CAN WE EXPECT NEW HERBICIDES WITH NOVEL MODES OF ACTION IN THE FORESEEABLE FUTURE?

Ken Pallett from Chelmsford, Essex, UK looks at the future prospects for new herbicide mode of action reflecting on 40 years of personal experience within herbicide research and development in both academia and industry explores the problems associated with new herbicide discovery

Ken Pallett

Introduction

The need for new products with novel mechanisms of action is the case for all crop protection chemicals particularly so for herbicides, where there has been no major herbicide with a novel mode of action commercialised in the last two decades. The reasons for this are a result of three overall factors, which are largely economic (Duke, 2012).

Firstly, the major economic influence has been adoption of herbicide tolerant (HT) GM crops, notably maize and soybean, two crops which previously had been priority targets for new selective herbicide discovery within the agrochemical industry. The introduction of glyphosate tolerance in these crops introduced relatively low cost and effective weed control, albeit part of the cost for the herbicide was switched to a technology fee for the seed. The expiration of the patent for glyphosate also reduced on the cost of weed control with this herbicide as it became generic. Evidence for the loss of attractiveness for new herbicide discovery by the industry is seen by the significant decreases in new herbicide patents following the introduction of HT crops in the mid-1990s (Gerwick, 2010, Duke, 2012).

The second factor is the consolidation of the companies involved in crop protection chemical discovery. During the heyday of new herbicide discovery in the 1980s, when the majority of today's herbicides were discovered there were over 20 separate agrochemical companies. Following a series of takeovers and mergers there were only the 'Big Six' multinational R&D-based companies remaining by the turn of the century (Copping, 2003). Herbicide discovery, as for insecticides and fungicides is based on empirical screening of diverse molecules. This coupled to different philosophical approaches from those engaged in new discovery in the 1980s, lead to the vast majority of herbicides that we have available for weed control today. Consolidation of companies conducting research since then, reduced the numbers of scientists involved and their approaches to new molecule discovery and as a result output of new molecules declined as seen by a significant reduction of new patents in the last decade (Ruegg *et al.*, 2007; Gerwick, 2010; Duke, 2012).

A third factor is the cost of bringing a new crop protection molecule into the market, also a reason behind the industry consolidation. Development costs to bring a new molecule have increased. By 1995, costs were estimated at $250 million approximately 3 times that of the decade earlier (Ruegg *et al.*, 2007). A breakdown of these costs shows all 4 major components: synthesis & screening; formulation, product analysis & process development; field trials & official testing; and regulatory studies (toxicology, eco-toxicology, environmental impact & metabolism) have all increased, those for the latter have increased more rapidly with 40% of the total costs in 1990–1995 were for regulation, whilst being 20–25% of the costs a decade earlier.

This explains how we have the downturn in new herbicides and I would now like to focus on how and when we can expect any new herbicides with novel modes of action. There have been several reviews in recent years highlighting the need for new herbicides with novel modes of action to meet resistance and legislative challenges and how this can been achieved (Cole *et al.,* 2000; Duke, 2012; Kraehmer, 2012)

This article builds on these, introducing personal experience over the last 40 years in herbicide R&D by providing some further historical perspective on herbicide mode of action discovery, on the status of existing herbicide targets and discusses the prospects and challenges to find them.

Historical perspective on mode of action discovery

All successful commercial herbicides developed to date have been identified from *in vivo* glasshouse screening, where the chemical was sprayed onto the plant or on soil in which seeds were sown. Their mode of action or target site was either identified afterwards, once the herbicidal potential of the molecule(s) was established or was assumed because the molecule had structural analogy to other herbicides, whose target site was already known. Given that there are now only very few industrial research groups conducting new herbicide discovery the prospects for new molecules seems bleak.

However, in the 1990s there was a switch from traditional glasshouse-based screening associated with conventional chemistry to high-throughput screening (HTS) with miniaturised biological and *in vitro* screens associated with combinatorial chemistry (Tietjen *et al.*, 2005). This enabled a far greater throughput of molecules in the screens from 1000s per year to tens or hundreds of 1000s and therefore, increased the chances of finding new leads (Berg *et al.*, 1999, Kraehmer, 2012). The reduction of new patents and the absence of new modes of action indicate that HTS was not commercially successful in delivering new herbicidal products. However, other factors have contributed to the lack of success such as the costs of bringing new products to the market, particularly those necessary for commercial product registration (Ruegg *et al.*, 2007).

There are around 20 targets for commercialised herbicides in plants, the last of which to be identified was in 1991 (Schulz *et al.*, 1991; Pallett, 1997; Duke, 2012). This is *p*-hydroxyphenyl-pyruvate dioxygenase (HPPD), the molecular target for the triketones (sulcotrione and mesotrione) and the benzoyl-isoxazoles (isoxaflutole) commercialized in the mid-1990s (Schulz *et al.*, 1991, Prisbylla, 1993; Pallett *et al.*, 1998). Two other herbicides, pyrazolinate and pyrazoxfen commercialised in the early 1980s for weed control in rice were also confirmed as HPPD inhibitors (Pallett, 2000).

This illustrates the historic status of herbicide mode of action know-how, prior to the onset of widespread herbicide resistance in the late 1980s and 1990s. Prior to this a detailed knowledge of mode of action was not a necessity in the discovery and development of new herbicides; however that situation no longer exists due to the need to classify herbicides based on their mode of action to assess the risk of resistance development. Furthermore, definition of mode of action also assists the registration process, particularly if the target site is plant-specific.

In an analysis of the mode of action of commercial herbicides and those in the development pipeline, based of patents and launch literature in 1997, over 50% of the commercial herbicides inhibit three molecular targets, namely photosystem II, acetolactate synthase and protoporphyrinogen oxidase, a situation that still exists in 2016 (Pallett, 1997). They are susceptible to a diverse range of chemistries able to interact with the target site at the molecular level (Table 1).

The status of existing herbicide target sites

Already in this article, I have used the terms target site and mode of action in relation to herbicide activity. The former is the specific molecular binding site of the herbicide molecule in the plant, whereas the latter takes this into account but also covers the processes which ultimately lead to the overall herbicidal activity. Table 1, illustrates the target sites of the major herbicides classes currently in commercial use. The primary and secondary effect(s) of target site interference are also listed which explains the overall mode of action, specifically the reasons for herbicide activity or catastrophic effect (Cole *et al.,* 2000).

Table 1. Examples of the major targets and the reason for overall herbicidal activity of major herbicides

Herbicide or herbicide class	Target site	Primary effect	Secondary effect or reason for toxicity (Catastrophic effect)
Glyphosate	Enoyl pyruvyl shikimate phosphate synthetase (EPSPS)	Inhibition of aromatic amino acid biosynthesis	Shikimate pathway deregulation accumulation of shikimate and shikimate-3-phosphate and depletion of phosphate Adverse impact of PEP accumulation (Cole *et al.*, 2000; de Maria *et al.*, 2006)
Glufosinate	Glutamine synthetase (GS)	Inhibition of the glutamine biosynthesis from glutamate.	Inhibition of photosynthesis due to accumulation of glyoxylate from the photorespiration pathway. (Wild & Wendler, 1993; Hess, 2000)
Sulfonylureas, imidazolinones, triazolopyrimidines and others	Acetolactate synthase (ALS)	Inhibition of branched chain amino acid biosynthesis	Accumulation of -ketobutyrate and its transamination product; disruption of photosynthesis transport and respiration (Zhou *et al.*, 2007)
Diflufenican, fluridone, fluorochloridone and others	Phytoene desaturase (PDS)	Inhibition of carotenoid biosynthesis	Absence of chloroplast development due to lack of pigments (bleached tissues) (Bramley & Pallett, 1993)
Ureas, triazines, nitriles, triazinones and others	D-1 protein of photosystem 2 (PSII)	Inhibition of photosystem 2 electron transport	Light-induced photo-oxidative damage due to singlet oxygen and free-radical production. (Pallett & Dodge, 1980; Hess, 2000; Rutherford & Liszkay (2001)
Diphenylethers, phenylpyrazoles and others	Protoporphyrinogen oxidase (PPO)	Inhibition of porphyrin biosynthesis	Accumulation of the photoreactive intermediate, protoporphyrin IX. (Dayan & Duke, 2010; Duke, 2012)
Aryloxyphenoxypropionates, cyclohexanediones and others	Acetyl CoA carboxylase (ACCase)	Inhibition of the biosynthesis of long-chain fatty acid components of membranes	Absence of membrane development due to inhibition of membrane lipids.
Triketones, benzoyl isoxazoles, benzoyl pyrazoles	Hydroxyphenyl pyruvate dioxygenase (HPPD)	Inhibition of homogentisic acid (HGA) biosynthesis	Prevention of chloroplast development due to reduced electron transport components (plastoquinone) and anti-oxidative systems and (tocopherols) plus an accumulation of tyrosine, likely to be toxic to plant cells. (Prisbylla, *et al.*, 1993; Pallett *et al.*, 1998)

There are two examples in Table 1 where the inhibition of the molecular target or primary effect directly leads to the toxicity and plant death; PDS and ACCase. For the other herbicides or herbicide classes, the primary interaction with the target site inhibition triggers secondary toxic or catastrophic effects that contribute to the overall herbicidal activity (Cole *et al.*, 2000). For example, the absence of protein synthesis was originally identified as the mode of action for glyphosate, glufosinate and sulfonylureas due to their respective inhibition of EPSPS, GS or ALS and the prevention of essential amino acid biosynthesis. However, it is now clear that other consequences also contribute to phytotoxicity and lead to the herbicidal effectiveness of these primary targets (Table 1). Similarly, as a result of HPPD inhibition the depletion of HGA interferes with both anabolic and catabolic metabolic processes contributing to the overall toxicity of HPPD inhibiting herbicides. Similarly, there are secondary toxic effects associated with PSII and PPO inhibitors, which have significance to the lethality of these herbicides.

These secondary effects are not always predictable or obvious and in many cases were only identified some time after the discovery of the herbicide and its target. This was particularly the case prior to the mid-1980s, after which biochemists were introduced into the Research Teams involved in herbicide discovery. Knowledge of plant metabolism increased and this, coupled to the introduction of molecular and some of the 'omic' technologies provided the tools to understand the consequences of disruption of a specific enzyme or metabolic process within a plant cell better and gain the full picture of plant susceptibility to herbicides.

The challenges of identifying new herbicide target sites

The onset of HTS in the 1990s introduced *in vitro* screening on isolated enzymes supported by molecular biology to produce recombinant proteins for the screens (Berg *et al.*, 1999; Cole *et al.*, 2000; Tietjen *et al.*, 2005). The selection of target site was a major challenge. Table 2, summarises the properties of effective herbicide targets site, some or all of which are necessary to lead to an effective herbicide.

It has been estimated that there are between 1000–2000 genes in *Arabidopsis thaliana* critical for plant growth, meaning that traditional herbicide discovery approaches has picked out 1–2% of potential herbicide targets (Cole & Rodgers, 2000). Today, genomic tools can help in new target site identity (Cole *et al.*, 2000; Duke, 2012), however some caution is necessary.

Genomic methods such as gene knock-out, antisense and RNAi methods have been used to determine if a target is essential for plant growth, however complete removal of gene expression with knock-out methods does not necessarily mimic the impact of chemical inhibition. As stated in Table 2, one of the desirable features of a good herbicide target site is that only a small proportion of the target site is inhibited. Antisense and RNAi methods can partially prevent expression or activity of putative molecular targets so this does offer potential (Cole *et al.*, 2000) and has lead to a number of potential new targets (Duke, 2012). However, they do not indicate if

Table 2. Some desirable features of an effective herbicide target (taken from Pallett, 1997; Cole *et al.*, 2001; and Duke, 2012).

Preferably plant specific to minimise toxicological issues
Critical for plant survival
No alternative isozyme of pathway to circumvent the inhibition
Irreversible inhibition
Low metabolic turnover
Inhibition of a relatively small proportion of the molecular target necessary to induce irreversible toxicity
Low abundance in the plant
Target inhibition is able to induce 'catastrophic' toxic effects on plant metabolism
Properties necessary for target binding also lead to optimum bioavailability*

*bioavailability covers the uptake or penetration of the herbicide into the plant, translocation or systemicity and metabolic persistence or degradation in the plant

the target is susceptible to small molecule interference or inhibition. Only screening of the target *in vitro* will confirm this. HTS methods, permit the screening of such targets with tens-hundreds of thousands of molecules, however even if potent inhibitors are identified, they may not have *in vivo* herbicidal activity due to limited bioavailability.

For many years, natural phytotoxins that reduce or prevent plant growth have been a focus for potential new herbicide targets. This is because of their obvious benefit of being identified as phytotoxic at the whole plant level. Investigations into their mode of action to identify new targets; the identity of the individual molecules leading to the phytotoxicity as well as chemical synthesis around the inhibitors, has been a focus for new herbicide discovery. (Duke *et al.*, 2000; Dayan *et al.*, 2012; Duke & Dayan, 2011). However, to date this has not led to any significant new targets or new herbicidal classes.

Selection of alternative enzymes in the same metabolic pathway as those of proven herbicides has been a further approach to identify new target sites. ALS is arguably the most effective target sites for herbicides due to its susceptibility to a number of chemical classes which possess excellent herbicidal activity at very low dose rates in the field (Wittenbach & Abell, 1999). Hoe 704 (2-dimethylphosphinoyl-2-hydroxyacetic acid) and IpOHA (*N*-hydroxy *N*-isopropyloxamate) are inhibitors of keto-acid reductoisomerase (KARI) the preceding enzyme in the pathway, but neither has been developed as a commercial herbicide (Schulz *et al.*, 1988; Dumas *et al.*, 1994). It was proposed that the low herbicidal potency of the two compounds appeared to be correlated with their slow rates of association with KARI *in vivo*, although it may also be that KARI inhibition may not lead to the accumulation of a toxin such as α-ketobutyrate (Table 1; Dumas *et al.*, 1994; Zhou *et al.*, 2007; Duke, 2012).

A further reason for the lesser herbicidal potential of the two KARI inhibitors could be associated with their bioavailability in the plant, i.e. less effective uptake or penetration into the plant, reduced translocation or systemicity and/or lower

metabolic persistence or degradation in the plant, compared to the commercialised ALS inhibitors.

This bioavailability 'black box' adds to the complexity of using *in vitro* methods to identify new herbicides. The physicochemical properties of herbicides for optimum uptake and translocation are well known and have been described for many years and these differ with foliage and soil applied herbicides (see Cobb & Kirkwood, 2000). However, the physicochemical properties of the active inhibitor are specific for binding into the inhibitor binding domain of the target and these may not be optimal for 'ideal' bioavailability in the plant, which differ depending on the type of herbicidal application and use.

Penetration through the lipophilic cuticle can be influenced by the formulation of the applied herbicide. Addition of surfactants can optimise foliar penetration and enhance foliar persistence and in cases where the active inhibitor is an acid, it can be applied as an ester to improve uptake into the cuticle followed by hydrolysis to the acid upon uptake. For soil applied herbicides the situation is more complex, in that the molecule needs to persist in the soil to provide residual weed control. Most residual herbicides tend to be lipophilic as more water soluble molecules tend to more susceptible to leaching and in addition to reducing herbicide activity could also lead to greater environmental issues such as ground and surface water contamination.

PDS is an example of a target site where its membrane-bound location in the chloroplast directly influences the bioavailability of the herbicidal molecule. PDS inhibitors tend to be highly lipophilic molecules in order to access the inhibitor binding domain of the enzyme (Bramley & Pallett, 1993). Although this is an excellent herbicide target, due to its plant-specific nature, only relatively few successful products exist, all of which are primarily soil acting residual herbicides and have very limited mobility from foliar application.

Selectivity of all but two major herbicides classes to date is linked to bioavailability. ACCase and auxin mimics (F-box protein) differ between grass or monocotyledonous and broad-leaved or dicotyledonous species leading to the selective uses of the former for grass weed control in dicotyledonous crops and the latter for post-emergence broad-leaved weed control in monocotyledonous crops. Crop insensitivity for other major selective herbicide classes is linked to differences in uptake, often morphological and particularly metabolic degradation mediated via cytochrome P450s and glutathione-S-transferases inactivation and compartmentalization in the insensitive crops.

To add further complexity, inhibition of the target site itself can influence bioavailability, e.g. PPO inhibitors lead to very rapid photoxidative damage of leaves in light which reduces, if not prevents, systemicity of the herbicide, even if the physicochemical properties of the inhibitor would mean that it would have phloem mobility. A similar situation exists for PSII inhibiting herbicides where rapid inhibition of photosynthesis reduces systemic activity following foliar application (Cobb & Kirkwood, 2000).

However, this is not the case for all herbicides. Even though glyphosate targets EPSPS, it does lead to relatively rapid Inhibition of photosynthesis following application (Barbagallo *et al.*, 2003) However, this presumably is sufficiently slow not to negatively impact phloem mobility and the systemicity of glyphosate.

Conclusions and prospects

The need for new herbicide modes of action remains today in order to cope with the continuing development of weed resistance. The challenge is not only to identify new targets but to identify inhibitors that can become commercial herbicides. The introduction of 'omics' will help in the identity of potential new target sites and will permit the production of the target sites for the screening of large numbers of molecules as potential inhibitors. One can hope that if sufficient numbers of diverse molecules are screened, new inhibitors will be found but the translation of potent *in vitro* target site interference to *in vivo* phytotoxicity will be the major challenge.

All successful commercial herbicides are a compromise between target site potency, induced secondary toxic effects with effective bioavailability in the target weeds and, for most selective herbicides, taking advantage of metabolic deactivation in crops. Understanding this compromise not only at the molecular, but also at the physiological level, will help move forward in the translation of *in vitro* activity. Furthermore, understanding the reasons why good inhibitors that do not translate to high *in vivo* activity can provide useful information. In the past, good *in vitro* inhibitors that do not translate, are simply ignored or forgotten, but I would argue that understanding why they are not good herbicides can improve the understanding bioavailability.

There is a considerable of literature on target site delivery; including plant uptake and systemicity (see Cobb & Kirkwood, 2000) and this should not be forgotten or ignored because it is 'old research'. The physicochemical properties necessary for phloem and xylem translocation whilst generated from traditional plant physiology studies are still valid today and need to be integrated into new herbicide discovery processes.

As stated previously, all current modes of action have been identified from molecules active in empirical whole plant screens. *In vitro* screening has become part of the HTS methodology today for new herbicide screening which will find good *in vitro* active molecules. However, miniaturised whole plant screening is also possible as part of the HTS toolbox, and this must be included as part of the approach to new molecule discovery coupled to use of 'omics' to identify the target sites.

A further challenge lies in the discovery of selective herbicides. Molecular biology can help by providing recombinant cytochrome P450's and glutathione-s-transferases responsible in target crops for the metabolic degradation of herbicides in crops as tools to understand selectivity mechanisms and possibly design potential selective molecules.

If highly effective non-selective herbicides are discovered then the option of engineering tolerance exists but given the current acceptance of such crops, particularly in Europe, the time for such herbicide to be registered and for the GM crop(s) to be commercialised, would be many years in the future.

To conclude, we should be optimistic that there will be novel herbicides in the future with new modes of action, discovered via an integrated approach of new biological

and 'omic' methods coupled with more traditional biology, biochemistry in close association with empirical screening – the question is when?

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