2020

Hedgehog Signaling Inhibators and Their Importance for Cancer Treatment

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ABSTRACT: The Hedgehog (Hh) signaling pathway plays a crucial role during embryonic development as well as tissue maintenances in adults. Deregulated Hh signaling has been implicated in different diseases and conditions including cancer. Previously, the mechanisms of Hh signaling pathway and its role in cancer formation have been studied and has led to the development of therapeutic drugs. Among those agents, vismodegib and sonidegib are FDA approved drugs to treat basal cell carcinoma and other cancers. Up to date, several hedgehogs (Hh) signaling inhibitors and drugs have been made and evaluating for cancer treatment. In this review, I will discuss the mechanisms of action of Hh signaling pathway, targeted components, and Hh inhibitors and clinical importance as well as side effects, current evidences, and drug resistance.

INTRODUCTION

The hedgehog gene was discovered first in 1980's by Christiane Niisslein Volhard and Eric Wieschaus when they studied segmentation in Drosophila melanogaster(*1*). Mutation of this gene affected the number of segments observed and polarity in fruit fly. After Christiane Niisslein Volhard and Eric Wieschaus discovery, hedgehog signaling has significantly been studied by different scientists and numerous efforts have been made to investigate the diseases associated with hedgehog signaling pathway. The hedgehog pathway plays a crucial role in embryonic development, cell growth and differentiation and segmentation in vertebrates as well as in annelid (*2, 3*). This signaling pathway is responsible for the correct formation of limbs, digits, brain, bone, oogenesis, spermatogenesis and many other cells and structures during early embryogenesis (*3*). In adult vertebrates, the hedgehog signaling pathway is mostly inactive and involves in tissues and organs(*4*). Mutations that occur during embryonic development that affect hedgehog signaling pathway are linked to developmental disorders and mutations that occur during somatic development in adults associated with multiple forms of cancers (*5*).

Researchers spent substantial amount of time targeting the hedgehog signaling pathway inhibitors to develop potential anticancer agents. Smoothened (SMO) is the primary regulatory protein that many researchers targeted including Ptch1, GLI and developed a lot of Hh signaling pathway inhibitors. Even if multiple Hh agents have been made, only two drugs get approved by FDA and the rest needs some type of modification and additional studies in order to use as a

cancer treatment. These drugs demonstrated and used as monotherapies and others in combination with other treatments. Most of them showed a promising result upon investigating on model organisms.

Hedgehog Ligands

The three orthologues that involve in hedgehog signaling pathway in vertebrates are Sonic Hh (Shh) the best studied pathway, Indian Hh (Ihh) and Dessert Hh (Dhh). Each Hh ligands displays different expression and function in the cell. For example, sonic hedgehog is very important for formation of limbs, neural tube, somites, and affects epithelial tissues developments. Indian Hh signaling is essential for bone growth, chondrocyte development and formation of the primitive endoderm. Dessert Hh expression mostly limited to gonads and Schwann cells formations (*3, 5*). These ligands mediate the Hh signaling pathway at the primary cilium where clusters of receptors found, and upon activation, signaling cascades takes place during embryogenesis and tissue homeostasis (*3, 6*). In the absence or overexpression of these ligands and defect at the primary cilia, the Hh signaling pathway causes developmental disorders and abnormalities in tissues and organs. Hh pathway requires primary cilia in order to regulate and cascade molecular signaling and induce gene transcription(*7*).

Hedgehog signaling pathway and Key Components

There are many positive and negative regulatory components of hedgehog pathway to transduce activation and inhibition of signaling pathway. Patched, GLI, smoothened (SMO) and SUFU are

some of the key components. Protein kinases like protein Kinase A, glycoprotein synthase Kinase 3 beta, and casein Kinase 1 also play a major role in the signaling and phosphorylation process of Hh signaling (*3, 8*). Understanding the basic concepts of Hh signaling pathway activation and it's components help to select and develop the appropriate drugs for specific cancer.

Patched is a 12-span transmembrane Hh receptor protein which has a negative regulatory function at the primary cilia. It is involved as a negative feedback mechanism to regulate Hh signaling activity in embryonic cell as well as tissue repairs. In the absence of Hh ligand, this transmembrane protein placed at the cilia prevents the accumulation of smoothened and therefore inhibit the Hh signaling pathway (*3*).

Smoothened (SMO) is a 7-transmembrane G protein-coupled like protein receptor which, leads to the activation of Hh pathway by activating GLI transcription factors. Smoothened is repressed by Ptch1 to inhibit Hh pathway (*8, 9*). Glioma-associated oncogene (GLI) is a transcription factor that mediates the response of transcription of the target gene. In mammals, GLI 1 and 2 are Hh signaling pathway activators. When these transcriptional activators are phosphorylated by protein kinases like PKA, GSK3β and CK1, lead to generate a GLI^R which is a transcriptional repressor and results in inhibitions of Hh pathway target gene transcription in the nucleus (*3, 9-11*).

SUFU is another intracellular component that negatively controls the activation of Hh signaling pathway. It directly binds to the GLI protein in the cytoplasm and blocks its activation and

nuclear localization and forms a repressor protein complex that can suppress the Hh gene expression (*12*).

Signaling pathway in the absence of Hh ligands

Hh signaling pathway is not always active in adult vertebrates. In the absence of Hh ligands, Ptch1 activated and repress smoothened by sitting and preventing its accumulation on the primary cilia. Primary cilium is where the activation and inhibition of Hh signaling started at the membrane before series of signaling cascades takes place in the cytoplasm and nucleus. When SMO inhibited by Ptch1, it cannot dislocate to the cytoplasm and dissociate SUFU-Gli complex. The SUFU and GLI complex in the cytoplasm then get phosphorylated by PKA, GSK3β and CK1which lead to proteolytic process to produce repressor protein (*3, 13*). SUFU is a negative regulator and suppress GLI and inhibiting its translocation to the nucleus. The SUFU and GLI complex generates a new repressor protein called GLI^R which transport to the nucleus and inhibit hedgehog signaling target gene expression (*3*). (Fig 1A below). Along with other Hh key components, signaling pathway inhibited.

Signaling pathway in the presence of Hh ligands

In the presence of Hh ligand, it's signaling pathway get activated and affect the cells activity. Hh ligand first converted to its active form and binds to the Ptch1 which is a 12-transmembrane protein and Hh ligand receptor (*12*). The inhibitory effect of Ptch1 removed from the SMO and then this 7TM translocate to the primary cilia and dissociate Suppressor of Fused (SUFU) and

GLI complex. SUFU-GLI complex was supposed to generate a repressor protein and inhibit the Hh signaling that target gene expression in the nucleus but when this complex dissociated, GLI, a transcription factor activate and translocate to the nucleus to induce a gene transcription for Hh target genes(*3*). (see Fig 1 B below). Including many other key components this is the activation pathway of Hedgehog signaling.

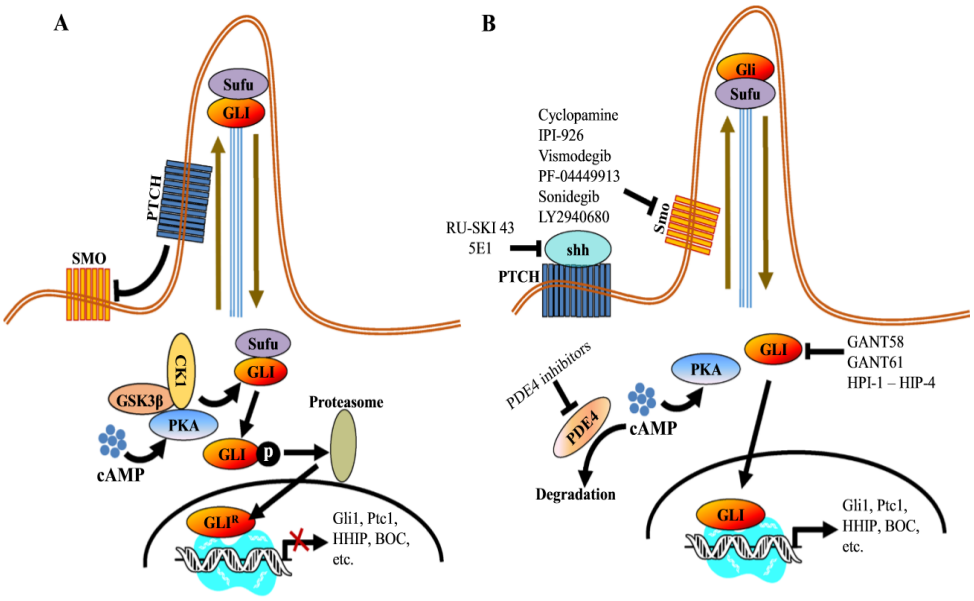


Fig. 1. General hedgehog signaling pathway: Activation and inhibition of hedgehog signaling pathway by different key components. (A) patch1 inhibit SMO at the primary cilia in the absence of Hh ligands and SUFU-GLI complex formed. When GLI phosphorylated by PKA, GSK3β and CK1 which generate a GLI^R which in turn inhibit Hh gene expression. (B) In the presence of Hh signaling ligands, Shh, Ihh or Dhh binds and inhibit Ptch1. SMO activated and dissociate SUFU and GLI complex and no GLI^R produced to suppress transcription but GLI move to the nucleus to induce activation of target gene.

Molecular mechanisms of Hh ligands and key components

The molecular mechanisms how Hh ligands interact with Ptch1 and inhibitory effect on smoothened is unclear. Recent study showed that the cytoplasmic tail contributed to inhibitory function of Ptch1 on smoothened and Hh ligands interaction (*13*). In this study, when the C-terminus amino acid truncated on the transmembrane segment, Ptch1 activity disrupted and smoothened suppression relieved and leads to constitutive activation of Hh signaling pathway. Ptch1 with truncated extracellular loop and normal cytoplasmic tail fully suppressed smoothened activity but Hh ligands did not bind. This research also discussed truncated tail and loop of smoothened impaired its accumulation at the cilia and downstream activation of Hh signaling pathway.

Cancer and Treatments Using Hh Inhibitors

The detailed study of activation and inhibition of the Hh pathway helped researchers to develop drugs and to turn their attention towards the cancer. Currently, the main target of cancer research regarding drug therapies including cancer prevention is smoothened. The Hh signaling is able to maintain cancer stem cells by feeding the surrounding microenvironments of the tumor to grow so, researchers targeted by inhibiting the over expression of Hh signaling pathway in different cancer and related diseases to treat the patients (*3*). Tumor invasion and progression inhibited by Hh signaling inhibitors in clinical patients and model organism. In some studies the hedgehog (Hh) pathway expression was limited in normal adults but in cancer cells Hh was overexpressed

and facilitates the growth of the tumor (*14*). Basal Cell Carcinoma (BCC) is the most common skin cancer in human and overexpression of Hh pathway is a key driven pathogenesis. Vismodegib and sonidegib are the only two FDA approved drugs. Nowadays BCC incidence is increasing in the world and different drug developers and researchers are doing tremendous effort to develop therapeutic drugs and showing promising results (*15*). Advanced BCC patients usually use vismodegib and sonidegib in combination or separately. As figure 2 indicated, the overexpression of Hh pathway in BCC tumor show regression and decrease in size after treatment. These drugs able to promote adaptive immune cells to the tumor microenvironment (*4*).

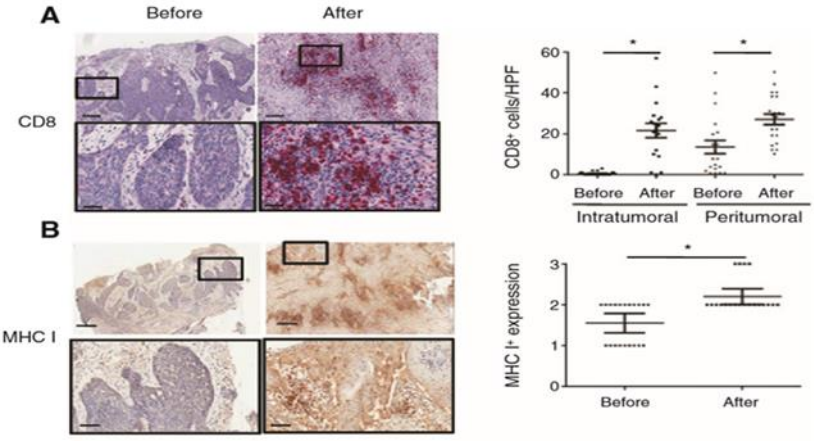


Fig.2. Immune cells infiltration after Hh pathway inhibitors treatments. A biopsy taken from patients before and after treatment. (A)Immunostaining of patients' biopsy revealed increaseCD8 cells in tumor environments. (B) upregulation of MHC class I cells expression in intratumor and peritumoral area.

Drugs that targeted Hh signaling pathway

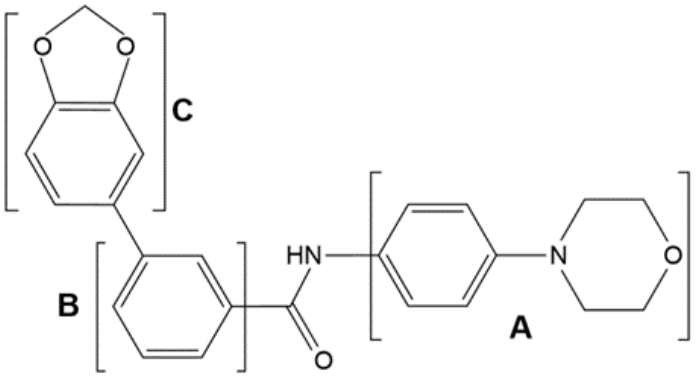
In recent years a major effort has been made and developed many inhibitors that blocked Hh signaling pathway at different steps. The Hh signaling pathway key components that targeted for these therapeutic agents are Smoothened, GLI, Hh orthologue and Hhat. As discussed above, most of the Hh signaling pathway inhibitors are targeted smoothened. Some cancers treated with single Hh pathway inhibitors and others treated with a combination of Hh antagonist drugs.

Therapeutic drugs target Smoothened

Most of those inhibitors are specifically acted on smoothened to block the expression of Hh signaling in tumor and its microenvironments (*16*). Among these therapeutic drugs Vismodegib and Sonidegib have been approved by Food and Drug Administration (FDA). Saridegib, Taladegib and Cyclopamine and its derivatives also target SMO receptor.

Vismodegib is a small molecule and the first hedgehog signaling pathway inhibitor which act on Smoothened protein to prevent its accumulation in primary cilia (*17*). This inhibitor approved by FDA for metastatic basal cell carcinoma. In one study involving 704 basal cell carcinoma patients showed a partial response rates of 62 % and currently Vismodegib is in clinical trials for other cancers as monotherapy and in combination with other drugs (*3, 17*). In some clinical trials of Medulloblastoma patients, Vismodegib showed an ineffective result because of mutation of SMO protein and in fact its low solubility (*12*).

Sonidegib is the second FDA approved smoothened inhibitor for basal cell carcinoma patients followed by surgery, radiation treatment and or patients who is not candidate for surgery and radiation (*12*). Otsuka et al studied 23 patients with basal cell carcinoma after Vismodegib and Sonidegib treatments(*4*). They found these drugs altered the tumor cell populations and promote adaptive immunity in tumor microenviroments. After these therapuatic drugs treatment, the patients showed influx of some immune cells in the tumor microenviroments (*10*). Both Vismodegib and Sonidegib are in clinical trials to treat lung and other solid tumors(*3*). Figure 3 below showed the modification of the orginal sonidegib compound in order to improve toxicity and solubility (*18*) and also chemical structure of vismdegib (*19*).



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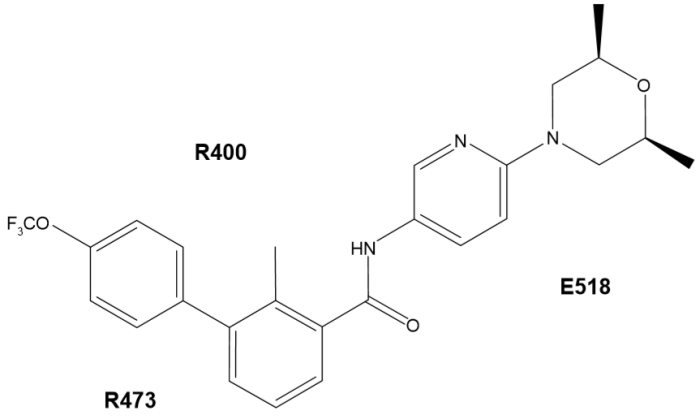

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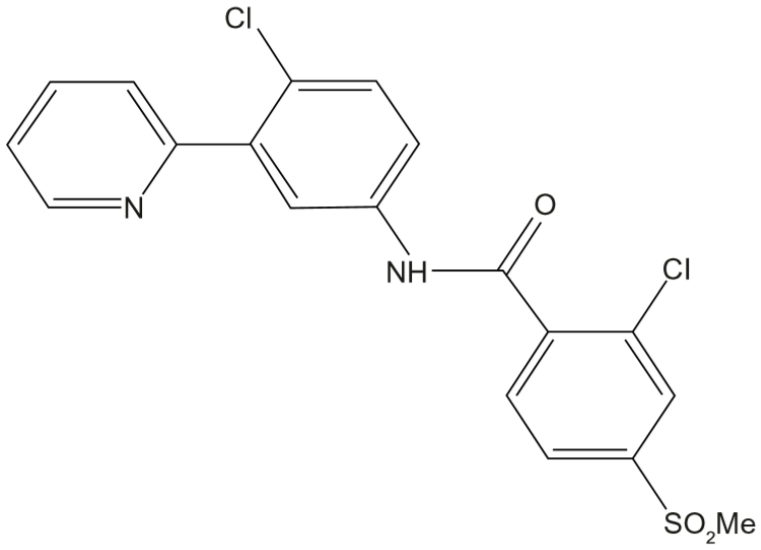

III

Fig. 3. Modification of sonidegib. 1. Three core regions namely A, B, and C of compound I get modified. 2. Region A modified with Nitrogen in the pyridine moiety and dimethyl group added to improve toxicity, Region B modified, and methyl added to increase potency and finally region C modified with F_3CO in order to favor absorption as shown on compound II. R400, R473, and E518 are drug binding pockets for SMO. Arginine (R) and Glutamic acid (E) are the amino acids associated with this binding site. Compound III is the first FDA approved drug, vismodegib.

Cyclopamine is naturaly occuring compound and effective smoothened inhibitor which is found in the corn lily. This compound found in 1950's in research done due to a lambs born with one

eye and it tooks scientists about ten years to figured it out (*17*). Cylopamine has been intensively studied and demonestrated to inhibit melanoma, colon, pancreatic, prostate, lung and other cancers but its therapauetic usage as Hh inhibitor for human is limited due to poor property and its side effects of low solubility in the body under normal condition (*3*). In order to overcome this limitation some other cyclopamine derivaties developed which include KAAD cyclopamine and Saridegib with a potentially improved soubility in model animals but iys clinical trials terminated due to inefficents clinical benefit and for patients saftey (*3, 9*).

Therapeutic drugs target Gli

The other target for hedghog signaling pathway inhibitors is Gli transcription factor (*3, 9, 20*). This target protein is important to overcome drug resistance property of smoothend. Among the Gli inhibitors, GANT58 and GANT61 are small molecules and suppressed the proliferation of rhabdomyosarcoma, prostate, colon, and ovarian cancers and further study showed potential distruption of Gli phosporaylation and Hh inhibition (*3, 21*). Arsenic trioxide (ATO) is also Shh inhibitor by blocking Gli protein and used to treat patients with white blood cells cancer and currently it is in clinical trials for solid tumors including pancreatic cancer (*3, 22*).

Therapeutic drugs target Hh Ligands

Hedgehog signaling pathway ligands (Shh, Dhh and Ihh) are also target for therapeutic agents to block abnormal proliferation of cancerous cells in tumor microenvironments (*3, 8, 16*). All the three ligands were able to block by a monoclonal antibody 5E1. 5E1 has been proved to suppress

pancreatic and medulloblastoma in mouse model. An acyltransferase (Hhat) which is essential hedgehog (Hh) signaling pathway enzyme targeted by a small molecule RUSKI-43 and RUSKI-201 (*3, 23*).

Table 1. Summary of hedgehog signaling pathway inhibitors and its implication in cancer.

Hh inhibitors	Target	Treatment/Repression	Side effects
Vismodegib	SMO	FDA approved for metastatic BCCs	FDA approved, ongoing clinical trials for other cancers and Resistance
Cyclopamine	SMO	Inhibit Tumor like glioma, melanoma, prostate, colon and lung cancers	Due to low solubility, it is not approved by FDA
Saridegib	SMO	Ovarian, medulloblastoma and pancreatic cancer	Potent and detrimental effects
Taladegib	SMO	Medulloblastoma in model animal	Effective only in BCCs (mice)
Sonidegib	SMO	BCCs (CR)[4], lung, and advanced solid tumors.	FDA approved, ongoing clinical trials for other cancers and resistance
Itraconazole	SMO	FDA approved for antifungal disease	Ongoing clinical trials.

GANT58 & 61	GLI	Prostate, colon and ovarian	Ongoing clinical trials
5E1	Hh ligands	Medulloblastoma and pancreatic	No clinical trial yet
RUSKI-43	Shh, Hhat & PDE4	Breast and pancreatic tumors	No clinical trial yet
Glasdegib	SMO	BCC	Under clinical investigation

Drug resistance challenges, and recent studies on small molecules

As I discussed above, SMO showed a resistance for vismodegib and sonidegib therapeutic drugs. It has been a major challenge for FDA approved Hh pathway drug users to overcome the drug-resistant property of SMO mutant (*12*). A recent research was done on Hh inhibitors and Hh dependent tumors, and they found a promising result for mutant SMO and sonidegib and vismodegib resistance tumors (*24*). In this study, a compound called 2', 4', 5', 3, 4-pentamethoxychalcone was found to be the most powerful Hh pathway inhibitor by binding at the same binding site as Cyclopamine. This drug acts on SMO receptor. They farther investigated on the Hh driven tumors in which constitutive activation of Hh target gene present and loss of Ptch1 function. Small concentration of this compound was able to decrease the mRNA levels of the downstream Hh target genes.

Many cancer patients who took vismodegib and sonidegib treatment showed drug resistance at the SMO receptor due to its rapid mutation, but this new drug is able to act on drug-resistant SMO mutant and it will have a potential impact for future cancer treatments. In recent study by F. Wang et al., derivative of vismodegib found to have a long-acting Hh inhibitor activity compared to vismodegib itself (*25*). In this study they synthesized a deuterium-hydrogen analogue of vismodegib and found to be more stable when administered in mice. As summarized in table 2 and Figure 4 below, a lot of small molecules and active plant products showed a significant inhibitory effect on Hh signaling pathway and further study and modification of these compounds while have great therapeutic treatment for Hh-driven cancers (*12, 23, 26-28*).

Basal Cell Carcinoma (BCC) is the most common skin cancer in human and overexpression of Hh pathway is a key driven pathogenesis. Vismodegib and sonidegib are the only two FDA approved drugs. Nowadays BCC incidence is increasing in the world and different drug developers and researchers are doing tremendous effort to develop therapeutic drugs and showing promising results (*15*). Advanced BCC patients usually use vismodegib and sonidegib in combination or separately. As figure 3 indicated, the overexpression of Hh pathway in BCC tumor show regression and decrease in size after treatment. These drugs able to promote adaptive immune cells to the tumor microenvironment (*4*).

Table 2. Summary of recent design of Hh signaling pathway inhibitors. The following small molecules and natural compounds are new and modified forms of known Hh inhibitors and needs further studies before clinical trial. These small molecules and active plant products might use as a source for development of newly synthetic Hh signaling pathway inhibitor.

Compound	Category/Source	Action and Target	Limitation and side effect
Eggmanone	Thiophenes	PDE4/SMO	Low solubility, potent
RUSKI 39, 41,43 and 50	Thiophenes	Hhat/Hhh	Potent and cytotoxicity
Bisamide compounds	pyridines	Kinases	Low solubility and strong bind with plasm
Tetrahydrothiazolo[5,4-c] pyridine	Pyridine	SMO	High potency and large size
Hh003	pyridine	SMO	Induce apoptosis, and strong inhibiting activity in mouse model compared to Vismodegib

HEK293 and NIH3T3-GRE	Pyridine	Hh/Wnt but specific target not identified	Inhibit Wnt and Hh pathway simultaneously
VD3	Vitamin D3 analogs	SMO	Promote hypercalcemia
SANT-2	Benzimidazoles	SMO	High potency
Robotnikinin	Macrolactone analog	SMO/Shh	Cytotoxic and poor solubility
LAB687	Biphenyl derivative	SMO	Weak affinity
Colubrinic and betulinic acid	Pentacyclic triterpenes	GLI/BCL-2	Good candidates for clinical trial
Genistein	Natural /plant	GLI1	Needs modification
Curcumin	Natural /plant	Shh, GLI	Needs modification
Resveratrol	Natural /plant	GLI	Needs modification

Epigallocatechin-3-gallate	Natural /plant	Ihh/GLI	Needs modification
Arsenic trioxide	Inorganic compund	GLI	FDA approved for Leukemia
Glabrescione B and Isoflavone	Extracted from seeds	GLI	Further study
LEQ-506 and TAK-441	Small molecules	SMO	Under clinical trials

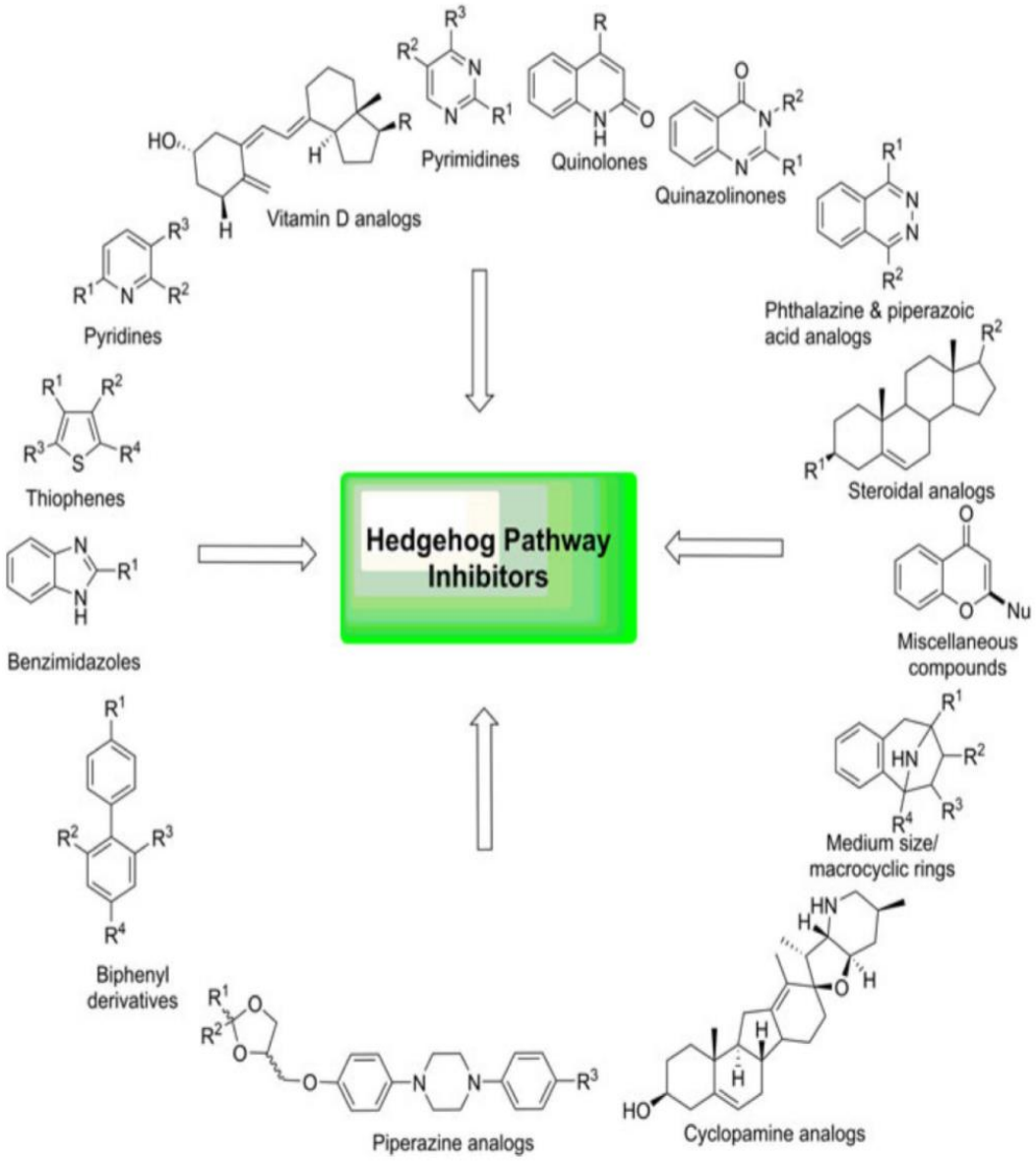

Fig. 4. Series of different groups of compounds that inhibit Hh signaling pathway. These chemicals showed a promising result by inhibiting the Hh pathway at different target regions of the cells. Modification of these compounds might result effective therapeutic drugs for Hh-dependent cancers.

Conclusion

The discovery of the Hh pathway played an important role in the understanding of normal vertebrate development (*29*). Abnormal activation of Hh is involved in cancer. Most studies focused on SMO and other key components to inhibit the Hh pathway. In recent years numerous hedgehogs signaling pathway inhibitors specifically target Smoothened have been came out and undergo preclinical and clinical trials. These Hh pathway inhibitors showed a significant blockage on the tumor growth and microenvironments in model organism (*30*). Vismodegib and sonidegib are the only FDA approved drugs for different cancerous diseases. Unfortunately Smoothened showed a drug resistance by changing its form after given multiple dosages. Scientists acquired a different mechanism to overcome this side effect and develop other drugs that target other than Smoothened like Gli, Hh orthologues, and Hh enzyme acyltransferase (Hhat). In order to overcome drug resistance, further research on the key components of signaling cascade might be crucial. In addition, studying other Hh signaling pathway components, developing new drugs and further clinical trials on the other drugs will lead to develop new effective drugs and better cancer treatment.

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