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Innovative applications for insect viruses: towards insecticide sensitization

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The effective management of emerging insect-borne disease is dependent on the use of safe and efficacious chemical insecticides. Given the inherent ability of insects to develop resistance, it is essential to propose innovative strategies because insecticides remain the most important element of integrated approaches to vector control. Recently, intracellular phosphorylation and dephosphorylation of membrane receptors and ion channels targeted by insecticides have been described as new processes for increasing the sensitivity of insecticides. An efficient method might be to infect host insects with recombinant viruses overexpressing specific protein phosphatases/kinases known to regulate specific insecticide-sensitive targets. This attractive strategy could lead to sensitization of the insects, thus reducing the doses of insecticides and increasing the efficacy of treatments.

Introduction

Emergence and/or resurgence of vector-borne diseases

The past 25 years have seen drastic changes of ecologic, climatic, demographic and economic determinants that have promoted the emergence and/or resurgence of vector-borne diseases (e.g. malaria, West Nile virus, rift valley fever, dengue fever, chikungunya virus), which account for ~17% of the estimated global burden of infectious diseases [1]. Climate change partly explains this trend because it contributes to an increase in the average exposure of vertebrate hosts to vector-borne diseases by changing the geographical distribution of conditions that are suitable for the vectors and disease pathogens [2]. For some diseases, such as chikungunya and West Nile, viruses have also undergone genetic changes that have helped them to jump vector host species and become more pathogenic [3,4]. In Europe, several arthropod-borne diseases have recently emerged: tick-borne encephalitis, transmitted by *Ixodes* sp. [5], the Bluetongue virus, transmitted by *Culicoides* sp. [6], and the West Nile and chikungunya viruses, transmitted by the mosquitoes *Culex* and *Aedes,* respectively [7,8]. Invasion and establishment of efficient disease vectors, such as the Asian tiger mosquito *Aedes albopictus* (Box 1), and steady import of their viruses, such as dengue and chikungunya, into Europe [7] raises the constant risk of tropical disease outbreaks in temperate regions.

Insecticide resistance mechanisms

Despite advances in therapeutic and vaccine research, the control of vector-borne diseases is still dependent to a large extent on vector control. However, few vector control strategies have been implemented with sustainable success. Failures are generally attribute to lack of knowledge of insect vector bio-ecology and vector–pathogen–human interactions, as well as unsuitable vector control strategies, which are partly responsible for the development of resistance to an increasing number of insecticides [9]. Indeed, it is assumed that the widespread use of chemicals for agricultural practices and/or domestic hygiene since the 1960s facilitated the evolution and spread of resistance in disease vectors, including mosquitoes [10]. Resistance mechanisms can be divided into two groups: metabolic (alterations in the levels or activities of detoxification proteins) and target site (non-silent point mutations within structural receptor genes) (Box 2; Table 1). Increased expression of the genes encoding the three major xenobiotic metabolizing enzymes, that is, the cytochrome P450 monooxygenases, glutathione transferases (GSTs) and carboxy/cholinesterases (CCEs), is the most common cause of insecticide resistance in insects. Genomic analysis of detoxification genes using so-called 'detox-chip' microarrays in the mosquitoes *Anopheles gambiae* [11] and *Aedes aegypti* [12] revealed an abundance of genes belonging to the CYP P450 classes (e.g. *CYP4*, *CYP9*, *CYP6*, *CYP12*) and the GST and CCE families that are overexpressed in resistant mosquitoes compared with susceptible insects. However, higher expression of detoxification genes in insecticide-resistant colonies does not necessarily correlate with insecticide resistance. Currently, only genes encoding CYP6

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Box 1. Emergence and spread of the Asian tiger mosquito vector

Recently, several vector-borne, parasitic or zoonotic diseases have (re)-emerged and spread into the northern hemisphere, where they have led to major health, ecological, socio-economical and political consequences. Among these, the establishment and spread of the Asian tiger mosquito (*Aedes albopictus*) is of particular concern [69]. Under experimental conditions, *Ae. albopictus* can be infected by at least 22 arboviruses, including dengue viruses, yellow fever virus, chikungunya virus and Ross river virus [70]. *Ae. albopictus*, originally originating from Asia, was first described by Skuse in 1894, and has spread to all continents in the past 30 years [7]. It was first recorded in Europe in Albania in 1979. In the USA, the species was first established in Texas in 1985 [71]. Since then, *Ae. albopictus* has been introduced into Italy, probably through shipments of used tyres from the USA. Since the first record of a breeding population on the outskirts of Padova (Veneto region) in 1991, It has quickly spread across the country, showing a great ability to adapt to different ecological situations. In Rome, *Ae. albopictus* has encountered particularly favourable conditions. Since its discovery in the capital city in 1997, the species has colonized the entire metropolitan area (Figure I), despite efforts to eradicate it. It is now present in all European countries around the Mediterranean Sea, being particularly abundant in Italy, and has become a major nuisance in South-East France along the Italian border since 2005 [7]. Based on a genetic algorithm (GARP) modelling system, Benedict and colleagues [72] recently predicted a global invasion of *Ae. albopictus* throughout the world, especially in Central American and sub-Saharan African countries, which show high numbers of potentially suitable niches for this species. Major outbreaks of chikungunya, in which *Ae. albopictus* was the presumed vector, have occurred between 2004 and 2007, causing more than 3 million cases in Kenya, the West Indian Ocean Islands, India and Indonesia. In 2007, a limited chikungunya outbreak occurred in Italy, with 205 human cases reported [8]. The spread of *Ae. albopictus* together with a mutation that helped the virus to adapt to the *Ae. albopictus* mosquito species has enhanced these epidemics and put the northern hemisphere at risk for this arthropod-borne disease [73,74].

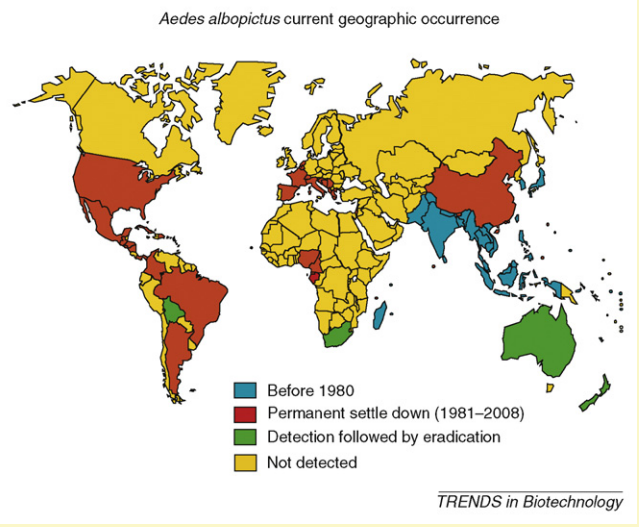

Figure I. Current geographic occurrence of *Aedes albopictus*.

Box 2. Diversity of insecticide resistance mechanisms in insects

To be effective, an insecticide has to come in contact with an insect, penetrate its body or be digested before it can reach its target site. Figure Ia illustrates the levels of penetration, excretion, enzymatic degradation and target interaction of an insecticide compound in a susceptible insect. Any event or mechanism that blocks one of these events can lead to resistance, as shown in Figure Ib. The first possible resistance mechanism by the insect is to avoid the insecticide, which can either be genetically determined or can be acquired by a learning process after previous contact with the toxic chemical. For example, insects avoid eating toxic plants as soon as they are able to detect them visually, through chemoreception or through contact. Contact avoidance can involve different behaviours. In many cases, genetically determined oviposition behaviour prevents females from laying eggs on unsuitable breeding sites. Insects can also escape to an insecticide-free site by shifting their resting behaviours or by exploiting the plants, animals or humans in different conditions [75]. Another resistance mechanism involves the excretion and possible metabolization of an insecticide after contact or ingestion. These detoxification metabolic pathways involve a variety of enzymes that can be induced upon contact with the insecticide [75,76]. Metabolic resistance is often a result of the overproduction of 'detoxification enzymes' that are able to metabolize insecticides. This mechanism could be associated with phenotypic plasticity because the production of detoxification enzymes is typically induced by the presence of insecticides in the insect environment. However, resistance to insecticide might also arise as a consequence of specific mutations in genes that enhance the catalytic activity of enzymes towards, for example, plant toxins [75].

Finally, mutation in the insecticide target itself can reduce or eliminate any of its deleterious effects. For instance, and highly relevant for public health concerns, is the observation that the major target site mutations are *Kdr*, which affects a gene encoding the sodium channel, and *Ace1*^{R}, which affects the gene encoding acetylcholinesterase. Because these mutations have been found in different mosquito species [59,77], these events are of great concern for the future efficacy of public health control programs based on the use of pyrethroids and organophosphates.

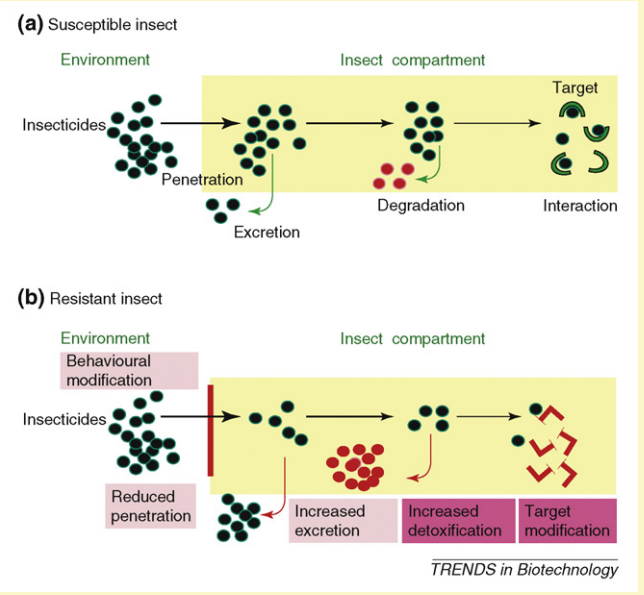

Figure I. Mode of action of an insecticide in **(a)** a susceptible and **(b)** resistant insect.

P450 enzymes have been clearly involved in cellular mechanisms known to metabolize DDT and pyrethroid insecticides in dipterans [13,14] and lepidopterans [15]. Further experimental validation, for example by enzyme characterization and RNA interference (RNAi), are necessary to deduce the identity of candidate genes in insecticide metabolism and resistance. By contrast, point mutations in genes encoding receptors such as the voltage-gate sodium channel (the well-known knock-down-resistant mutations *kdr*), the GABA receptor (*Rdl*) and acetylcholinesterases (*ace-1*^{R}, *ace-2*^{R}) have been shown to confer cross-resistance to all chemical classes of insecticides

Table 1. Mechanisms of insect resistance relevant in the main insecticide families

Insecticides	Molecular target	Resistance mechanisms		Refs
		Target site	Enzymatic overproduction	
Pyrethroids, type I	Sodium channel	*Kdr* and super *Kdr* mutations	Monooxygenases + esterases	[10,57,58]
Pyrethroids, type II	Sodium channel	*Kdr* mutations	Monooxygenases + esterases	[10,57,58]
Organochlorates	Sodium channel	*Kdr* mutations	GS-tranferases + monooxygenases	[10,57,58]
N-alkyl-amides	Sodium channel	No resistance reported against insect of public health importance		
Organophosphates	Acetylcholinesterase	$Ace1^R$ mutation	Esterases + GS-tranferases + monooxygenases	[10,59–64]
Carbamates	Acetylcholinesterase	$Ace1^R$ mutation		[10,59–64]
Neonicotinoids	Nicotinic acetycholine receptor	Not reported	Monooxygenases	[65,66]
Spinosad	Nicotinic acetycholine receptor	Not reported		
Cyclodienes, lindane, bicyclic phosphates	GABA receptor	*Rdl* mutation	GS-transferases	[58,67]
Phenylpyrazoles	GABA receptor	*Rdl* mutation	GS-transferases	[58,67]
Avermectines	GABA receptor	Undescribed	Monooxygenases + esterases	[58,67]
Insect growth regulators	Ecdysone agonist/disruptor or inhibitor of ATP synthase, chitin biosynthesis or lipid synthesis	No resistance reported against insect of public health importance		
Bacillus thuringiensis var. *israelensis*	Microbial disruptors of insect midgut membranes	Reported against *Culex pipiens s.l.* but not described		[68]

acting on the same target sites [10], including the organochlorates, the organophosphates, the carbamates and the pyrethroids (Table 1).

Current strategies for vector control

The arsenal of usable insecticides for vector control has dwindled considerably owing to environmental and toxicological considerations. Economic factors have also limited investment into research and development of new compounds and their applications in controlling public health pests and vectors. For these reasons, the management of vector populations and any strategies to potentially slow down the evolution of pesticide resistance are currently based on a rational use of existing compounds that are acting on different target sites [16,17]. With regard to diseases relevant for public health, appropriate rotations of different insecticides have allowed a reduction in the pressure of selection on black fly larvae and hence an effective and sustainable control of the burden of river blindness has been maintained in West Africa for more than 30 years. In mosquito control, two different strategies have shown promising results for malaria vector control and offer a potential tool for resistance management: (i) the use of 'two-in-one' combinations of different chemicals, such as insecticides (pyrethroids, organophosphates or carbamates) and repellents (e.g. DEET or Icaridin) applied to bednets [18–20], and (ii) the use of systematic rotations and mosaics for insecticide residual spraying [16]. However, questions remain concerning the residual activity of these 'cocktails' of chemicals (e.g. insecticides might have different decay rates), their cost-effectiveness and their potential toxicity for mammals [21].

In the same context, genetic control of insect pests is an old concept that has been the subject of renewed interest in the public health sector over the past 10 years [22]. In theory, genetic control of vectors has several advantages compared to classical insecticide use, such as that no compounds are released into the environment. Furthermore, a genetic strategy can be species-specific. Two main approaches are currently under development: the release of genetically modified insects that are unable to transmit diseases (e.g. malaria or dengue) to humans [23] and the 'Sterile Insect Technique' (SIT), which consists of releasing factory-produced sterile insects to control field populations of important vectors [22]. Unfortunately, only very few studies have provided any evidence for effective and sustainable control of mosquitoes using these techniques, as discussed in Ref. [24]. Concerning transgenic mosquitoes, several constraints need to be overcome before implementing open field releases, including the identification of genes of interest, their transformation and expression in mosquitoes, the fitness and specificity of the resulting insects and, last but not least, ethical aspects (e.g. cost-effectiveness) and risk management (e.g. adverse consequences).

Viruses as innovative vector-control strategies

Viruses as bioinsecticides

Among insect pathogenic viruses, densoviruses and baculoviruses are those that offer realistic potential as biological control agents for insects. Densoviruses have been isolated in seven different insect orders to date, including Diptera. They are small, non-enveloped DNA viruses that belong to the family Parvoviridae and are relatively stable in the environment. Despite their high virulence and infectivity for their natural hosts, densoviruses are not known to infect mammalian cells. Many insect hosts for densoviruses, particularly mosquitoes and cockroaches, have medical or economic importance, but so far only few studies have tested densoviruses as candidates for biological control [25,26].

Another class of viruses that have been widely researched and that might have a greater potential as bioinsecticides are the baculoviruses. Indeed, they are highly selective for insect species and do not replicate in vertebrates and in plants. Baculoviruses are predominantly pathogenic for insects belonging to the order Lepidoptera, and at least a hundred isolates have been reported. Interestingly, they have been identified in mosquitoes, being the only example for Diptera. The family Baculoviridae comprises two genera, the *Nucleopolyhedroviruses* (NPVs) and the *Granuloviruses* (GVs), based on the morphology of their occlusion bodies (OBs). The OBs are

responsible for the survival of the virus in the environment and the spread of the virus from insect to insect. The NPVs produce large OBs, also called polyhedra, that contain many occlusion-derived virions (ODVs), which are surrounded by a crystalline matrix protein composed mainly of the OB protein polyhedrin. These ODVs can either have only one nucleocapsid, so-called single NPVs (SNPVs), or multiple nucleocapsids, so-called multiple NPVs (MNPVs). The GVs, however, produce small granular occlusion bodies, the granula, that normally contain only a single virion that is surrounded by the structural OB protein granulin, which is only found in Lepidoptera [27].

In the case of NPVs, OBs are ingested by insects and dissolved by the alkaline pH of their midgut. This leads to a release of the ODVs, which pass through the peritrophic membrane and enter cells of the midgut epithelium. Virus replication takes place in the nucleus of the epithelium cells and results in the production of nucleocapsids that are released from the nucleus and bud through the plasma membrane to form a second baculovirus phenotype, the so-called budded virus (BV), which typically contains a single nucleocapsid. These BVs are responsible for the systemic spread of the virus within an infected insect. During later phases of infection, a switch occurs from production of BVs to production of ODVs, which are produced by interaction of nucleocapsids with the nuclear membrane. ODVs then become occluded by the very late OB protein-polyhedrin to form OBs. Finally, nuclei of the host cell become packed with OBs, which are released into the environment upon host death [28].

Baculoviruses found in mosquitoes have so far been restricted to the NPV group. They are specific for the midgut tissues of mosquito larvae, but infections have also been found in the midgut of adults that have emerged from surviving larvae [29]. Since the discovery in 2001 of a baculovirus isolated from the mosquito *Culex nigripalpus*, the so-called CuniNPV, new information has become available, allowing us to understand the interaction between CuniNPV and the mosquito at the molecular level. The major OB protein of CuniNPV does not seem to be homologous to either polyhedrin or granulin found in other baculoviruses [30]. Laboratory assays have shown that CuniNPV is specifically transmitted to *Culex* mosquito species and does not infect other species, such as *Aedes* or *Anopheles* [31–33], meaning that this virus is unsuitable for an effective management of all types of mosquito-borne diseases.

Some natural baculoviruses from the NPV and GV families have a registration number and are used successfully against Lepidopteran pests, such as *Helicoverpa armigera*, *Anticarsia gemmatilis*, *Spodopera exigua* and *Cydia pomonella*. For example, commercial products are used in the protection of crops, fruit orchards and forests in North America (Canada and USA), South America (Brazil and Argentina), Europe and China [34–37].

However, despite their potential, the widespread use of natural baculoviruses as bioinsecticides has been limited particularly by their slow speed in killing the targeted insects. Indeed, it can take days to weeks from virus application to insect death. During this time, the insects can still cause significant damage, which incurs considerable costs, limiting the commercial use of viral insecticides. These limitations of natural viruses could be overcome by genetic engineering strategies, and several approaches have been used to produce more potent viral insecticides with a drastically reduced kill time [35].

Among these genetically modified insect viruses, a few recombinant densoviruses have been constructed for biological control. However, to date, their use has been restricted to research applications. Furthermore, their major limitation is their small genome size, which is imposed by the geometry of the particle. Because all of the viral gene products are necessary for viability, the length of any inserted foreign DNA sequence is greatly restricted. This limitation can be overcome with the use of so-called 'helper viruses', which allow the replication of recombinant virus genomes and their packaging into virions, which are then able to infect host cells [25]. Recently, a novel densovirus (AgDNV) has been isolated that is capable of infecting and spreading within *Anopheles gambiae*. Using the 'two-plasmid helper–transducer system' method, AgDNV has been genetically modified, and the resulting virus has been shown to allow expression of a foreign gene in mosquitoes [38].

Baculoviruses are also commonly used as expression vectors for the production of a variety of recombinant proteins in insect larvae or insect cells. Genetic engineering of the well-characterized *Autographa californica* NPV has led to the improvement of baculoviruses already in use as biopesticides [35] via a reduction in the time required for the virus to kill the host insect. The insecticidal activity of wild-type baculoviruses can be improved either by insertion of foreign genes or by deletion of baculovirus-encoded genes. Using the first approach, recombinant baculoviruses have been constructed containing genes for insect-specific toxins (e.g. from scorpions, mites, spiders, sea anemones and *Bacillus thurengiensis*), hormones (e.g. diuretic hormone, neuropeptide hormone, juvenile hormone esterase [JHE], eclosion hormone [EH]) or enzymes such as proteases [34]. The most successful example to date of deleting a viral gene to enhance baculovirus pathogenecity has been provided in the approach described by O'Reilly and Miller [39]. The authors of this study have shown that larvae infected with a mutant baculovirus, in which the ecdysteroid UDP-glucosyltransferase (*egt*) had been deleted, displayed considerably reduced feeding behaviour and earlier mortality than larvae infected with wild-type baculovirus [39,40]. Such genetically modified baculoviruses have been shown to significantly improve insecticidal effect, and trials have demonstrated that they can compete with fast-acting chemical insecticides under field conditions in terms of preventing economic loss [34,35,41,42]. Such field trials have been conducted in the USA, the UK and China [28]; however, for unknown reasons, no genetically modified baculovirus-based insecticides have so far been registered for commercial use in these countries.

Recombinant viruses able to sensitize mosquitoes to chemicals

As we will argue, the application of viral strategies for increasing the sensitivity of mosquitoes to chemicals is an

alternative and promising method that could decrease the delivery load of chemical pesticides.

It is well known from the literature [43,44] that important targets for the most commonly used insecticides include voltage-dependant sodium channels (which have fundamental roles in the electrical activity of excitable cells), acetylcholine receptors and GABA receptors (which are crucial elements of excitatory and inhibitory synaptic transmission relays, respectively) and acetylcholinesterase (which is involved in the hydrolysis of acetylcholine within the synaptic cleft of the synaptic transmission). However, the significant levels of use of such neuro-insecticides over the past 20 years has led to the development of resistance in many insect species, particularly in those of medical importance (see Box 2). To overcome resistance, novel insect-control compounds that are active against resistant pest strains have been developed [45]. However, recent studies reported the emergence of strains that have become resistant also to these new compounds [46].

More recently, the intracellular regulation (i.e. phosphorylation process) of plasma membrane receptors and/or ion channels that are targeted by insecticides has been elucidated, raising hope for a new means to affect the sensitivity of insecticides [47]. A recent study reported, for the first time, evidence for posttranscriptional modifications, such as RNA editing, that resulted in the expression of modified insect GABA receptors, which led to a decreased sensitivity of *Drosophila melanogaster* to the insecticide fipronil [48]. Furthermore, an initial search for patterns of conserved amino acid residues that have been associated with phosphorylation sites in voltage-dependent sodium channel α subunits and in nicotinic acetylcholine receptor subunits showed that they possess potential phosphorylation sites for protein kinase A (PKA), PKC and PKG and for calcium/calmodulin-dependent serine/threonine-protein kinase (CaM-kinase). This is important because it has been clearly demonstrated that in insect neurons, elevation of the intracellular cAMP concentration through a calcium-CaM-sensitive adenylate cyclase modulates the opening of the nicotinic acetylcholine receptor by activating PKA, which phosphorylates the molecule and maintains the nicotinic acetylcholine

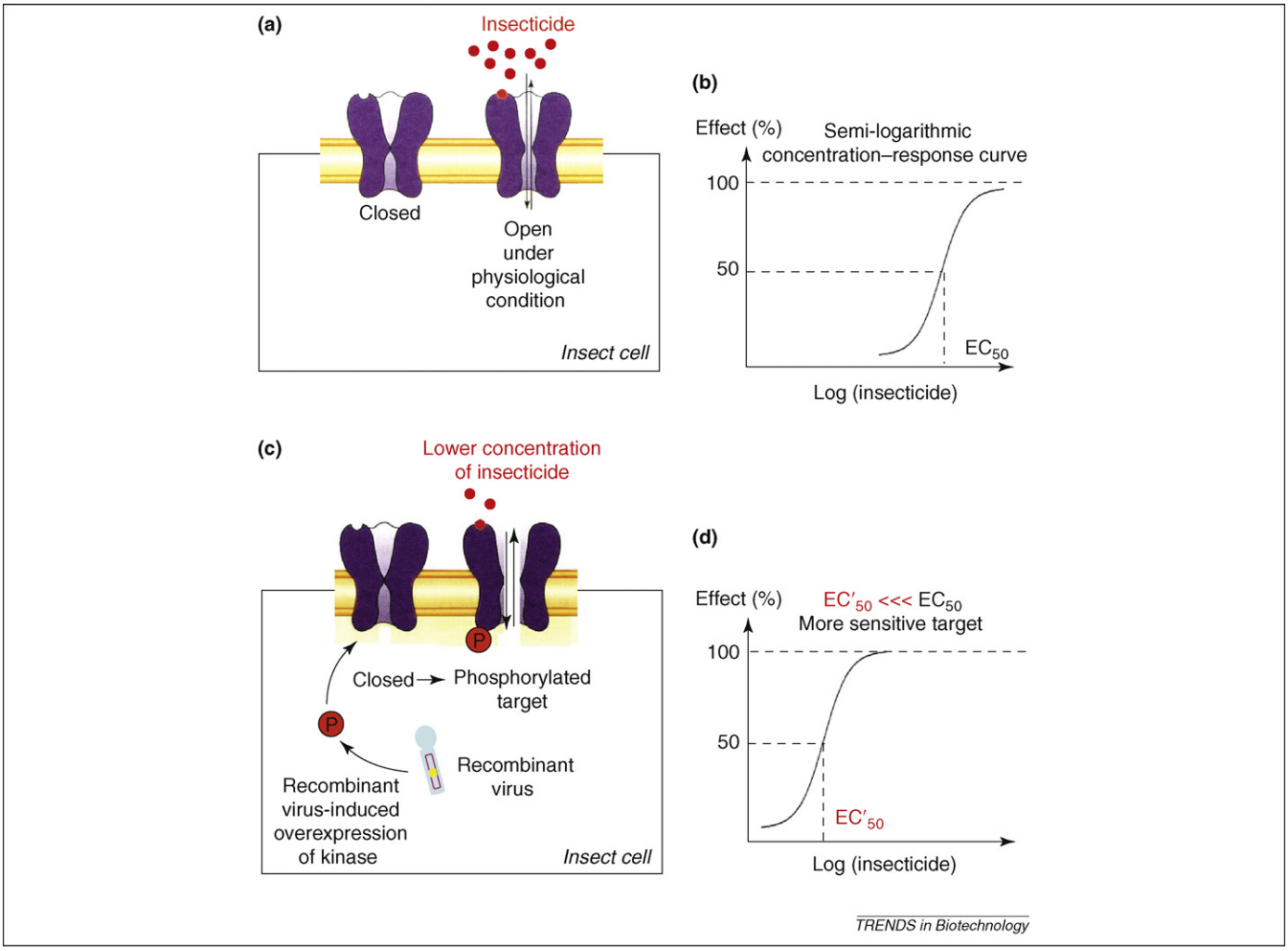

Figure 1. Hypothetic effect of a recombinant virus on increasing insecticide sensitivity by modifying an insect membrane target. **(a)** Membrane ion channel and/or receptor are targeted by an insecticide, which can either activate or inhibit the receptor or enter through an already open ion channel. **(b)** This panel demonstrates the quantitative effect of a given insecticide on the membrane target. Plotted are the different concentrations of insecticide against the logarithm of the non-cumulative concentration of insecticide. The EC_{50}, that is, the effective concentration of an insecticide, which produces 50% of its maximal effect, is calculated from the corresponding sigmoid curve. **(c)** An insect cell is infected with a recombinant virus, which leads to overexpression of, for instance, a specific kinase that is involved in the phosphorylation process, which will induce a conformational change. This renders the membrane target more sensitive to a given insecticide. **(d)** A corresponding semi-logarithmic concentration–response curve will yield a lower EC_{50}, which results from a shift of the sigmoid curve towards lower concentrations than those in an uninfected insect cell (b).

receptors in a functional form. Phosphorylation is counteracted by a dephosphorylation mechanism, which renders this type of receptor non-functional [49]. Because the effects of neonicotinoid insecticides, which are known to specifically bind on nicotinic acetylcholine receptors, depend on receptor openings, the phosphorylation process that modulates nicotinic acetylcholine receptor openings thus affects the mode of action and efficiency of neonicotinoid insecticides [49]. Similarly, an increase in intracellular Ca^{2+} concentration results in the formation of the calcium–CaM complex that activates CaM-kinase. Expression of this kinase affects the inactivation properties of the insect voltage-dependent sodium channels, which therefore facilitates inhibitory effects by the insecticide oxadiazine. Under this condition, it has been reported that the sensitivity of sodium channels to oxadiazine insecticides was increased by ~1000-fold (C. Lavialle-Defaix, PhD Thesis No. 701, University of Angers, 2005).

From these results it is apparent that manipulating intracellular regulatory mechanisms of insecticide targets can have fundamental consequences for the sensitivity of insects towards insecticides. This opens up a new exciting research area that could lead to significant improvements in the efficiency of certain insecticides. Based on the above findings, overexpression of phosphatases and/or kinases in the insect with the help of recombinant viruses might be able to increase the sensitivity of mosquito targets to insecticides (Figure 1).

To reach this objective, a possible strategy might include the following different steps. The first step consists of the construction and production *in vitro* of genetically modified viruses that will express a certain kinase and/or phosphatase after infecting insect cells and thus might be able to modify the insecticide target. To achieve this goal, a rapid and efficient method will be needed to produce recombinant viruses, for example by site-specific transposition in *Escherichia coli* (for details, see Figure 2) [50]. The second step will include infection of mosquito cells (e.g. neuronal cells) with these recombinant viruses to overexpress in these model cells the kinase or phosphatase that is involved in the intracellular regulatory pathway and that is known to affect the insecticide-sensitive target protein. The infection with genetically modified viruses combined with an insecticide should lead to higher susceptibility of the insect cells and might allow the use of a lower insecticide concentration. In the third step, and based on the results obtained in the previous steps, whole insects (i.e. larvae and adult mosquitoes) will be exposed to the geneti-

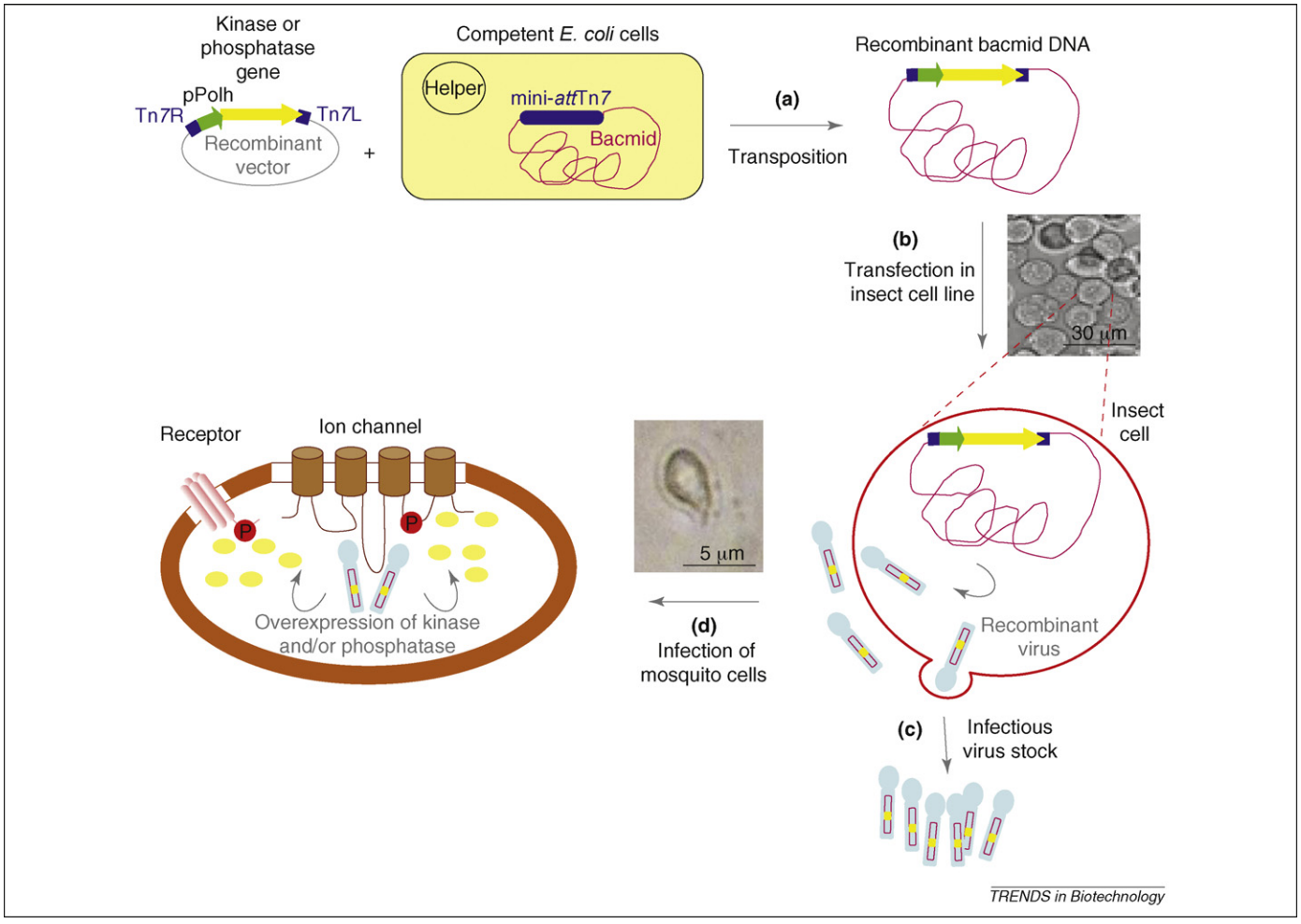

Figure 2. *In vitro* construction of recombinant genetically modified viruses encoding a kinase and/or phosphatase to modify the insecticide target. **(a)** Recombinant bacmids are constructed in *Escherichia coli* by transposing a mini-Tn7 from a recombinant vector containing the gene of interest (inserted downstream from the promoter for polyhedrin) to the mini-*att*Tn7 attachment site on the bacmid. Tn7 transposition functions are provided in *trans* by a helper plasmid. **(b)** Purified bacmid DNA is transfected into insect cells. **(c)** Recombinant viruses are obtained and amplified to produce a virus stock. **(d)** Mosquito cells (e.g. neurons) are infected with genetically modified baculoviruses, which leads to overexpression of a kinase and/or phosphatase, which can subsequently modify insecticide targets, such as receptors or ion channels.

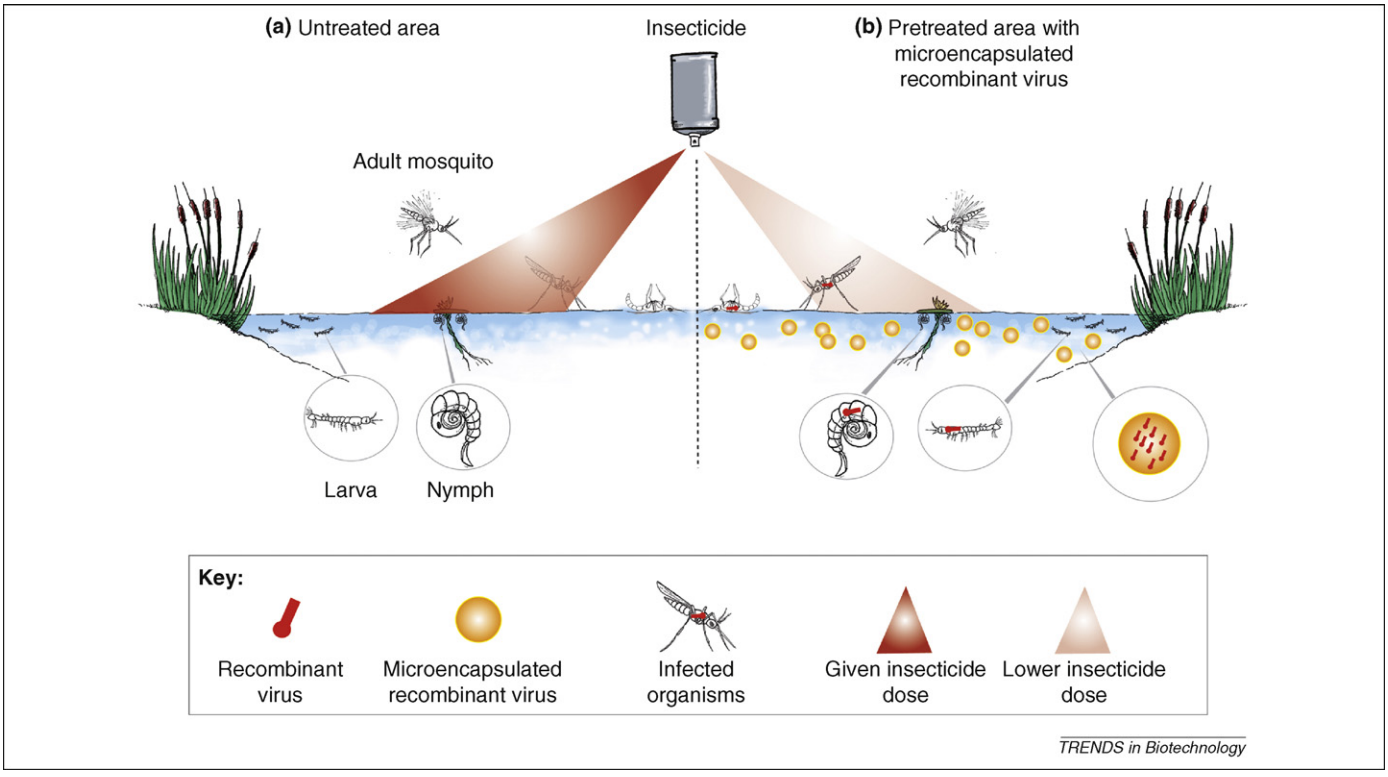

Figure 3. Innovative vector control strategy using microencapsulated recombinant viruses. **(a)** In an untreated area, a given insecticide dose is applied to kill larvae, nymphae or adult mosquitoes. **(b)** In areas that have been pretreated with microencapsulated recombinant virus, overexpression of a particular kinase and/or phophatase in the insects is induced, which will increase the sensitivity of the insecticide target. Therefore, a lower dose of insecticide will be sufficient to kill insects that have been infected with recombinant viruses.

cally modified viruses, and the effects of overexpressing a particular kinase and/or phosphatase in the insect will be evaluated using classical *in vivo* toxicological studies (e.g. LD_{50}) to establish increased sensitivity towards insecticides.

Outstanding challenges and perspectives

Innovative, safe and cost-effective measures are urgently needed to ensure effective management of arthropod vectors and thus of the diseases they transmit. Important steps towards this goal are to delay the selection of resistance genes that shorten the period in which conventional pesticides are useful. Arthropod-borne diseases are expanding rapidly in several continents and are also spreading into more temperate regions (e.g. Southern Europe) where they did not exist before.

Current knowledge of regulatory intracellular pathways in insects shows that phosphorylation and dephosphorylation events can strongly affect the sensitivity of insect ion channels and receptors that are often targeted by commonly used insecticides. These new data allow us to consider the development of novel strategies for controlling disease-bearing vectors and particularly mosquitoes. Recombinant viruses that are able to induce overexpression of kinases and/or phosphatases in the insect might provide an attractive tool for controlling mosquito-borne diseases.

A first requirement for the production of virus as bioinsecticides is their host specificity. One product, 'Viroden', which contains an *Aedes* densovirus, has the potential of becoming commercially used. It has been evaluated as a microbial pesticide in Ukraine [51] but, to date, it is not yet available on the market. Several wild-type viruses that are infectious against mosquitoes are described by Becnel and White [52], and the new understanding of virus–mosquito interactions can be used to develop novel control strategies. As already indicated above, the mosquito baculovirus CuniNPV represented an attractive tool for mosquito control, but its transmission has only been successful to mosquitoes within the genus *Culex*. However, the identification and use of *Aedes*-specific baculoviruses will counteract this problem in the control of mosquito-borne diseases.

Another important consideration is that viral preparations should be stable in storage and resistant to unfavourable environmental factors (e.g. water quality, UV radiation, dilution in water, rain and heavy dew). Based on these limitations, the microencapsulation of virus particles represents an effective alternative for virus preparations for the following reasons. Microencapsulation will avoid loss of virus activity by protecting them from these unfavourable conditions and an appropriate formulation will allow the virus to remain in the mosquito feeding zone. Furthermore, compounds that promote a long and stable shelf life, resistance in water and UV-blocking, as well as those capable of attracting insects, could be incorporated in microencapsulated formulations [53,54]. In addition, microencapsulation can also allow a rapid delivery of acute doses of viruses, which will allow the optimization of virus infectivity. Finally, because the efficient infection of mosquito requires magnesium, an appropriate virus formulation will therefore

need to contain such divalent cations within their OBs [55,56].

In the near future, genetically modified viruses, possibly in microencapsulated form, could be spread in aquatic breeding sites (Figure 3), where they would infect larvae and adult mosquitoes after ingestion of virus-containing water. Expression of recombinant proteins, such as kinases and/or phosphatases in the insect might increase the sensitivity of infected mosquitoes to conventional pesticides applied in the same area. Overall, this strategy would result in a requirement for lower doses of chemical insecticides and hence reduce the adverse environmental, human health and ecological effects of these compounds.

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