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Beyond the semi-synthetic artemisinin: metabolic engineering of plant-derived anti-cancer drugs

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The discovery and supply of plant-derived anti-cancer compounds remain challenging given their low bioavailability and structural complexity. Reconstituting the pathways of these compounds in heterologous hosts is a promising solution; however, requires the complete elucidation of the biosynthetic genes involved and extensive metabolic engineering to optimise enzyme activity and metabolic flux. This review describes the current strategies and recent advancements in the production of these valuable therapeutic compounds, and highlights plant-derived immunomodulators as an emerging class of anti-cancer agents.

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Plant-derived anti-cancer drugs: a recurring problem of supply

Plants produce a chemically diverse range of anti-cancer compounds — the major classes of which are summarised in Table 1, and their therapeutic properties have been reviewed elsewhere [1]. Cancer is a leading cause of mortality, resulting in a high demand for novel therapeutics. Recently, plant-derived immunomodulators have emerged as a new class of anti-cancer compounds. Unfortunately, many plant-derived anti-cancer compounds are synthesised and accumulated at low quantities *in planta*. Additionally, their structural complexity renders them inviable for mass-production by chemical synthesis. Therefore, to meet supply demands, recent efforts have focused on the production of these natural products (NPs) in heterologous hosts (Figure 1). Re-engineering of host organisms to express native plant biosynthetic enzymes allows *de novo* synthesis of anti-cancer compounds. Here we discuss the current state and limitations of the elucidation and heterologous reconstitution of plant biosynthetic pathways to produce endogenous and novel therapeutic NPs, with an emphasis on emerging immunomodulatory anti-cancer compounds.

Identification of missing enzymes in plant specialised metabolism

Biosynthetic pathways of plant-derived anti-cancer NPs are often complex — for example, there are over 31 known enzymatic steps from the primary metabolite geranyl diphosphate to the therapeutic compound vinblastine in the source plant Madagascar periwinkle (*Catharanthus roseus*). Identification of biosynthetic enzymes remains a major bottleneck in pathway reconstitution; however, recent methodological advancements have accelerated their discovery.

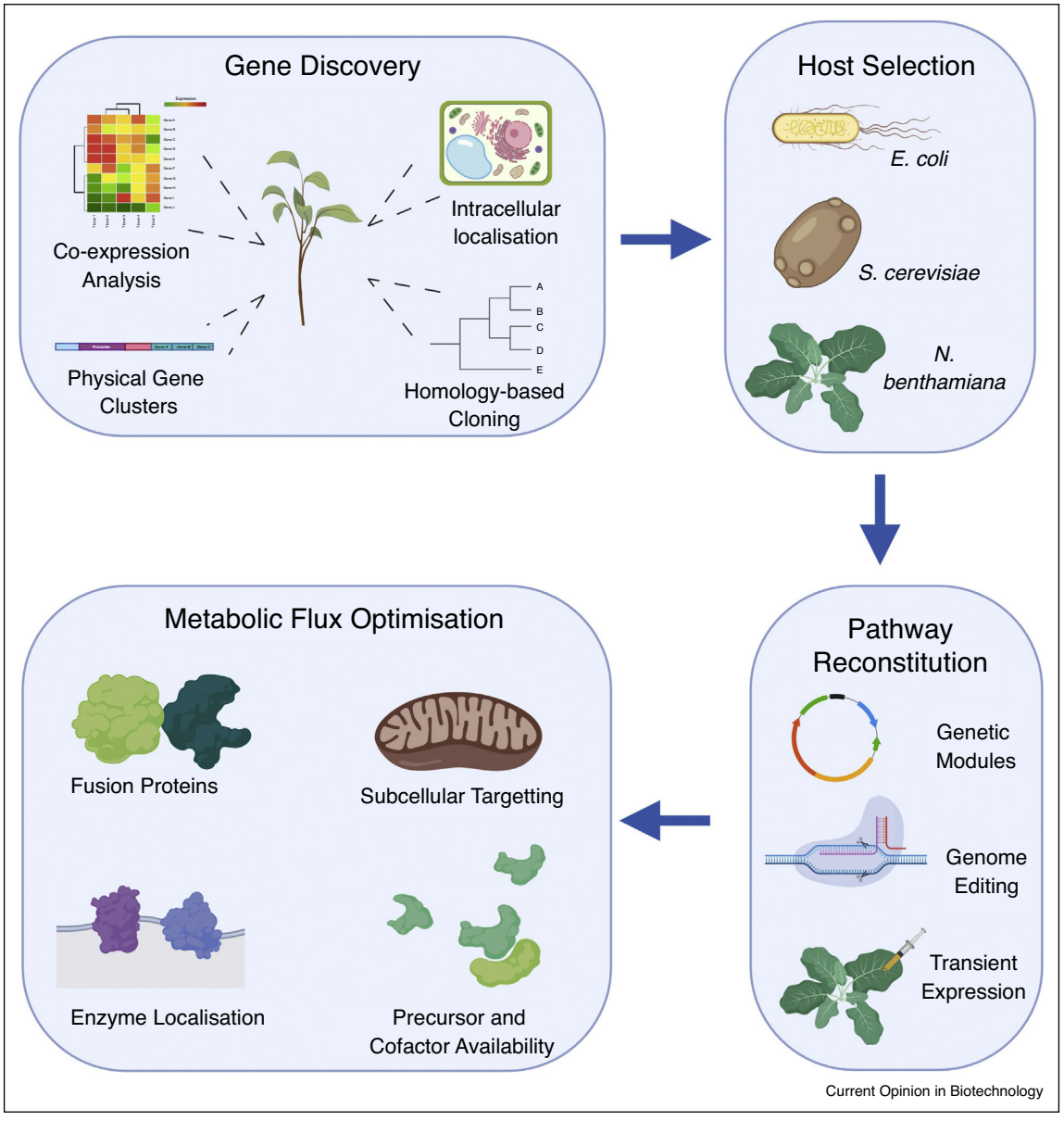
Sequencing and transcriptomic advances have enabled enzyme discovery by co-expression analysis, in which gene candidates are identified by similarities in tissue expression patterns with known pathway enzymes — demonstrated by recent discoveries in *C. roseus* and *Podophyllum peltatum* [4••,5]. Machine learning approaches such as self-organising maps have further refined gene candidates [6]. These methods, in combination with a greater understanding of the *in planta* localisation of biosynthesis, and the development of techniques such as single-cell metabolomics have further improved candidate selection, accelerating enzyme discovery [7]. Identification of physical gene clusters aided by the use of OMICs-based tools such as plantiSMASH can help elucidate missing biosynthetic enzymes, as in the noscapine and vinblastine pathways [8–10]. However, this approach is limited, with many plant biosynthetic pathways having few or no gene clustering, such as the camptothecin biosynthetic pathway [11]. Homology-based cloning can accelerate the discovery of genes with orthologous functions to known biosynthetic enzymes, as in the identification of a *C. roseus* decarboxylase orthologue in the ibogaine biosynthetic pathway in *Tabernanthe iboga* [12]. However, pathway complexity often necessitates a combinatorial approach, exemplified by the discovery of the *Gelsemium sempervirens* oxindole pathway [13].

Table 1

Overview of major classes of plant-derived anti-cancer compounds

Compound class	Anticancer agents	Plant derived from	Anti-cancer activity	Reference
Monoterpene indole alkaloids (MIAs)	Vincristine, vinblastine	*Catharanthus roseus*	Anti-mitotic - microtubule destabilising agents	[1]
	Camptothecin	*Camptotheca acuminata*	DNA damage inducers	[2]
Taxane derivatives	Paclitaxel, docetaxel, cabazitaxel	*Taxus genus*	Anti-mitotic – microtubule stabilising agents	[1]
Lignan derivatives	Etoposide, teniposide	*Podophyllum hexandrum*	DNA damage inducers	[3]
Benzylisoquinoline alkaloids	Noscapine	*Papaver somniferum*	Anti-mitotic – microtubule binding agent	[1]

Figure 1



Overview of approaches discussed in this review that are used to discover biosynthetic enzymes, and reconstitute and optimise natural product pathways in heterologous hosts to produce anti-cancer compounds Created with Biorender.com.

Once candidate enzymes have been selected, their enzymatic activities are characterised using functional assays in conjunction with metabolomic techniques to detect product formation. While being efficient, such assays can be complicated due to unknown or unpurifiable reaction substrates and therefore requiring the use of either crude whole-plant or tissue extracts. Alternatively, virus-induced gene silencing (VIGS) can be used to characterise gene function *in vivo*, but requires plant-specific development as demonstrated in *C. roseus* and *Camptotheca acuminata* [14,15].

Selecting a suitable heterologous host organism

Biosynthetic gene discovery enables engineering of NP pathways to produce pharmaceutically valuable compounds. A key factor in successful pathway reconstitution is the choice of an appropriate heterologous host based on the host's characteristics, as summarised in Table 2. Plant NP pathway reconstitution poses a unique challenge due to the often high number of enzymatic steps, complex tissue and subcellular localisation of intermediates, and intricate regulation networks, exemplified in the vinblastine biosynthetic pathway [7]. In addition, the scalability and economic-viability of production must be considered, taking into account the choice of substrate and the ease of downstream product purification.

The bacterium *Escherichia coli* is a common heterologous host for plant NP biosynthesis due to its established genetics toolbox, well-understood native metabolism and fast doubling time. *E. coli* has been used for the production of many NPs including taxadiene and reticuline [19,20]. However, there are significant physiological differences between plants and *E. coli*, such as the lack of eukaryotic cell architecture and protein post-translational modification mechanisms. These differences can disrupt the metabolic flux of the reconstituted pathway and reduce product titres, as demonstrated by the transfer of semi-synthetic artemisinin production from *E. coli* into the eukaryotic *Saccharomyces cerevisiae* host [21].

S. cerevisiae is a typical eukaryotic host that has been used in the reconstitution of cannabinoids, noscapine and monoterpene indole alkaloids (MIA) pathways [22•,23,24•,25,26], and its use has been extensively reviewed elsewhere [17]. A key advantage is its eukaryotic cell architecture, allowing the expression of membrane-localised proteins commonly found in NP pathways such as cytochrome P450's [27].

Nicotiana benthamiana is the most widely used plant chassis for heterologous NP biosynthesis; however, alternative systems such as the false flax *Camelina sativa*, microalgae, and the moss *Physcomitrella patens* have also been used [28–30]. Plant systems have the advantages of better replicating native pathway compartmentalisation at both the tissue and cellular levels, and have the required cofactors and post-translational mechanisms for proper enzyme expression and activity [18]. Unfortunately, the limited number of developed genetic modification tools and the organism's longer generation time hamper their use in NP pathway reconstitution.

The use of hosts with edited or minimal synthetic genomes is currently being explored to improve product yields compared to their native host counterparts [31,32]. Gene editing allows the targeting of destabilising elements such as enzymes that derivatise biosynthetic precursors [33]. Minimal synthetic genome organisms have been developed that only retain genetic elements of known function, greatly decreasing the risk of metabolic flux shifting [34]. This enables impact modelling of the introduced heterologous pathway on native host metabolism, allowing for better design of the reconstitution process [35,36]. However, due to the complexity of higher plants, synthesis of minimal genomes is currently only available in microbial organisms.

Heterologous reconstitution of plant biosynthetic pathways

Recent advances in molecular techniques have accelerated the heterologous reconstitution of NP pathways. Technologies such as Golden Gate cloning and Gibson assembly enable segmentation of pathways into clonable modules [37,38]. In accordance with these systems, syntaxes containing standardised parts have been developed for their use in different hosts [37,39]. Empirical combinations of standardised parts, especially regulatory elements, can then be tested to optimise pathway flux. Screening of resulting genetic modules can be further accelerated through the use of automated biofoundries based on the design-build-test (DBT) cycle, outlined in Figure 2 and reviewed elsewhere [40].

Novel genome editing technologies have increased the ease of integrating longer pathways into a host's genome. Combinatorial toolkits such as the Easy-Clone system use homologous recombination and CRISPR-Cas for markerless, single-step genome integration of multiple biosynthetic modules into pre-defined sites [41]. This approach lowers the risk of recombination within the target constructs, and thus the loss of their parts [42]. The hierarchical organisation of constructs and availability of online regulatory sequence libraries allows for easy selection and swapping of parts in *S. cerevisiae* [43,44].

Transient expression remains the most widely used reconstitution platform in plant hosts, successfully producing medicinally-active terpenes [45••]. *Agrobacterium*-mediated co-infiltration of plants allows rapid expression of biosynthetic enzymes. However, the system's lack of protein stoichiometry control and the limited number of genes that can be expressed make it less suitable for reconstituting longer pathways [18]. A possible solution to

Table 2

The advantages and disadvantages of commonly used host organisms for heterologous NP pathway reconstitution

Organism Domain	Heterologous host	Advantages	Disadvantages	Ref.
Prokaryotic	Bacteria for example, *Escherichia coli*	- Fast generation times - Well established genetic toolboxes - Efficient gene-editing tools - Ease of culturing on cheap carbon sources	- Lack of eukaryotic cell architecture for expression of plant enzymes - Lack of post-translational modifications needed for expression of plant enzymes - Lack of certain biosynthetic precursors and co-factors	[16]
Eukaryotic	Yeasts for example, *Saccharomyces cerevisiae*	- Fast generation times - Eukaryotic cell architecture - Well-established genetic toolboxes - Efficient gene-editing tools - Ease of culturing on various cheap carbon sources	- Lack of tissue compartmentalisation (single cell organism) - Different post-translational mechanisms compared to plants	[17]
	Plants for example, *Nicotiana benthamiana*	- Eukaryotic cell architecture - Pathway compartmentalisation possible at both tissue and cellular level - Presence of relevant post-translational modifications and enzyme co-factors - Possibility of rapid transient expression of pathways - No need for exogenous carbon source	- Longer generation times - Lack of well-established genetic toolboxes - Efficient genome-editing tools still at early stages of development - Lack of detailed knowledge of metabolism - Costly growth facilities and upscaling of production	[18]

these challenges may lie in the stacking of target biosynthetic genes into a single vector [39].

Because of the complexity of their genomes, gene-editing technologies in plant systems are still being developed. The relative simplicity of plastomes compared to their nuclear counterparts renders them more amenable to gene editing and therefore these have been targeted for NP pathway reconstitution [46]. However, recent developments in CRISPR-Cas technologies have enabled whole plant genome editing to eliminate endogenous NP derivatives [47]. Although still in their infancy, these gene-editing techniques pose exciting prospects for the future of *de novo* production of plant-derived NPs.

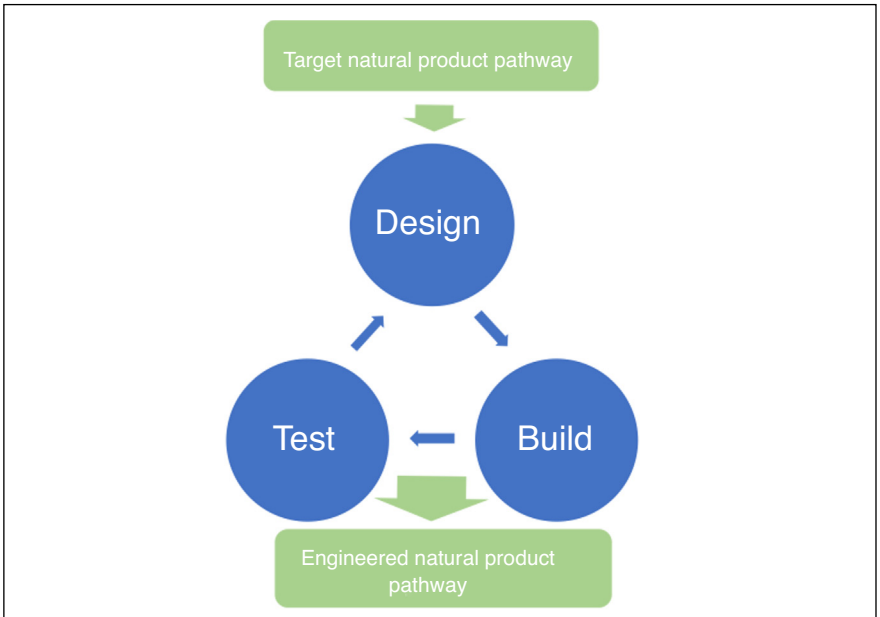
Metabolic flux and process optimisation

Heterologous reconstitution of long pathways often causes metabolic cellular stress, reducing host growth and thereby production efficiency. Low or absent activity in bottleneck enzymes can lead to the build-up of pathway intermediates, thereby affecting cell fitness. Protein engineering and the introduction of suitable exogenous enzymes are commonly used strategies to improve enzyme activity, alleviating bottlenecks and thereby increasing pathway flux [24•]. The identification and modulation of protein–protein interactions are also important factors, as exemplified by the engineering of cytochrome P450 enzymes and their reductase partner to improve oxygenated taxane production [48]. An alternative strategy is to reduce the spatial dispersion of pathway intermediates by fusing downstream enzymes, as achieved in taxol and geraniol biosyntheses [19,49•].

The availability of metabolic precursors or enzymatic cofactors is also an important component in metabolic flux optimisation. In the recent cannabinoid biosynthesis reconstitution, engineered upregulation of the mevalonate pathway in primary metabolism increased the metabolic flux towards the target NP pathway [21]. Precursor availability can also be increased by knocking-down non-essential competing enzymes, or by degron-tagging essential competing enzymes [24•,50•]. The presence of growth media additives such as glycerol may also improve cofactor availability, demonstrated in the reconstitution of the noscapine pathway in *S. cerevisiae* [24•].

Heterologous NP biosynthesis can also be hampered by improper host post-translational modification mechanisms, causing protein misfolding or mistargeting and thereby affecting enzyme activity. Modification or addition of localisation sequences is a commonly used strategy to improve enzyme targeting within the host at both the cellular and subcellular levels. This prevents the loss of pathway intermediates by diffusion and reduces metabolic flux into undesired pathways [21,24•]. This technique was exemplified in the recent targeted production of the MIA precursor geraniol in *S. cerevisiae* mitochondria, whereby exploiting the host's lack of intermediate-specific catabolic enzymes in the organelle enabled increased intermediate accumulation and thereby higher end-product titres [49•].

Figure 2



Stage of the cycle	Factors to consider
Design	-Mode of reconstitution (pathway integration into the genome/transient expression) -Choice of the cloning syntax -compartmentalisation of the pathway on tissue/sub-cellular level -Directing metabolic flux through enzyme expression control -Addition of other enzymes for flux optimisation
Build	-Throughput of the assembly process -Ease of modification of already assembled parts -Automation of the assembly
Test	-Screening method for product formation -Use of bio-sensors to detect product formation

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Design Build Test (DBT) cycle for pathway reconstitution. Each cycle phase is split into three main steps, which is then further split into automatable subsections to accelerate the process of NP pathway engineering.

Intermediate localisation and accumulation can also be manipulated by transporter protein engineering; however, few have been identified in plant NP pathways. In heterologous hosts, this technique has been used to introduce the uptake of alternative carbon sources [51]. Additionally, transporter engineering can increase flux into a biosynthetic pathway by reducing the production of unwanted by-products as exemplified by the fusion of a xylose transporter to a xylose isomerase [52].

New-to-nature plant-derived anti-cancer compounds

Advances in heterologous pathway reconstitution have recently turned to the production of new-to-nature plant-derived compounds to expand metabolic diversity. A common strategy to produce novel NPs is by reengineering or exploiting an enzyme's natural substrate promiscuity to allow the feeding of non-natural precursors. Feeding of halogenated substrates has produced novel compounds with new or improved pharmacological properties such as in the opiate, MIA and phenylpropanoid pathways [24•,53,54]. This technique has also been used in the feeding of fatty acids to produce unnatural cannabinoid analogues [22•]. Knowledge of the biosynthetic pathway also enables a combinatorial approach to produce novel NPs by swapping, omitting or introducing new enzymatic steps in a non-canonical fashion. This was exemplified by the use of non-native cytochrome P450 enzymes to oxidise β-amyrin in *N. benthamiana*, resulting in novel triterpenes [45••].

Table 3

Summary of the mode of action, origin and activity of major plant-derived immunomodulators

Immunomodulatory agent	Mode of action	Plant derived from	Activity	Reference
Artemisinin	Immunosuppressant	*Artemisia annua*	Reduction of TNF-α and IL-6 secretion, regulation of NF-κB signalling	[57]
Berberine	Immunosuppressant	*Berberis spp.*	Activation of mitochondrial apoptotic pathways, reduction of TNF-α production	[55,58]
Curcumin	Immunostimulant	*Curcuma longa*	Restoration of $CD4^+/CD8^+$ T cells, suppression of T cell apoptosis	[59]
Matrine	Immunosuppressant	*Sophora flavescens*	Inhibition of JAK/STAT3 signalling pathway	[60]
Tryptanthrin	Immunostimulant	*Strobilanthes cusia*	Regulation and recruitment of regulatory T cells	[61]

Plant-derived immunomodulatory agents: an emerging class of anti-cancer compounds

Plant-derived immunomodulatory agents are an emerging class of anti-cancer compounds that are currently largely unexploited due to their low bioavailability. Unlike cytotoxic anti-cancer compounds (summarised in Table 1), immunomodulators target specific constituents of the cancer development process through various mechanisms, outlined in Table 3. Detailed mechanisms of major plant-derived immunomodulators have been reviewed elsewhere [55]. In clinical trials, these compounds show high cell type interaction specificity, potentially enabling more predictable and targeted treatments [56]. Immunosupressants such as artemisinin and its derivatives including artemisone, artesunate, and dihydroartemisinin are being investigated as tumour-inhibiting therapeutics due to their modulation of various signaling cascades involved in the spread of cancer [57]. Matrine is another example of a plant-derived immunosuppressant that acts by inducing the expression of anti-tumour immune defence activating ligands [60,61]. Plant-derived immunostimulants such as curcumin stimulate immune cell production involved in tumour defence, increasing immune system identification of tumour cells and the subsequent activation of apoptotic pathways [59]. The alkaloid tryptanthrin is also an effective stimulant of T cell recruitment, triggering the immune inflammatory response against cancer cells [61].

As the biosynthesis of more NPs are elucidated, new potentially therapeutic chemical scaffolds are becoming available. The recent elucidation and heterologous reconstitution of the immunosuppressant alkaloid berberine enables the production and development of novel derivatives with improved pharmacokinetic properties [58,17]. Although this class of anti-cancer therapeutics remains understudied, use of NP pathway discovery and reconstitution techniques will enable advances towards the production of novel immunomodulatory compounds. Reaching a high-level production of these compounds will require complementary pathway engineering. For terpenoid compounds, a fine tuning of the metabolic flux can be obtained by limiting competing metabolic branches through the downregulation of the expression of the yeast gene *ERG9* for instance. The resulting rerouting of the flux towards isopentenyl diphosphate (IPP) thus favours artemisinic acid production by decreasing ergosterol synthesis [62]. Engineering of enzyme specificity and efficiency for acceptance of orthogonal substrates that do not compete directly with endogenous pathways of the host organism can be also explored as recently demonstrated for monoterpenoid biosynthesis in yeast [63].

Concluding remarks

Significant developments over the last few years have enabled the heterologous reconstitution of increasingly complex plant biosynthetic pathways to produce anti-cancer compounds. Improvements in gene discovery techniques in conjunction with sophisticated synthetic engineering tools such as genome editing have accelerated the process of pathway elucidation and heterologous reconstitution. Increased screening of plant-derived NPs has revealed new classes of anti-cancer therapeutics such as immunomodulatory compounds. Despite recent biotechnological improvements, significant limitations remain, preventing the mass-production of these plant-derived NPs in heterologous systems. Securing and increasing the supply of these compounds are vital to meet both current demands, and will also expand the panel of highly toxic compounds used to generate antibody drug conjugates for immunotherapies.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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