Chapter 1. Introduction

1.1 Cancer

Cancer is defined as a group of diseases characterized by uncontrolled cell growth. These abnormal cells multiply uncontrollably, forming tumours that can invade healthy tissues and spread throughout the body. In earlier days, cancer treatments were limited and with minimal success hence, cancer diagnosis was often synonymous to death sentence.[1]

However, 20th and 21st centuries have witnessed a revolution in cancer research and treatment. Today, a wider range of treatments *viz.* surgery, radiation therapy and targeted medicines offer a greater chance of survival and remission compared to earlier days. Although, after years of research and availability of new invented therapies, 9.7 million cancer-related deaths were reported worldwide in 2022 which makes cancer still a leading cause of death worldwide.[2] Novel targeted therapies to enhance survival rate and reduce the global mortality rate is an unmet need specially in the area of solid tumours like Pancreatic ductal adenocarcinoma (PDAC).

1.2 Pancreatic ductal adenocarcinoma (PDAC)

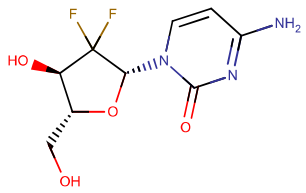
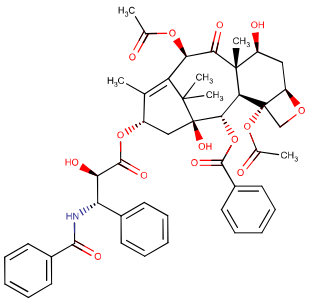
Pancreatic ductal adenocarcinoma accounts for more than 90% of pancreatic malignancies.[3] It is highly aggressive and lethal malignancy originating from exocrine cells. With a 5-year survival rate of less than 12%, it is one of the leading causes for cancer related deaths worldwide.[4] Majority of PDAC patients are diagnosed at advanced unresectable stages, which contributes to its poor prognosis and low survival rates.[5]

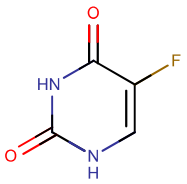
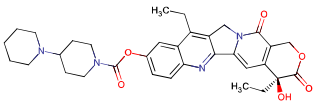
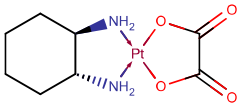
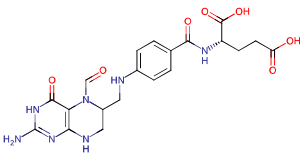
1.3 Current therapies for PDAC

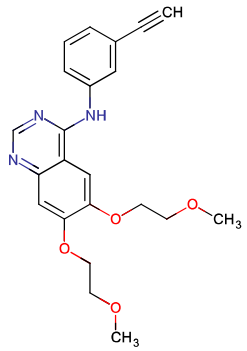
Though the diagnosis and treatment of PDAC remain a significant challenge, a few chemotherapeutic treatments with modest success rates are available for treatment of PDAC.

FDA approved therapies available for the treatment of PDAC are summarized in **Table 1.1** in which mechanism of action of the respective chemotherapeutic agent is highlighted.

Table 1.1: Current therapies for the treatment of PDAC

Sr. No.	Approved drug	Mechanism of action	Stage of treatment
1	 Gemcitabine	Gemcitabine interferes in the synthesis of DNA as an antimetabolite to kill cancer cells.	Gemcitabine is a chemotherapy drug that has been a standard treatment for PDAC for many years. It is used in both localized unresectable and metastatic PDAC. It is often combined with other drugs to improve effectiveness.
2	 Nab-paclitaxel	Nab-paclitaxel leverages albumin for delivery and targets microtubules within cancer cells to halt their division and promote cancer cell death.	Nab-paclitaxel is another chemotherapy drug that is commonly used in combination with gemcitabine for advanced PDAC.

Sr. No.	Approved drug	Mechanism of action	Stage of treatment
3	FOLFIRINOX [ 5-Fluorouracil (5-FU),  Irinotecan,  Oxaliplatin and  Leucovorin]	It is a combination of four different drugs. 1. 5-Fluorouracil (5-FU): Pyrimidine analogue that interferes with thymidylate enzyme to disrupt DNA synthesis; 2. Irinotecan: Topoisomerase enzyme inhibitor that disrupts duplication of DNA during cell division; 3. Oxaliplatin: Platinum based drug that forms bonds with DNA, hindering its repair mechanism leading to cell death; 4. Leucovorin: Enhance the effect of 5-FU.	FOLFIRINOX is a combination chemotherapy regimen that includes 5-fluorouracil, irinotecan, oxaliplatin and leucovorin. It is used for advanced PDAC and has shown improved survival outcomes compared to gemcitabine alone. However, it is generally used in patients with good overall health and functional status due to its increased toxicity.

Sr. No.	Approved drug	Mechanism of action	Stage of treatment
4	 Erlotinib	Erlotinib inhibits epidermal growth factor receptor (EGFR) leading to disruption of growth signals required for uncontrolled proliferation of cancer cells.	Erlotinib is a targeted therapy that inhibits the Epidermal Growth Factor Receptor (EGFR). It is used in combination with gemcitabine for the treatment of locally advanced, unresectable or metastatic PDAC.
5	Atezolizumab + Bevacizumab (Monoclonal antibodies)	Atezolizumab: Immune check point inhibitor that blocks interaction between PD-L1 and PD-1 by binding to PD-L1, leading cancer cell death through T cell activation; Bevacizumab: Anti-angiogenic drug that targets Vascular Endothelial Growth Factor-A (VEGF-A) to starve tumours by limiting their blood supply.	This combination is approved for use in advanced PDAC patients who have not received prior treatment.

Although survival rates of patients with hepatic, gastric and colorectal cancers are increasing due to advancements in treatment, no significant improvement in survival rate is reported for PDAC patients. However, in PDAC, there is a substantial unmet clinical need for novel targeted therapies which could enhance survival and reduce the global mortality rate.[6]

1.4 Reprogrammed tumour metabolism and introduction to malic enzymes

In more than 90% of PDAC cases, mutation in *Kirsten Rat Sarcoma Virus* (*KRAS)* oncogene has been observed. This activates downstream signalling pathways which subsequently regulate cell proliferation, differentiation and apoptosis. PDAC is also associated with deletion of tumour suppressor genes like *INK4a/ARF*, tumour protein 53 (*TP53*), and SMAD family member 4 (*SMAD4*).[7] Tumorigenesis requires adequate supply of energy in the form of ATP and biosynthetic precursors like proteins, nucleic acids and fatty acids.[8] This enhanced requirement in neoplastic cells is satisfied through metabolically rewired processes like aerobic glycolysis, glutaminolysis and elevated fatty acid synthesis. With stimulation of glucose uptake, enzymes involved in glucose metabolism pathway including ones involved in mitochondrial tricarboxylic acid (TCA) cycle are upregulated.

Malic enzymes (MEs) catalyze oxidative decarboxylation of L-malate in to pyruvate and simultaneously reduce nicotinamide adenine dinucleotide phosphate $NAD(P)^+$ to NAD(P)H as depicted in **Figure 1.1**.[9] Three isoforms of malic enzyme have been identified in mammalian context, classified based on their cofactor and subcellular localization. Malic enzyme 1 (ME1) is localized in cytosol and uses $NADP^+$ as cofactor while Malic enzyme 2 (ME2) is localized in mitochondria and uses $NAD(P)^+$ as cofactor. Malic enzyme 3 (ME3), a paralogue of ME2, is localized in mitochondria and uses $NADP^+$ as cofactor.[10] Through pyruvate production MEs support generation of lipids and other cellular building blocks. On the other hand, MEs play a crucial role in energy production and redox balance via production of NAD(P)H.

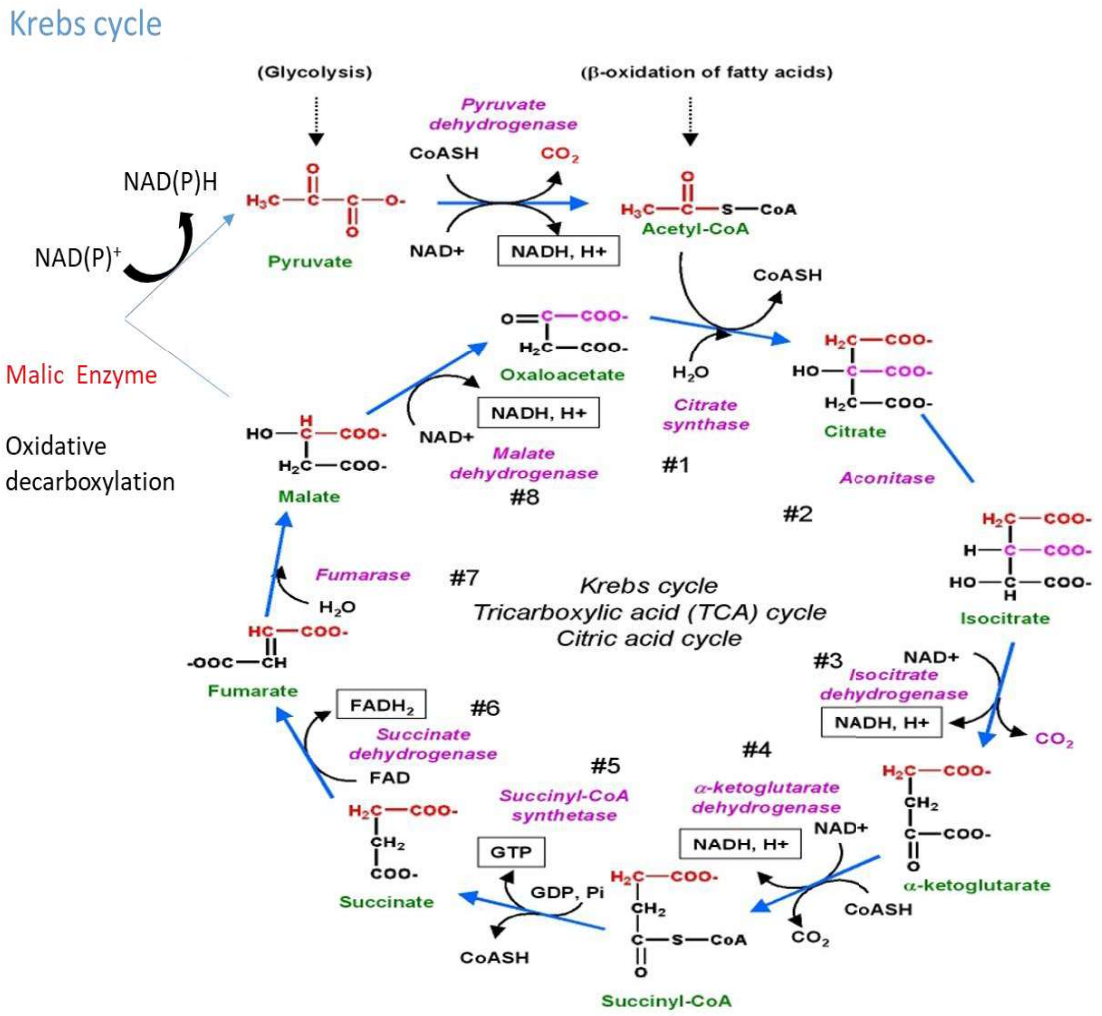


Figure 1.1. Krebs cycle and role of Malic enzyme in pyruvate and NAD(P)H production

While ME1 expression positively correlates with tumorigenesis in cancers like gastric, breast and squamous cell carcinoma (oral, head and neck) through regulation of NADPH,[11,12,13] ME2 is known to promote survival and proliferation of cancer cells in lung cancer, glioblastoma and oral squamous cell carcinoma.[14,15,16] ME3 overexpression boosts proliferation, migration and invasion capacity of PDAC cells.[17]

1.5 Collateral lethality and role of malic enzyme 3 in PDAC

In 30% of PDAC patients, the tumour suppressor gene *SMAD4* is deleted along with its chromosomal neighbour *ME2*. In this situation ME3 takes over role of ME2. In such patients, inhibition of ME3 will lead to selective death of cancer cells over normal cells where ME2 is operative. Using *in vitro* and *in vivo* experiments, Dey *et. al.* have demonstrated that depletion of ME3 in ME2 null PDAC cells (**Figure 1.2**).[18]

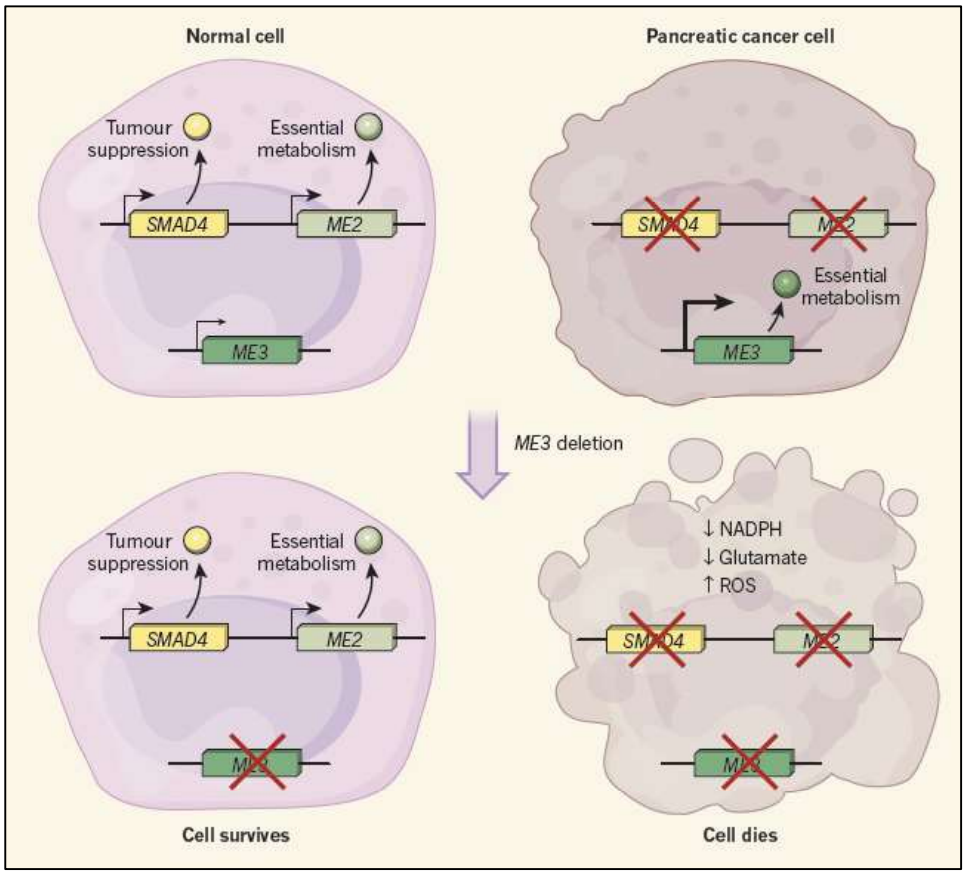


Figure 1.2: Collateral lethality and role of ME3[18]

ME3 catalyzes the conversion of malate in to pyruvate and in this process, it generates NADPH. NADPH is essential in maintaining cellular oxidative stress and redox balance. Inhibition of ME3 will diminish NADPH production and consequently increase reactive oxygen species (ROS) in cancer cells that could possibly lead to apoptosis and cell death. Increased ROS also activates Adenosine monophosphate (AMP) activated protein kinase (AMPK) which supresses Sterol Regulatory Element-Binding Protein 1 (SREBP1)-directed transcription of branched chain amino acid transaminase 2 (BCAT2). BCAT2 is a transaminase required for branched chain amino acid (BCAA) catabolism hence diminished BCAT2 levels will block nucleotide synthesis which in turn will block proliferation of cells as depicted in **Figure 1.3**.[18]

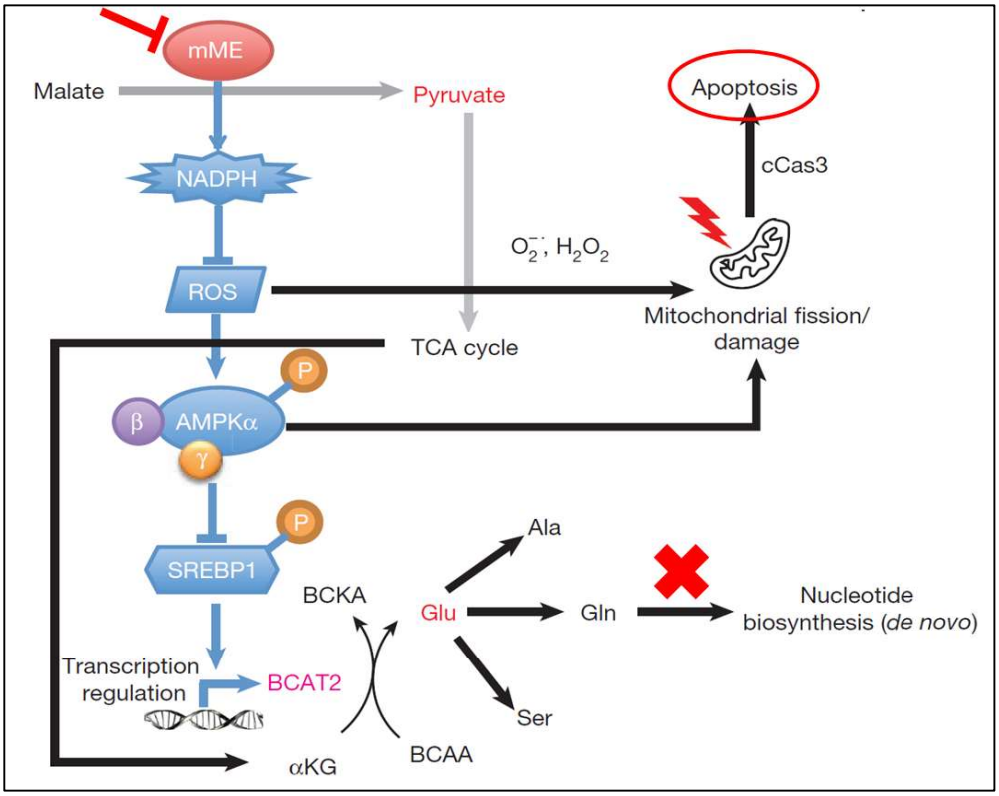


Figure 1.3: Effect of ME3 inhibition on tumorigenesis in PDAC[18]

1.6 Objective of the research project

PDAC is mostly refractory to currently available chemotherapeutic treatments because of limited efficacy. Hence the response rates are often very low. Identification of new molecular targets and subsequent development of effective targeted treatment could increase overall survival rate in PDAC.

In one third of PDAC patients, homozygous deletion of *SMAD4* is a frequent event which often results in co-deletion of neighbouring mitochondrial *ME2* gene. Through their systematic *in vitro* and *in vivo* experiments, Dey *et. al.* have demonstrated that genetic depletion of *ME3* in *ME2* null, but not *ME2* intact, cells resulted in apoptosis and blocked tumorigenic potential in PDAC.[18]

Based on these compelling data published by Dey *et al.*, it was envisaged that selective inhibition of ME3 through small molecule pharmacological inhibitors would create cancer specific metabolic vulnerability leading to cell death. Highly specific ME3 inhibitors could provide an effective targeted therapy for substantial number of PDAC patients with deletion of *SMAD4/ME2* genes. Hitherto, no small molecules have been reported as ME3 inhibitors in

context of PDAC, hence research was planned towards identification of first in class hit molecules as ME3 inhibitors which could subsequently be optimized for potency and selectivity.

1.7 Strategy for identification of hits for ME3 as target

Steps taken for identification of hit molecules for ME3 protein are depicted in **Figure 1.4**.

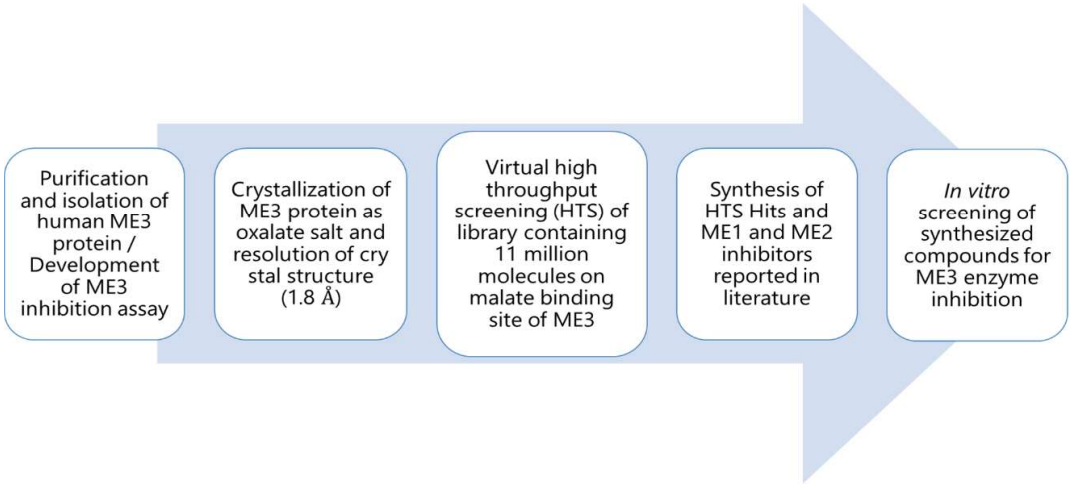


Figure 1.4: Steps take for identification of HIT molecules

1) ME3 protein crystal structure was not available in protein data base (PDB) so at first human ME3 protein was isolated and purified.[19]
2) Enzyme inhibition assay for ME3 was developed using the purified protein.
3) After that ME3 protein was crystallized as oxalate salt and crystal structure was resolved (1.8 Å).[19]
4) Using this crystal structure, commercial library of 11 million molecules along with Sun Pharma Advanced Research Company Ltd. (SPARC) library of 11500 molecules having different chemical scaffolds were docked on malate binding site of ME3 protein.
5) Chemical structures showing good binding affinity and consistent binding mode at malate binding site of ME3 were synthesized using reported methods.
6) Inhibitors of ME1 and ME2 enzymes reported in literature were also synthesized using described methods.[20,21,22,23]
7) Hits from virtual high throughput screening (VHTS) described in step-4 and reported inhibitors of ME1 and ME2 were screened in *in vitro* for ME3 inhibition.

Structures of the molecules which exhibited inhibition of ME3 enzyme in *in vitro* screening along with their IC_{50} values are presented in **Figure 1.5**.

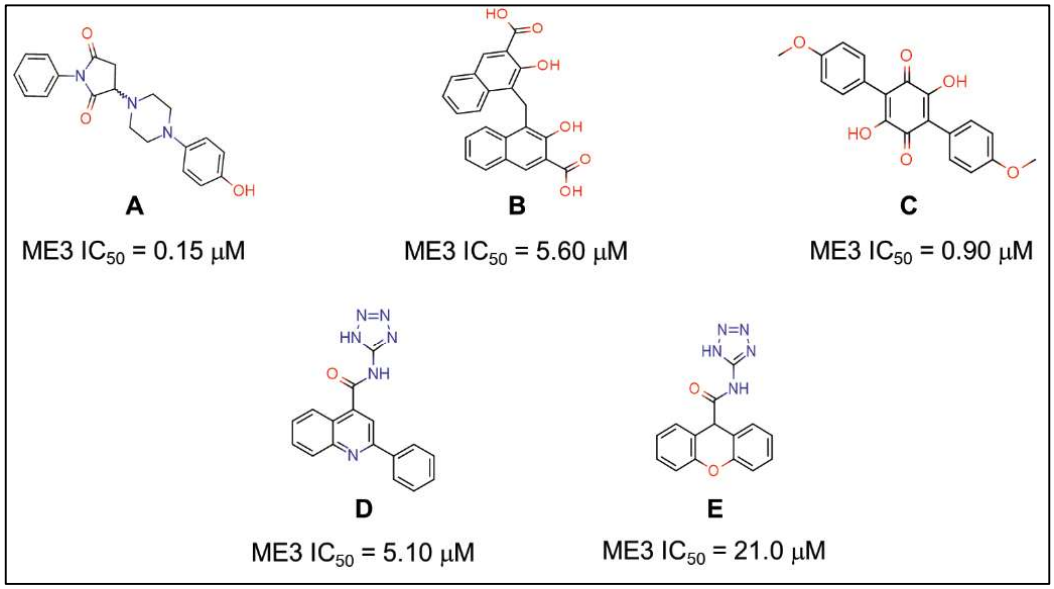


Figure 1.5: Structures of ME3 inhibitors

1.8 Selection of ME3 hit for further optimization in to lead molecule

Among the molecules identified and validated as ME3 inhibitors, compound **A** and compound **C** exhibited potent inhibition of ME3 enzyme with an IC_{50} value of < 1 μM. From toxicological perspective, quinones are known for producing *in vivo* toxicity. Hence, compound **A** was selected as appropriate scaffold for further optimization in terms of potency and safety over compound **C** which was a symmetrical 1,4-benzoquinone derivative.