Natural products as Ras inhibitors

Subjects: Oncology

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RAS genes encode signaling proteins, which, in mammalian cells, act as molecular switches regulating critical cellular processes as proliferation, growth, differentiation, survival, motility, and metabolism in response to specific stimuli. Deregulation of Ras functions has a high impact on human health: gain-of-function point mutations in *RAS* genes are found in some developmental disorders and thirty percent of all human cancers, including the deadliest. For this reason, the pathogenic Ras variants represent important clinical targets against which to develop novel, effective, and possibly selective pharmacological inhibitors. Few druggable sites have been identified for wild type and some oncogenic Ras mutants, and few natural compounds able to attenuate Ras signaling have been identified so far. Natural products represent a virtually unlimited resource of structurally different compounds from which one could draw on for this purpose, given the improvements in the isolation and screening of active molecules from complex sources, which can now be exploited for the selection of potential Ras inhibitors from natural sources.

Ras signaling | cancer | Ras inhibitory strategies | Ras druggable pockets | natural products | anticarcinogenic effect

1. Introduction

1.1. Ras Proteins

Ras proteins are eukaryotic small guanine nucleotide-binding (G) proteins that, by cycling between the GDP-bound inactive state and the GTP-bound active state, act as molecular switches in signaling pathways regulating many cellular processes including proliferation, growth, survival, adhesion, migration and metabolism in mammalian cells [1]. Ras proteins are endowed with low intrinsic GTPase activity and a very slow rate of spontaneous nucleotide exchange. The Ras activation state is finely regulated, in response to different specific extracellular stimuli, by the competitive interplay of upstream regulators: Guanine nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs). GEFs activate Ras proteins by promoting nucleotide dissociation, and thereby preferentially GDP/GTP exchange due to GTP being 10-fold more abundant in cells than GDP, while GAPs inactivate them by providing an essential catalytic group for GTP hydrolysis [2][3][4][5]. In the active GTP-bound state Ras proteins increase their affinity for many effectors that initiate downstream signal transduction [6][7] (Figure1). In human cells, three *RAS* genes (*HRAS*, *NRAS* and *KRAS*), encode four homologous but functionally distinct isoforms, HRas, NRas, KRas4A and KRas4B, the two latter ones deriving from alterative splicing of the *KRAS* gene [8][9]. Notably KRas4B is usually referred as KRas.The four isoforms share 90% of sequence identity in the first 166 residues and

mainly differ in the carboxyl-terminal hypervariable region (HVR) that contains sites for posttranslational modifications (PTM). This region is responsible for membrane tethering of Ras proteins that is required for correct membrane trafficking and localization, and function of each isoform [10][11].

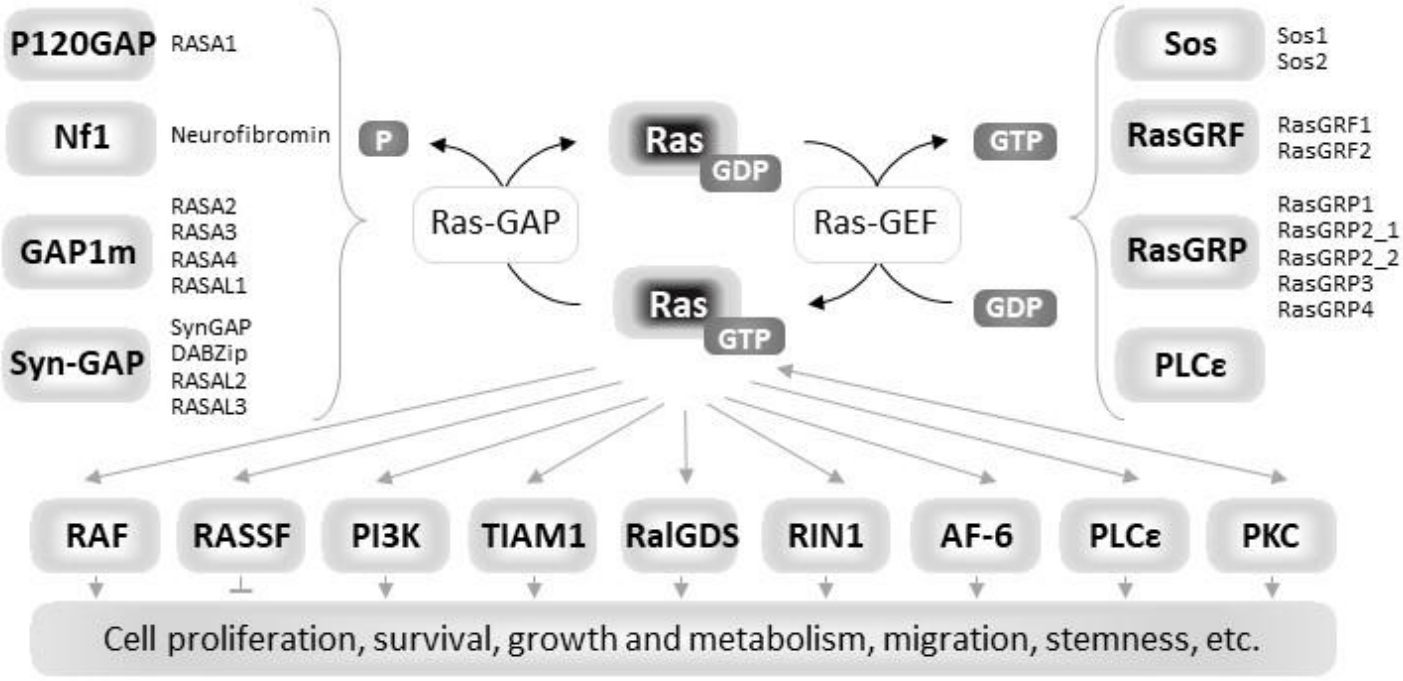

Figure 1. Diagrammatic representation of the functional cycle, upstream regulators and downstream effectors of Ras proteins

1.2. Ras mutants in Human Diseases

Deregulation of Ras activity has a driving role in the pathogenesis of several human diseases, including developmental disorders, known as RASopathies [12][13], and thirty percent of all human cancers, including the deadliest. *KRAS* gene is the most frequently mutated isoform (21%), followed by mutations in *NRAS* (8%), and in *HRAS* (3%) (www.sanger.ac.uk/genetics/CGP/cosmic/). A mutationally activated KRas oncoprotein is present in almost all pancreatic ductal adenocarcinomas and in up to 50% colorectal cancers. The large majority of gain-of-function missense mutations that constitutively activate Ras oncoproteins map at codons 12 (89%), 13 (9%), and 61 (1%) [14], which encode critical residues involved in the interplay between Ras, nucleotides, and modulators. Each oncogenic mutation alters the functional cycle of Ras through a distinct mechanism depending on the conformational change induced by the presence of the mutated amino acid. For example, the G12V substitution abolishes the intrinsic and GAP-mediated GTP hydrolysis due to interference with the allosteric switch [15][16], while G13D mutation determines the self-sufficiency in nucleotide dissociation, even maintaining the sensitivity to GEFs and at least one GAP [15][16][17][18][19][20]. The Q61L mutation reduces the intrinsic, in both free and Raf-bound Ras, and GAP-mediated GTP hydrolysis and accelerates nucleotide exchange [16]. Regardless of the activation mechanism, all oncogenic Ras mutants show an altered residence time in the GTP-bound active state [15], and

aberrantly transduce downstream signals contributing to tumor onset, maintenance, and progression [21], impinging on most cancer hallmarks [22], such as growth signal-independent sustained proliferation, resistance to apoptosis, the ability to migrate and to invade/metastasize, the ability to promote angiogenesis, and ability to elude the immune response, as previously reviewed [23]. Oncogenic *KRAS* activation also induces significant changes in cell metabolism, including enhancement in glucose transport and aerobic glycolysis [24][25][26] that determine the acquisition of the hyperglycolytic phenotype known as the Warburg effect [27][28], anaplerotic usage of glutamine [29][30][31], altered sulfur amino acid metabolism [32], altered mitochondrial morphology and function, and production of large amounts of reactive oxygen species (ROS) [33][34]. Ras GAPs and members of the RASSF family constitute a barrier to Ras-dependent transformation in cells. However most Ras oncoproteins are insensitive to GAP, and loss of function of Ras GAPs or RASSFs is common in tumors [35].

2. Natural Products Targeting Biosynthesis, Processing, Activity, and Signaling of Ras Oncoproteins

Due to the critical role of Ras oncoproteins in cancer, many efforts, mostly promoted by the RAS initiative (https://www.cancer.gov/research/key-initiatives/ras), have been devoted to explore different direct and indirect strategies for attenuating their aberrant signaling, as recapitulated in several recent reviews [36][37][38], and schematically depicted in Figure 2. Bioactive natural products identified in some of these strategies are reported in the figure.

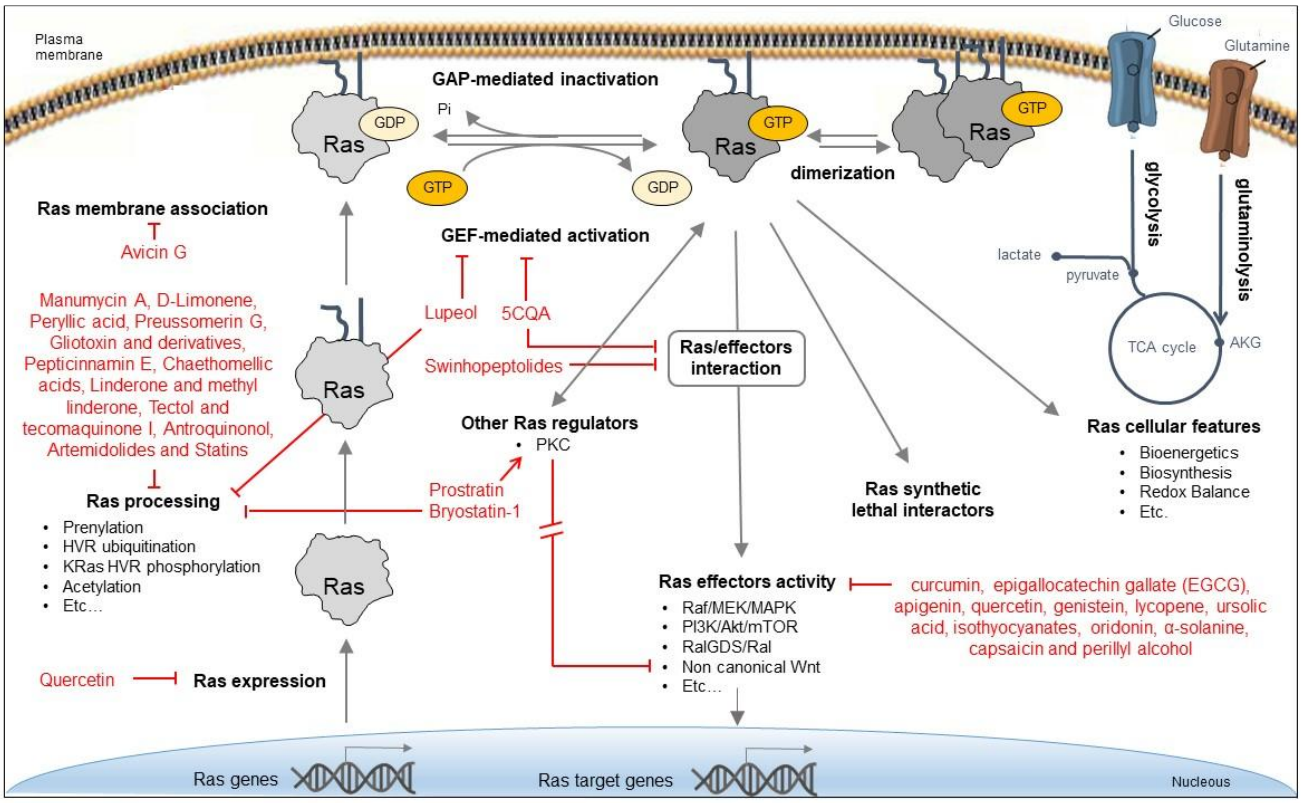

Figure 2. Approaches for inhibiting Ras oncoproteins biosynthesis, processing, activity, and signaling. Indirect Ras inhibitory approaches include the interference with different processes: expression of Ras oncogenes; Ras

processing and membrane localization; activity of Ras regulators; activity of downstream effectors; Ras-dependent cellular features; and activity of synthetic lethal interactors. Direct Ras inhibitory approaches include the interference with Ras/GEF interaction and exchange activity, Ras/effector interaction, and Ras dimerization.

2.1. NPs Indirectly Targeting Ras Function

2.1.1. NP Inhibiting Ras Expression

- Quercetin

Quercetin is a dietary flavonoid found in tea, onions, grapes, wines, and apples, and the anti-cancer activities of this compound have been previously explored in breast and colon cancer cells [39]. Quercetin reduced the expression of numerous prostate tumor-associated microRNAs (miRs) [40]. Quercetin regulated cisplatin sensitivity of human osteosarcoma cells by modulating the miR-217-*KRAS* axis [41]. Consistently, quercetin reduced the steady state levels of K-, H-, and N-Ras mRNAs and proteins in both colon cancer cell lines and primary colorectal tumors [42].

2.1.2. NPs Inhibiting Ras Regulation and Membrane Association

- Avicin G

Avicin G is a family of natural plant-derived triterpenoid saponins from *Acacia victoriae*, which mislocalizes KRas from the plasma membrane and disrupts plasma membrane spatial organization of KRas and HRas oncoproteins by depleting phosphatidylserine and cholesterol contents, respectively, at the inner plasma membrane leaflet [43]. Avicin G inhibits oncogenic K- and H-Ras signal output and the growth of KRas-addicted pancreatic and non-small cell lung cancer cells. Avicin G also perturbs lysosomal activity and disrupts cellular localization and activity of sphingomyelinases, resulting in altered cellular sphingomyelin levels and distribution.

- Bryostatin-1

Bryostatin-1 is a cyclic macrolide isolated from the marine bryozoan *Bugula neritina* that acts as a protein kinase C (PKC) agonist, activating PKC isozymes at nanomolar concentrations [44][45]. PKC-mediated phosphorylation of the C-terminal segment of KRas regulates its association with the plasma membrane. In particular, bryostatin-1 induces a rapid translocation of KRas to intracellular membranes such as endoplasmic reticulum (ER) and Golgi apparatus, but also to the outer mitochondrial membrane where K-Ras stimulates apoptosis [46]. Bryostatin 1 is in clinical development as an anti-leukaemic agent and is also in Phase II clinical trials against melanomas, lymphomas, and renal cancer [47].

- Prostratin

Prostratin is a phorbol ester found in the bark of the mamala tree of Samoa, *Homalanthus nutans* (Euphorbiaceae), acting as an activator of atypical PKCs. It can efficiently reduce the interaction of KRas and CaM, rewire non canonical Wnt/Ca^{2+} signaling, and suppress malignancy mediated by oncogenic KRas in pancreatic cancers [48].

2.1.3. NPs Targeting Ras Processing

- Manumycin A

Manumycin A is a natural macrolide antibiotic isolated from *Streptomyces parvulus* that acts as a potent peptidomimetic inhibitor of Ras farnesylation [49][50][51][52]. Manumycin A significantly inhibits the proliferation and migration of vascular smooth muscle cells (VSMCs), reduces the amount of Ras protein localized at the cytoplasmic membrane, inhibits the phosphorylation of MAPK, and disorganizes the actin fibers [53]. In addition, manumycin A decreases exosome biogenesis in prostate cancer cells and in myofibroblasts primarily via targeted inhibition of the Ras/Raf/ERK1/2 signaling [54][55].

- D-Limonene and peryllic acid

D-Limonene is a common monoterpene, found in essential oils of orange, lemon, mandarin, lime, grapefruit and many other plants, with antiproliferative, apoptosis-inducing and chemopreventive effects and, as similar monoterpenes, inhibits Ras prenylation [56][57][58]. The related compound peryllic acid is able to inhibit Ras prenylation targeting both farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) [59].

- Preussomerin G

The preussomerins and deoxypreussomerins are phenolic fungal metabolites extracted from the coprophilous fungus *Preussia isomera* and the endophytic fungus *Harmonema dematioides* with FTase and GGTase inhibitory properties [60][61][62][63]. Low toxicity synthetic esters derived from these compounds require reductive activation specifically at the cancer cells, resulting from hypoxia and overexpression of reductases. The anticancer activity was determined in cancer cell lines with reported reductase activity such as BC-1 cells and NCI-H187 [64].

- Gliotoxin and derivatives

Gliotoxin is a sulfur-containing mycotoxin, produced by various pathogenic fungi, including *Aspergillus fumigatus*, that inhibits Ras farnesylation and cell growth [49][50]. Some derivatives were developed as GGTase specific inhibitors [65].

- Pepticinnamin E

The natural product pepticinnamin E was reported to inhibit protein farnesyl transferases and cell proliferation almost 30 years ago [66][67]. Pepticinnamin E contains a rare N-terminal cinnamoyl moiety as well as several nonproteinogenic amino acids, which mimics the two substrates of FTase, CAAX, and FPP. Its biosynthetic pathway has only recently been characterized due to loss of the original producer organism [68]. A library of 51 analogues was generated from pepticinnamin E and screened for FTase inhibitory activity [69].

- Chaethomellic acids

Chaetomellic acids are a class of alkyl dicarboxylic acids, isolated from *Chaetomella acutiseta*. They are potent and highly specific farnesyl-pyrophosphate (FPP) mimic inhibitors of Ras FTase with lower specificity for GGTases [70][71]. Long-term treatment with chaethomellic acid A can attenuate Ras-dependent progression of renal fibrosis in a murine model of chronic kidney diseases [72].

- Linderone and methyl linderone

The cyclopentenediones linderone and methyl linderone isolated from the fruits of *Lindera erythrocarpa* (Lauraceae) showed FTase inhibitory and anti-tumor activity [73][74].

- Tectol and tecomaquinone I

Tectol and the related compound tecomaquinone I were isolated in a screening for FTase inhibitors; tectol also exhibited significant activity against human leukemia cell lines HL60 and CEM [75][76].

- Antroquinonol

A compound with anti-inflammatory activities extracted from the mycelium of *Antrodia camphorate* antroquinonol has been shown to exert anticancer effects in lung cancer, liver cancer, and leukemia by inhibiting the activity of both Ras FTase and GGTase [77][78][79].

- Artemidolides

Arteminolides (A-D) are dimeric sesquiterpene lactones isolated from *Artemisia spp.* with inhibitory activity on FTase [80][81]. These compounds and other similar sesquiterpene lactones from *Artemisia* inhibited tumor cell growth in a dose-dependent manner [82][83]. In particular, arteminolide C blocked in vivo growth of human colon and lung tumor xenograft [84].

- Statins

Several statins, comprising natural ones (lovastatin, simvastatin), efficiently inhibited KRas protein trafficking from the cytoplasm to the cell membrane of pancreatic cancer cells due to depletion of the mevalonate pathway's intermediates [85].

2.2. NPs Inhibiting Ras Effectors

- Several compounds

Many Ras effectors play a relevant role in the onset and progression of Ras-dependent disorders and therefore represent attractive therapeutic targets for drug development. Several inhibitors of Ras-ERK signaling have been developed, including Raf inhibitors and MEK inhibitors, as reviewed [86]. Several natural products were reported to inhibit ERK signaling although the mechanisms of action are often unclear. Among these, we can enlist sulphoraphane, epigallocatechin gallate (EGCG), isothyocyanates, genistein, and perillyl alcohol, (see [87] for a review). Also the inhibition of PI3K-AKT-mTOR pathway has been widely experimented, even with natural products such as lycopene, curcumin, resveratrol, genistein, apigenin, oridonin, α-solanine, and capsaicin [88][89][90][91][92].

2.3. NPs Targeting Ras Activity Directly

- 5-O-caffeoylquinic acid (5-CQA)

The first natural compound reported to directly target Ras activity was a chlorogenic acid and was identified on the basis of its structural resemblance to previously identified synthetic Ras inhibitors [93][94]. The chlorogenic acids (CGAs) occur ubiquitously in food, representing the most abundant polyphenols in the human diet. Particularly high levels of chlorogenic acid (5-O-caffeoylquinic acid, 5-CQA) were found in coffee beans used to prepare green

coffee and, after roasting, black coffee, a widespread drink worldwide. A number of CGA beneficial biological effects, including anti-inflammatory activity, anti-carcinogenic activity, and protection against neurodegenerative diseases were reported. Its mechanism of action is based on the inhibition, upon direct binding to the target, of Ras interaction with both activators and effectors. In addition, viability and MAPKs activation/phosphorylation assays performed on $KRas^{G13D}$ expressing breast cancer cells, MDA-MB-231, suggested its capability of reducing cancer cells growth [95].

- Lupeol

The triterpenoid lupeol was reported to inhibit farnesyl transferase [96] and thus to inhibit the growth of KRas mutant cancer cell lines but not of wild-type KRas-expressing cells [97]. Lupeol was identified as a KRas directly binding compound in an in silico screening of a library of the triterpenoid class of molecules and its binding results in inhibition of GDP/GTP exchange [97].

- Swinhopeptolides

Two new cyclic depsipeptides named swinhopeptolides A and B have been isolated from the marine sponge *Theonella swinhoei cf. verrucosa*, collected from Papua, New Guinea. These compounds contain 11 diverse amino acids and 13-carbon polyketide moieties attached at the N-terminus. They can impede the interaction between Ras and Raf, a serine/threonine protein kinase. Swinhopeptolides A and B showed significant inhibition of the Ras/Raf signaling pathway with effectiveness in the micromolar range [98].

3. Conclusion and Perspectives: Natural Products as a Source of Selective Inhibitors of Ras Oncoproteins

Although various strategies to inhibit Ras have been explored over three decades, success has only recently been achieved in human clinical trials, in particular with small molecules capable of directly inhibiting the activity of specific Ras oncoproteins, which, although important, represent only a small percentage of those involved in human pathologies. Therefore, there is a strong need for effective inhibitors which target the other pathogenic Ras variants. Several natural compounds has been successfully applied to different direct and indirect strategies of Ras inhibition, however natural product research has not been prioritized in the research and development of Ras inhibitors so far. Since natural products provide a virtually limitless source of structurally novel, highly diverse natural compounds, they would be a promising approach to discover novel molecules with higher affinity for specific pathogenic Ras mutants. Three points are worth mentioning on this subject:

- the improvement of techniques that allow to isolate, purify and structurally characterize new molecules of natural origin, often already available in large libraries [99][100];
- the simultaneous development of experimental and computational approaches for their high-throughput screening (HTS) on targets of clinical relevance;

- the availability of virtual screening allowing to identify the structurally most promising compounds for a target of interest, thereby reducing the research costs.

Both structure-based virtual screenings and HTS approaches with Ras oncoproteins as targets will now be able to take advantage of the newly-discovered druggable pockets available in specific oncogenic Ras isoforms and mutant proteins to isolate, characterize and iteratively improve Ras-specific inhibitors (Figure 3).

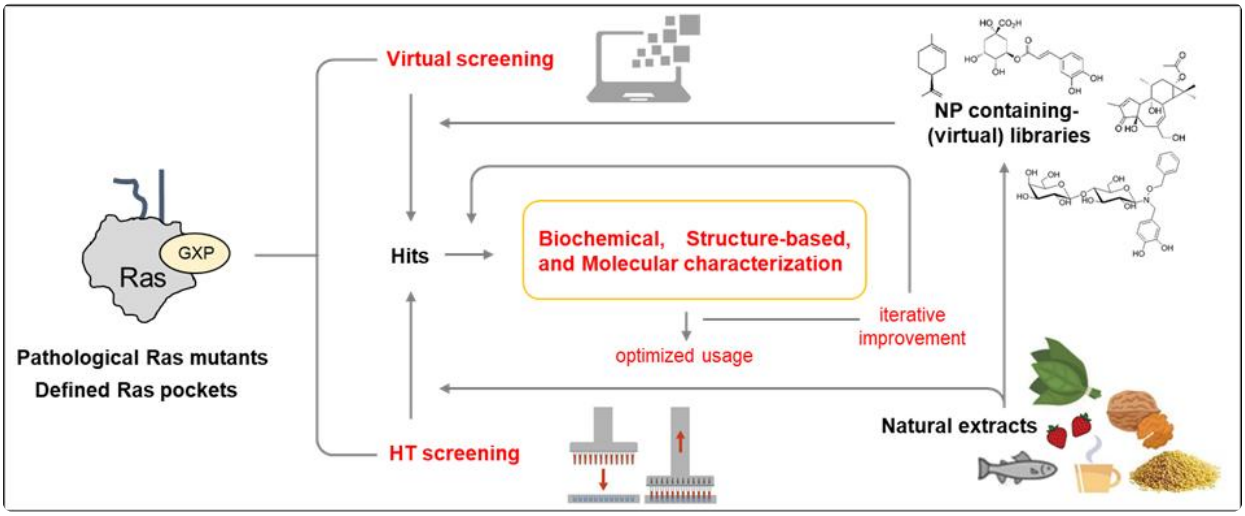

Figure 3. Approaches for the identification and development of Ras inhibitors from natural sources.

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