acid sequence. As ERK1 and ERK2 belong to the mitogen-activated family, thus they are also acknowledged as MAPKs (mitogen-activated protein kinases) (Pouysségur and Lenormand 2016). In cancer, the proto-oncogenic drivers like mutation leads to dysregulation and increased kinases activity. Due to this increased phosphorylation, ERKs have been involved in cancers of different tissues like breast, lung, prostate, etc. Therefore, both ERK1 and ERK2 are one of the promising targets involved in cancer, which seek the attention of many researchers (Braicu, Buse et al. 2019). Till date, several ERK inhibitors have been reported; based on their mechanism, ERK inhibitors have been categorized into reversible/ATP-competitive, covalent and allosteric ERK inhibitors (Kidger, Sipthorp et al. 2018). All these inhibitors either alone or in combination have aimed to prevent the phosphorylation of ERK1/2 and inhibition of catalytic activity of ERKs. **PD098059**, an allosteric inhibitor was the first reported ERK inhibitor in the year 1995 (Alessi, Cuenda et al. 1995). In 2014, SCH772984, a potent and selective ERK1/2 inhibitor with anti-cancer activity in BRAF or RAS mutations harboring MAPK inhibitor-resistant cells was also reported (Wong, Robert et al. 2014). Later on, several ERK inhibitors have been established, few are FDA- approved and some are in various phases of clinical trials (**Figure 1.6.**) but overall, the total number of ERK1/2 inhibitors in clinical trials is still less corresponding to other such targets. Ulixertinib is one such selective, reversible, ATP-competitive and orally active ERK1/2 inhibitor in clinical trials (Sullivan, Infante 0et al. 2018). Although Ulixertinib possess potent activity, it causes some adverse effects such as rashes/acneiform, diarrhea, fatigue and nausea. Natural products including astragaloside IV, a triterpenoid present in Radix astragali suppressed MAPK family members like ERK1/2 and JNK (Li, Hou et al. 2017). Two other natural product, 20(S)-protopanaxadiol and platycodin D also showed the inhibition of metastasis in TNBC by inhibiting EGFR mediated MAPK pathway (Gao, Lv et al. 2013). Honey from strawberry tree has also been found to inhibit downstream markers such as p-

p38MAPK and p-ERK1/2 in colon cancer cell proliferation. Existing ERK inhibitors reveal the presence of various heterocyclic scaffolds in them, which are essential for their inhibitory property. In 2007, pyrrole-based ERK inhibitors appeared and since then various other heterocycles containing inhibitors have been developed including SCH772984, an indazole-based ERK1/2 inhibitor which selectively targets the ERK signaling pathway. Other derivatives include 1,2,4-trisubstituted imidazolinone moiety, 3(S)-thiomethyl pyrrolidines, 1,2-bis-quinolinyl-1,4-naphthoquinones etc.

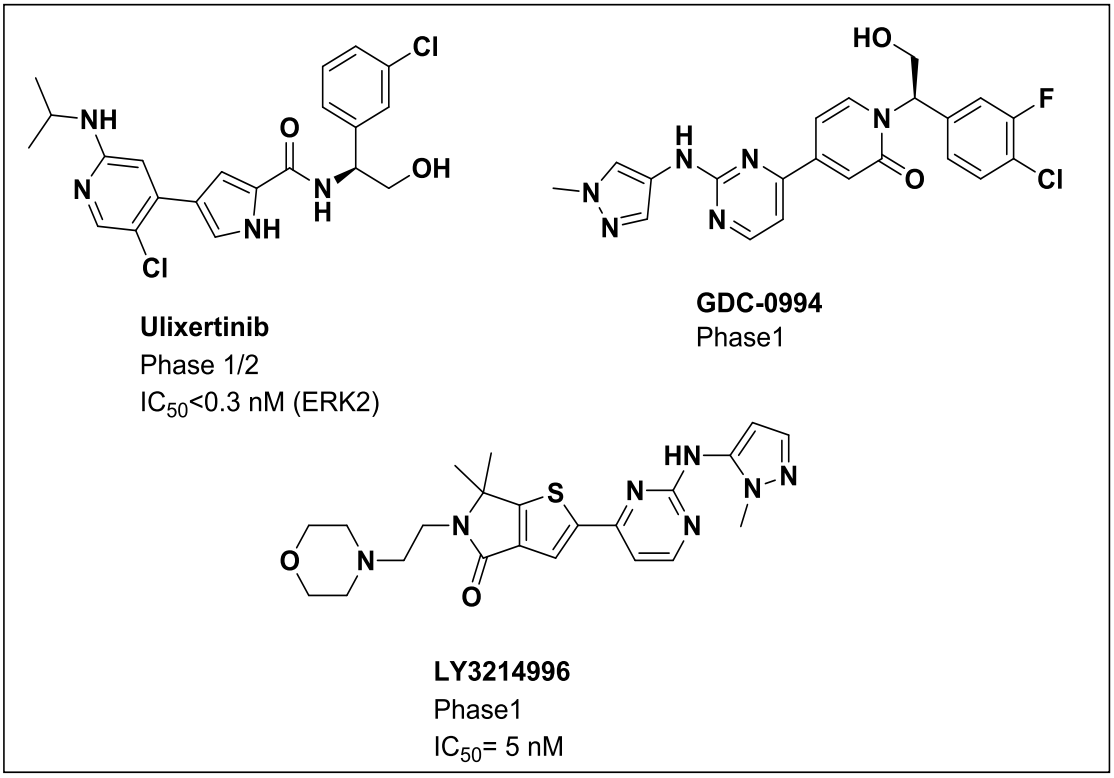

Figure 1.6. Some ERK1/2 inhibitors in clinical trials.

1.6. SUBSTITUTED PYRIMIDINES AS A SOURCE OF DIVERSE PHARMACOPHORE

Pyrimidine, an aromatic heterocyclic molecule, constitutes an interesting class of biologically active compounds. It presented itself as a privileged scaffold in medicinal word due to its wide range of activities (Chiacchio, Iannazzo et al. 2019). The

presence of pyrimidine moiety in DNA bases, such as thymine, cytosine and uracil, describe the importance of this scaffold which also might be major reason behind pharmacological activity of pyrimidine-based compounds (Yerragunta, Patil et al. 2013). Pyrimidine is found to have large abundance in nature and present as an important pharmacophoric part in thiamine, alloxan, orotic acid and other isoforms of nucleotide bases. As a synthon, pyrimidine present large number of possibilities for structural modifications which have attained the attention of a lot of researchers for development of new molecules with diverse spectrum of biological profiles (Kumar and Narasimhan 2018). Structure activity relationship studies of explored derivatives demonstrated that slight modifications at pyrimidine ring can deviate biological activity to large extent. The fusion of pyrimidine ring with various other heterocyclic and aromatic moieties further enhances the possibility of structural modifications which can lead to development of novel molecules to target disease conditions (Mohana Roopan and Sompalle 2016). Pyrimidine-condensed derivatives are known to possess broad spectrum pharmacological profile (**Figure 1.7.**) including anti-viral, anti-tubercular, anti-fungal, anti-bacterial, anti-malarial, anti-inflammatory, anti-cancer and anti-diabetic (Joshi, Nayyar et al. 2016). There are many retrosynthetic strategies reported in the literature for the development of pyrimidine-fused/coupled derivatives with improved medicinal chemistry. Due to this very reason, pyrimidine and its derivatives are considered most useful heterocyclic core and has attracted attention of almost every medicinal chemist. Since their existence, there role in improving efficacy of biologically active molecules have been recognized. Thus, in our work we intend to perform diversity-oriented synthesis of pyrimidine derivatives as anticancer agents.

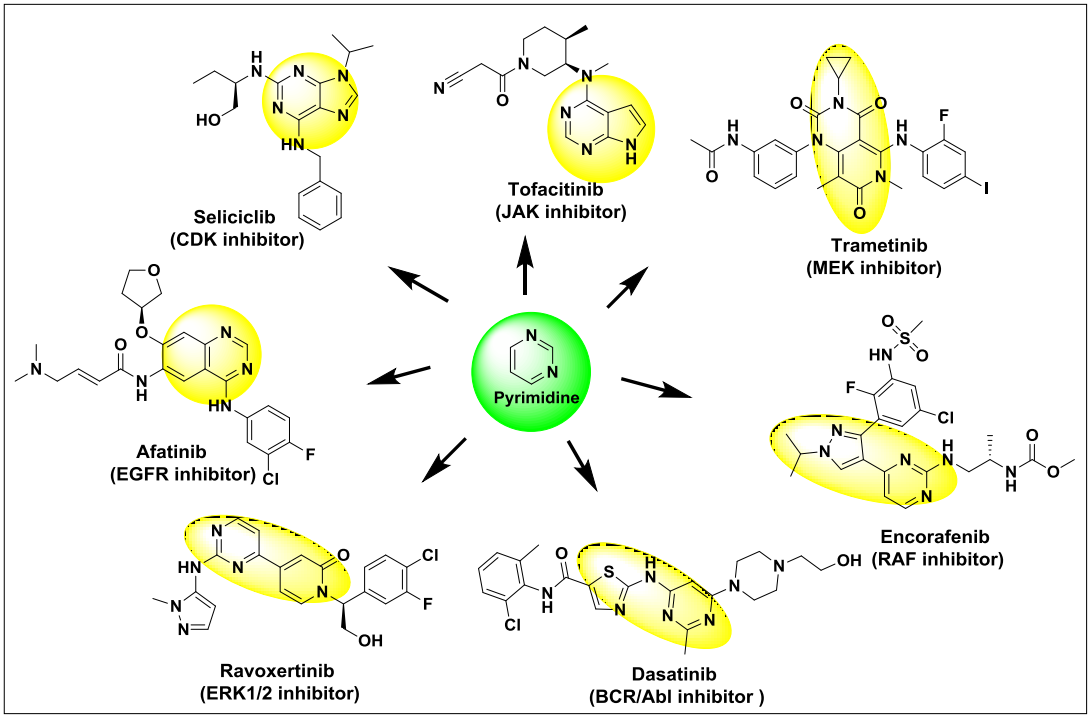

Figure 1.7. Substituted pyrimidines lead to diversified derivatives against different targets of some diseases.

1.7. COMPUTER-AIDED DRUG DESIGN (CADD)

Discovery of new drugs is a cumbersome process and requires significant economic input with an approximate expenditure of US$ 1 billion (Morgan, Grootendorst et al. 2011). To speed up this process in a cost-effective manner, pharmaceutical companies and research groups have now incorporated computer aided molecular modeling techniques in their protocols. These techniques form a rational drug design approach that can yield valuable information about the interaction patterns between protein and ligand as well as their binding affinity (Kapetanovic 2008). CADD techniques are broadly categorized into structure based and ligand based drug design approach (Macalino, Gosu et al. 2015).

1.7.1. Scaffold hopping

Scaffold hopping is defined as the process of identification of iso-functional central cores with diverse backbones and in the process improving or maintaining the pharmacological and pharmacokinetic profiles. This term was given by Gisbert Schneider, former researcher at Hoffmann-La Roche. Scaffold hopping is performed to speed-up the drug discovery process. The concept of scaffold hopping is based on the principle of similarity, which means that compounds that are structurally similar show similar pharmacological profile (Macalino, Gosu et al. 2015). This concept is usually applied to modify an already known pharmacologically active molecule to either improve its potency or to remove its toxicity *via* substituting its central core with an iso-functional moiety. This process is very useful in diversifying the heterocyclic cores of the lead molecules to avoid unwarranted properties of a specific heterocyclic core (Zhao 2007). There are several such issues related to the central core of a pharmacologically active molecule which warrant scaffold hopping exercise to find more suitable core without altering the activity. First could be poor solubility, which could be improved by substituting lipophilic scaffold with more hydrophilic scaffold. Second could be metabolic liability, which require substituting a more stable scaffold. Another one could be optimizing the flexibility of the core to optimize the fitting of the core in the binding pocket of the target protein. A more commercial use of scaffold hopping is that it provides a window to generate a patentable molecule with similar architecture and pharmacological profile (Zhao 2007). Briefly, scaffold hopping provides a way to increase the number of structurally diverse small molecule heterocycle within a similar spectrum of activity and therefore improves the chances of identifying clinically relevant drug molecules (Hu, Stumpfe et al. 2017). Molecular modeling researchers nowadays have developed several computational tools and procedures to execute scaffold hopping and identify molecules with high structural similarity scores. Computational approaches to perform scaffold hopping include:

i) Pharmacophore searching

ii) Recombination of ligand fragments

iii) Molecular similarity method

1.7.2. Fragment based drug discovery (FBDD)

Fragment based drug discovery (FBDD) is an approach of breaking down the binding pocket into smaller sites and identifying sub-structures which have higher affinity for these smaller regions in the binding pocket. It is believed that sometimes due to the conformational restrictions of the "bigger" drug-like molecules which do not fit properly we discard some key sub-structures which make excellent contacts, therefore in this technique first smaller fragments which make good contact and have high affinity in their specific sub-region of the pocket are identified and later they are connected or coupled *via* rigid bonds to avoid any alterations in the interaction network. Similar to the screening criteria of drug like molecules, the smaller fragments in FBDD also have selection criteria known as 'Rule of Three' which means their molecular weight should be less than 300, number of H-bond donor and acceptor should be less than 3, their CLogP should be less than 3, number of rotational bond should be less than 3, and the polar surface area should be less than 60 (Rees, Congreve et al. 2004). Keeping in mind that the fragments are to be coupled for them to be of any use, it is advised that fragments with simpler chemistry should be preferred. The success of any FBDD exercise relies on the correct selection of binding pocket hot spots which are to be targeted by the fragments. Regions which supply crucial H-bond interactions, salt bridges, hydrophobic interactions etc., to the already known inhibitors of the target protein are usually selected as hot spots. Upon identifying high affinity fragment, the drug like molecules can be obtained by following several approaches including growing fragment, linking fragments, or merging them. It requires generating a library of fragments which could be substituted or coupled to the high affinity fragment to maintain affinity and selectivity (Hubbard, Jahnke et al. 2016). Computational approaches to perform FBDD include:

i) Fragment-based molecular evolutionary approach
ii) Construction and deconstruction approach
iii) Computational functional group mapping
iv) Multitasking computational model approach

1.7.3. Molecular docking

One of the conventional and most reliable method of structure-based drug design (SBDD) is molecular docking. This *in-silico* approach provides insight into how a drug or a drug-like molecules interacts with its biological target. It is one of the most informative experiments which gives the basic idea of how and if the newly designed molecules are fitting and interacting properly with its target. In technical terms, this exercise search for the conformation of the designed molecule which it will attain in the binding pocket of the target (Hammes 2002). A set of rules called *sampling algorithms* are utilized to generate conformations. Thereafter a score is generated utilizing a scoring function to rank and filter the poses (conformation of the designed molecules) and to perform these calculations (**Table 1.1.**). In a routine molecular docking exercise, the target structure is considered rigid while only the ligand is allowed to alter its conformation, keeping its internal bonds fixed. While, nowadays several advancements allow even the target structure to be flexible to mimic the induced fit model of the receptor-ligand interactions. Nonetheless, molecular docking analysis has been the integral component of drug discovery approaches and virtual screening exercises (Goodford 1985). Different conformation sampling algorithm available in various docking tools include:

i) Shape matching algorithm
ii) Multiple copy simultaneous search
iii) Incremental construction
iv) Monte Carlo (MC) method
v) LUDI

vi) Genetic algorithms

Different scoring functions available in various docking tools include:

i) Force field-based scoring functions
ii) Empirical scoring functions
iii) Knowledge-based scoring functions
iv) Consensus scoring

Table 1.1. List of some common molecular docking tools used for SBDD.

Name of software	Developer	License	Year of release
Autodock	The Scripps Research Institute	Freeware	1990
AutodockVina	The Scripps Research Institute	Freeware	2010
Glide	Schrödinger	Commercial	2004
DOCK	University of California-San Francisco	Freeware	1988
MOE	Chemical Computing Group	Commercial	2005
GOLD	University of Sheffield, Glaxosmith Kline, and CCDC	Commercial	1995
Flex	BioSolveIT	Commercial	2001
Surflex-Dock	Trios	Commercial	2003
Swissdock	Swiss Institute of Bioinformatics	Freeware	2011

1.7.4. Molecular dynamic simulations

Molecular dynamic (MD) simulations are still developing field of computational drug discovery field. Out of all the *in-silico* methodologies, MD simulations is the closest to the wet lab experiments. Therefore, this method is extensively utilized to study biological systems and their real life parameters on a time dependent manner

(Goodford 1985). It was initially developed to study solid spheres in 1950s by Alder and Wainwright. Later on, it was improved to study liquids like water and finally it entered the field of drug discovery with the simulation of trypsin inhibitor in 1977 (Rahman 1964)(Stillinger and Rahman 1974)(McCammon, Gelin et al. 1977). Since then, like molecular docking analysis, MD simulation has become an integral component of every drug discovery approach and virtual screening protocol. Briefly, a MD simulation experiment involves subjecting a biological assembly to a state of motion and calculating its parameters *via* laws of motion. Principally, MD simulations is based on Newton's second law. It attempts to calculate the acceleration and related coordinates of each atom which is subjected to a force under physiological conditions (Coveney, Giordanetto et al. 2002). The most important parameter determined after solving these equations of motion include the trajectory of the atoms of the biological assembly. A set of rules called *force fields* are utilized to simulate the biological assembly in an environment similar to physiological conditions such as pH, temperature, water, etc. Therefore, details about the state of these assemblies under study is must to accurately perform a MD simulation experiment. These simulations are run for a given time period in which the system should get stabilized so that every conformational change and fluctuations can be thoroughly studied (Wilde and Singh 1998).

1.8. RESEARCH ISSUES/GAPS

As discussed above, breast cancer is one of the most common types of cancer observed in women. Out of all sub-types of breast cancer, TNBC is the most aggressive one due to the absence of molecular targets such as HER2, ER and PR. However, TNBC shows overexpression of different signaling pathways, one of which is MAPK pathway. Several studies have identified and validated the key role of MAPK signaling pathway in breast cancer. It is a well-known signaling pathway which is involved in various cellular functions including growth, while dysregulation in MAPK signaling results in the development and progression of different types of

cancers. MAPK pathway is composed of RAS, RAF, MEK and ERK. Many of these MAPK pathway members, upon mutations and overexpression, have an established role in the progression of breast cancer, for example upregulated RAS activity is involved in the progression of TNBC.

However, targeting RAS *via* small molecule heterocycles has been futile. Mutated RAS have been found to be insensitive to GTP hydrolysis making them a constitutively active protein and more importantly the RAS has astonishingly high affinity for GTP, limiting the possibility of a competitive inhibitor. Similarly, mutations in the B isoform of RAF are very commonly observed in breast cancer and although BRAF inhibitors have achieved early clinical benefits, they suffer from acquired resistance due to point mutation in the target protein on longer run. With the emergence of problems with RAS and RAF inhibitors, researchers shifted their focus towards targeting MEK1/2. However similar to BRAF inhibitors, within several months treatment with MEK inhibitors also resulted in the development of acquired resistance in the patients. However, targeting ERK may provide a possible way to bypass resistance mechanisms against other members of the MAPK pathway (Inamdar, Madhunapantula et al. 2010) but the drug discovery research on ERK1/2 selective inhibitors is still in lagging behind with only handful of inhibitors in clinical trials and none approved.

1.9. RESEARCH PROBLEM/PROBLEM FORMULATION

Detailed analysis of literature focused on targeting MAPK pathway members imply that either solely targeting ERK, the tail member of the MAPK pathway or targeting ERK in combination with other members of the MAPK pathway such as MEK and RAF, can provide an efficient approach of managing TNBC and other form of breast cancer (Hatzivassiliou, Liu et al. 2012). In MAPK pathway ERK1/2 is positioned at a unique place. The signaling protein, RAF, upstream to it has only limited effectors besides MEK, which does not have any other substrate besides ERK1/2 and therefore ERK1/2 are the prime activators to execute the functional outcome of the MAPK

pathways (Montagut and Settleman 2009). Accordingly ERK1/2 inhibitors are reported to overcome the mutated upstream signaling molecules induced activation of MAPK pathway (Liu, Yang et al. 2018). Additionally, other over-activated pathways in cancer also execute their functional outcome *via* cross-talk with ERK1/2, which enhance the clinical relevance of ERK inhibition in the management of cancer (Von Kriegsheim, Baiocchi et al. 2009). Supporting this hypothesis, studies show that treatment with selective ERK inhibitors reversed acquired resistance to MEK and BRAF inhibitors. (Von Kriegsheim, Baiocchi et al. 2009). Therefore, considering limitations of MEK and RAF inhibitors, ERK1/2 inhibitor discovery seems both rational and interesting to study the impact of inhibiting MAPK pathway in TNBC and cancer, in general.

1.10. AIM AND OBJECTIVES

The aim of the study is to design pyrimidine-based molecules targeting ERK1/2, to manage various types of MAPK-dependent cancer. Following objectives have been put down to achieve this aim:

1. To design pyrimidine containing derivatives using molecular modelling tools.
2. To synthesize the designed compounds.
3. To characterize the synthesized molecules using spectral techniques.
4. To evaluate *in vitro* anti-proliferative potential of the synthesized compounds *via* enzymatic assays and cancer cell lines (MCF-7, MDA-MB-231 and A549).

1.11. PLAN OF WORK

Following work was planned to achieve the above-mentioned objectives as follows:

A. Design of pyrimidine derivatives

- Thorough literature survey on pyrimidine derivatives with ERK inhibitory potential.

- Preparation of libraries of different molecules.
- Selection of target protein and preparation of ligands.
- *In-silico* studies including scaffold hopping, fragment-based drug designing, molecular docking, molecular dynamics, MM-GBSA calculations and DFT study.
- Validation of the *in-silico* results.
- *In silico* ADMET prediction.

B. Synthesis of designed compounds

- Selection of suitable synthetic schemes.
- Procurement of chemicals and reagents.
- TLC analysis and purification of synthesized compounds *via* column chromatography.

C. Spectral characterization of synthesized molecules *via*

- ^{1}H-NMR
- ^{13}C-NMR
- Mass spectrometry

D. Biological evaluation of designed compounds using *in-vitro* assay and cell lines

- Z'-LYTE® kinase assay against ERK2.
- MTT assay using MCF-7, MDA-MB-231 and A549 cancer cell lines.

E. Evaluation of synthesized compounds for normal cell toxicity

- MTT assay using HBL-100 cell line.

F. Compilation of results and thesis writing