ARTICLES

A new class of anthelmintics effective against drug-resistant nematodes

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Anthelmintic resistance in human and animal pathogenic helminths has been spreading in prevalence and severity to a point where multidrug resistance against the three major classes of anthelmintics—the benzimidazoles, imidazothiazoles and macrocyclic lactones—has become a global phenomenon in gastrointestinal nematodes of farm animals. Hence, there is an urgent need for an anthelmintic with a new mode of action. Here we report the discovery of the amino-acetonitrile derivatives (AADs) as a new chemical class of synthetic anthelmintics and describe the development of drug candidates that are efficacious against various species of livestock-pathogenic nematodes. These drug candidates seem to have a novel mode of action involving a unique, nematode-specific clade of acetylcholine receptor subunits. The AADs are well tolerated and of low toxicity to mammals, and overcome existing resistances to the currently available anthelmintics.

The nematodes, or roundworms, comprise a large number of pathogens of man and domestic animals. Gastrointestinal nematodes, such as the blood-sucking *Haemonchus contortus*, are major parasites of ruminants that cause substantial economic losses to livestock production worldwide. In the absence of vaccines for gastrointestinal nematodes, control of infections relies mainly on chemotherapy. Anthelmintic chemotherapy is limited to three major chemical classes, and, inevitably, drug resistance has emerged in human[1–4] and animal[5,6] pathogenic helminths against each class. The appearance of multidrug-resistant nematodes that withstand all available classes of anthelmintics[7,8] further underscores the need for new drugs[9]. No new anthelmintic class has reached the market during the past 25 yr with the exception of the cyclodepsipeptides represented by emodepside, which is indicated for use in cats but not in livestock. We have pursued extensive *ex vivo* screening followed by lead optimization in rodent models and evaluation in farm animals, and identified AADs as resistance-breaking drug development candidates. Their mode of action was investigated using drug-resistant mutants of *Caenorhabditis elegans* and *H. contortus*.

Synthesis and anthelmintic potential of AADs

The AADs are a class of low molecular mass compounds that are easily accessible by alkylation of phenols with chloroacetone, Strecker reaction and acylation of the amine with aroyl chlorides (Fig. 1a). Over 600 compounds bearing different aryloxy and aroyl moieties on an amino-acetonitrile core have been synthesized and evaluated for anthelmintic activity. Some exhibited high activity against *H. contortus* and *Trichostrongylus colubriformis*, with EC_{100} (the minimum effective concentration that eliminates 100% of nematodes) values for the best molecules in the range of 0.01–0.032 parts per million (p.p.m.) in an *in vitro* larval development assay (LDA). After chiral resolution (Fig. 1b) a significant difference in the potency of each enantiomer was observed (see Supplementary Information). Promising AADs were validated in a rodent model using *Meriones unguiculatus* infected with *H. contortus* and *T. colubriformis*. Although the original lead compound, AAD 450, was fully active against *H. contortus* at 10 mg racemate kg^{-1} (not shown; where racemate is an equimolar mixture of a pair of enantiomers) but not at 3.2 mg racemate kg^{-1} after oral application, the compounds obtained at the end of the optimization process (AADs 1336 and 1470; Fig. 2) cured infected rodents at 3.2 mg racemate kg^{-1} (Fig. 2). These compounds were also effective against both species when applied subcutaneously, indicating systemic anthelmintic activity (Fig. 2). At doses up to 32 mg racemate kg^{-1} the AADs 1336 and 1470 did not show insecticidal (*Ctenocephalides felis*) nor acaricidal (*Rhipicephalus sanguineus*) activity (data not shown).

Efficacy of the AADs in ruminants

When tested in ruminants, all AADs were able to eliminate fourth larval (L4) stages of *H. contortus* in sheep and *Cooperia oncophora* in cattle at a single oral dose of 20 mg racemate kg^{-1} (corresponding to 10 mg active enantiomer per kg). In addition, the optimized AADs 1336 and 1470 were able to eliminate L4 stages of *T. colubriformis* in sheep and *Ostertagia ostertagi* in cattle (Fig. 2). Compound AAD 1470 administered orally at a dose of 10 mg racemate kg^{-1} to sheep ($n = 6$), infected simultaneously with L4 stages of five major nematode species, was 100% effective based on faecal nematode egg counts (EPG, eggs per gram faeces) and 90–100% effective based on nematode counts for *Nematodirus spathiger*, *H. contortus*, *Teladorsagia circumcincta* and *T. colubriformis*. Furthermore, compound 1470 was more than 98% effective at a dose of 10 mg racemate kg^{-1} against adult stages of *H. contortus*, *Trichostrongylus axei*, *T. circumcincta*, *T. colubriformis*, *Cooperia curticei*, *N. spathiger* and *Chabertia ovina* in sheep.

The anthelmintic efficacy of AAD 1470 was also tested in naturally parasitized sheep. Ten diseased sheep were used in a trial in the Ivory Coast with the study inclusion criteria of EPG above 500 (to ensure a high infection with gastrointestinal nematodes) plus the presence of *Moniezia expansa* (sheep tapeworm) proglottids. Eight sheep were treated orally at 40 mg racemate kg^{-1} body weight. Two untreated control sheep were necropsied and found to be positive for

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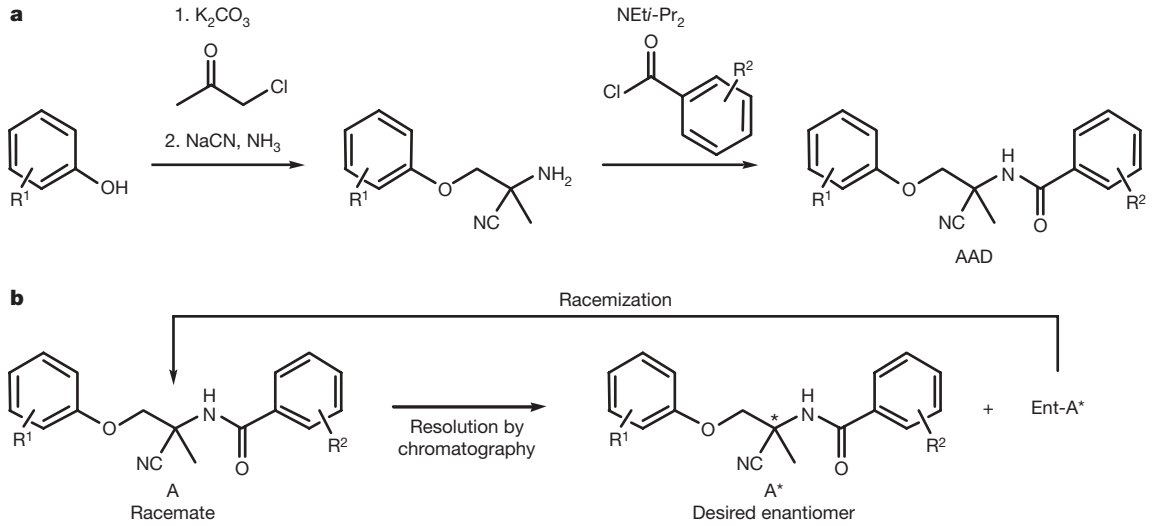

Figure 1 | Amino-acetonitrile derivatives (AADs). a, Chemical synthesis (for details see Supplementary Methods and Supplementary Fig. 1). R^1 and R^2 represent different possible chemical substituents on the phenyl rings, as detailed in figure 2. **b**, Enantiomerically pure AADs are obtained by chiral chromatography of the racemic mixtures followed by recycling of the undesired isomers via base-catalysed racemization. This route was used on a 100-g scale for AAD 1470.

H. contortus, *T. colubriformis*, *Trichostrongylus* sp., *Oesophagostomum columbianum* and *M. expansa*. AAD 1470 eliminated all gastrointestinal nematodes (on the basis of EPGs and nematode counts at necropsy), but was ineffective against *M. expansa* and coccidia (on the basis of faecal examination). The nematicidal efficacy of AAD 1470 was further demonstrated in cattle. When treated orally at 20 mg racemate kg^{-1} ($n = 3$) or topically at 40 mg racemate kg^{-1} ($n = 7$), all cattle were cured from infections with adult *O. ostertagi* and *C. oncophora*.

Pharmacokinetics and tolerability

Comparison of the pharmacokinetics in sheep showed that AADs 1336 and 1470 are eliminated more slowly than the other AADs (Fig. 3a). Unlike the situation with other molecules of the class, the pharmacokinetic disposition of AAD 1470 is not enantioselective in sheep. Therefore, the blood profiles displayed in Fig. 3b as well as the calculated pharmacokinetic parameters are valid for either enantiomer. The kinetic profile of AAD 1470 is characterized by a rather slow clearance of 0.11 l kg^{-1} h^{-1}, a very high volume of distribution ($V_{D\ area}$ of 33 l kg^{-1}; V_{ss} of 27 l kg^{-1}; where $V_{D\ area}$ is the apparent volume of distribution during the terminal phase and V_{ss} is the apparent volume of distribution at steady state), a rapid decline of the blood levels below 20 ng ml^{-1} within 2 days owing to distribution into peripheral compartments (mainly fat) followed by a slow elimination (terminal half life of 215 h), and a good oral bioavailability of 57%.

All AADs tested were well tolerated and of low toxicity to rodents and ruminants. The micronucleus test (*in vitro*, using L5178Y mouse lymphoma cells) and Ames test screens for the racemate of AAD 450

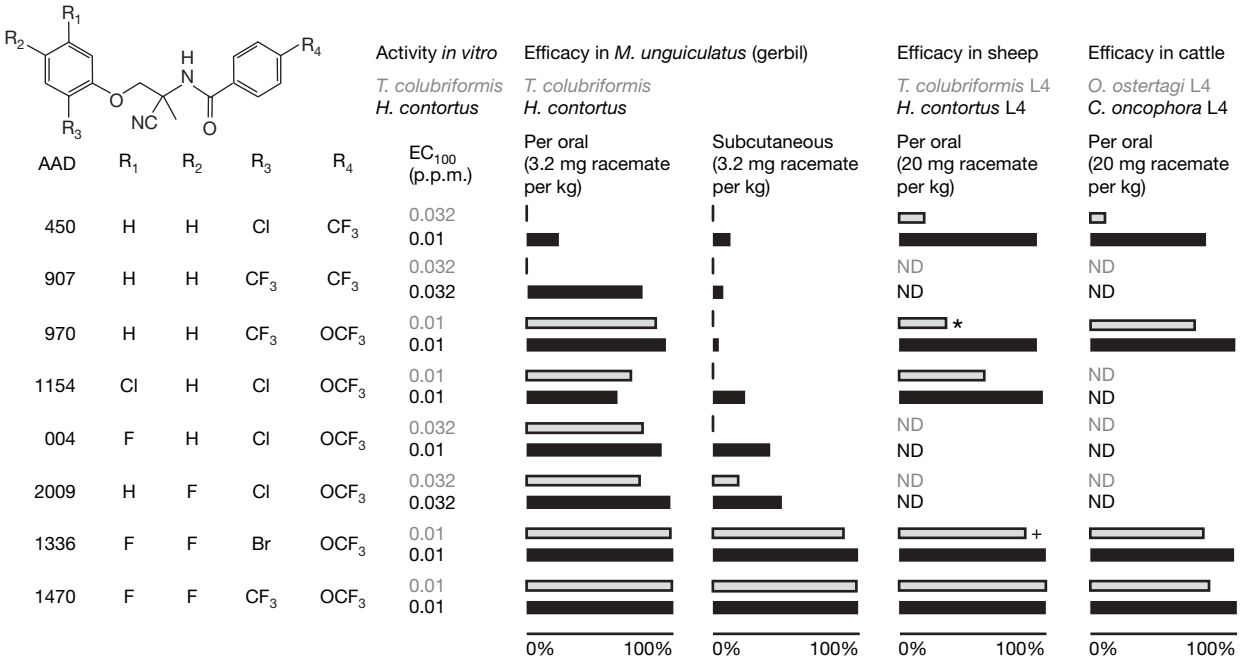

Figure 2 | Selected AADs, their structures and the corresponding anthelmintic efficacies *in vitro* and *in vivo*. Efficacy is given as percentage based on nematode count reduction. Asterisk, 40 mg racemate kg^{-1}; plus symbol, 10 mg racemate kg^{-1}. All compounds were fully effective in sheep against adult stages of *H. contortus* (not shown). ND, not determined.

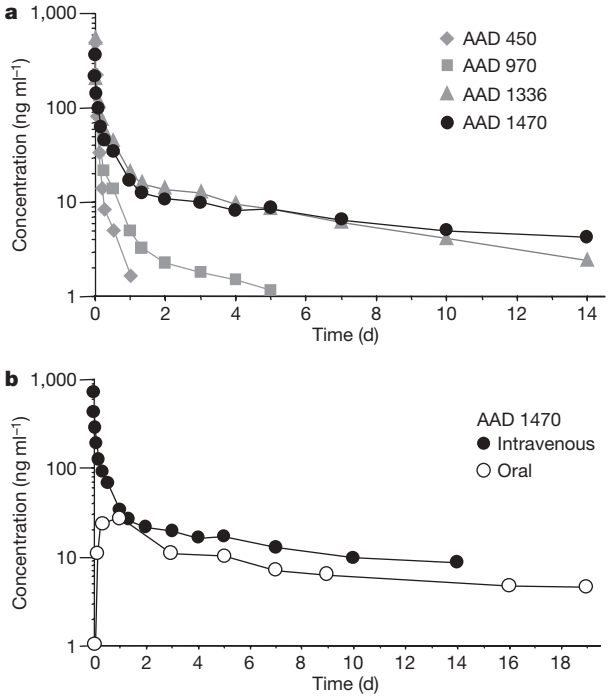

Figure 3 | Pharmacokinetics of AADs. a, Group mean blood profiles ($n = 3$) of AADs after intravenous administration as a cocktail at 0.5 mg racemate kg^{-1} in sheep. **b**, Group mean ($n = 2$) blood profiles of AAD 1470 after oral application at 20 mg racemate kg^{-1} and intravenous application at 0.5 mg racemate kg^{-1} in sheep (both profiles normalized to 1 mg kg^{-1}). For the chemical structures, see Fig. 2.

and for both enantiomers of AAD 1470 revealed no clastogenic, aneugenic, or mutagenic potentials (data not shown). The oral lethal doses in rats of these compounds were above 2 g kg^{-1}, indicating low acute toxicity to rodents. The AADs 450, 970, 1336 and 1470 were tested in sheep up to an oral dose of 200 mg kg^{-1} without any adverse clinical effects. Overall, the toxicity and tolerability studies showed that AADs are well tolerated and of low toxicity to mammals.

Activity against drug-resistant nematodes

AADs active against parasitic nematodes also exerted marked effects on the movement, growth and viability of the free-living nematode *C. elegans*, inducing a pleiotropic combination of phenotypes distinct from the effects of any single known anthelmintic. The AADs cause hypercontraction of the body wall muscles leading to paralysis, spasmodic contractions of the anterior portion of the pharynx and ultimately death (phenotypic effects are observed at AAD 1336 or AAD 1470 concentrations of ≥50–100 ng ml^{-1}, with full lethality occurring above 1 μg ml^{-1}). Similar effects were observed for *H. contortus* (adults). AAD-exposed *C. elegans* also exhibited moulting defects and growth-arrested nematodes frequently developed large vacuoles characteristic of necrosis. Similar phenotypes were also observed after exposure of *C. elegans* to the general nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP)[10] but not to the anthelmintic levamisole, which agonizes a specific subtype of nicotinic acetylcholine receptor (nAChR)[11]. AAD 1470 showed similar activity against ivermectin-, benzimidazole- and levamisole-resistant *C. elegans* strains as against the wild-type strain (Supplementary Table 1). Notably, resistance to the AADs was not conferred by loss of any of the three required nAChR subunits of the levamisole receptor (UNC-29, UNC-38 and UNC-63; refs 12, 13).

AADs 450 and 1470 cured sheep infected with drug-resistant field isolates of *H. contortus*, *T. circumcincta* and *T. colubriformis* (Table 1), completely inhibiting egg shedding within 2 days after treatment, whereas the recommended doses of the three major classes of anthelmintics failed. AAD 1470 was effective against levamisole-, benzimidazole-, macrocyclic-lactone- and multidrug-resistant pathogenic nematodes at 10 mg racemate kg^{-1} (Table 1). The resistance-breaking activities of the AADs and the unique suite of phenotypes induced in *C. elegans* suggest that these compounds act by a novel mode of action, different from those of the currently used anthelmintics.

A unique nAChR is required for anthelmintic action

To explore further the AAD mode of action, we performed a forward genetic screen for AAD-resistant *C. elegans* mutants. Of 44 AAD-resistance alleles isolated, 36 fell into a single genetic complementation group that was mapped by genetic recombination to a ~5-map-unit interval on chromosome V. Two genes in this interval, *acr-17* and *acr-23*, encode predicted nAChR subunits. DNA sequencing of the corresponding regions from individual mutants revealed 27 independent mutations in *acr-23* (Fig. 4a and Supplementary Table 2), identifying *acr-23* as a major contributor to the AAD response in *C. elegans*. The ACR-23 protein belongs to the DEG-3 group of nAChRs, a nematode-specific subfamily that is not present in mammals[14]. Its role in AAD sensitivity is the first biological function to be described for ACR-23. The *acr-23* mutants did not exhibit any overt phenotypes other than AAD resistance and were fully susceptible to levamisole, ivermectin, benomyl, aldicarb and DMPP (Supplementary Table 3). Eleven AAD-resistance mutations result in truncation of the predicted ACR-23 protein (Fig. 4a and Supplementary Table 2) and are probably null alleles that do not produce any functional protein. The finding that high-level (>1,000-fold) AAD resistance is the major phenotype resulting from loss of functional ACR-23 protein is most simply explained by hypothesizing that AADs are direct agonists of ACR-23-containing ion channels. However, it remains possible that pathway activation results from compound interaction at another point, for example, through inhibition of a negative regulator of ACR-23.

To investigate whether related nAChRs may function in the AAD response in pathogenic nematodes, we probed for nAChR genes in *H. contortus* mutants selected for AAD resistance. Starting from the *H. contortus* isolates CRA and Howick, respectively, two independent lines were obtained by *in vitro* selection that were resistant to a full dose of AAD 1470 (2.5 mg active enantiomer per kg) in sheep. A third line, selected *in vivo* from *H. contortus* Courtion, was resistant to a one-quarter dose. Candidate nAChR genes were identified by polymerase chain reaction (PCR) with primers corresponding to conserved regions of the proteins and from the *H. contortus* genome

Table 1 | Efficacy of AADs against adult stages of drug-resistant *H. contortus*, *T. circumcincta* and *T. colubriformis* in sheep

Species and isolate	Drug	Dose (oral drench)	Number of animals cured/number of animals treated
H. contortus	Levamisole	7.5 mg kg^{-1}	0/2
Baton Rouge	Levamisole	15 mg kg^{-1}	0/2
	AAD 450	10 mg racemate kg^{-1}	2/2
H. contortus	Ivermectin	0.2 mg kg^{-1}	1/4
Howick	Levamisole	7.5 mg kg^{-1}	1/2
	AAD 1470	5 mg racemate kg^{-1}	6/6
	AAD 1470	10 mg racemate kg^{-1}	2/2
	Combination of: ivermectin + albendazole + levamisole	0.2 mg kg^{-1} + 3.8 mg kg^{-1} + 7.5 mg kg^{-1}	1/2
H. contortus	Albendazole	3.8 mg kg^{-1}	0/3
Courtion	AAD 1470	10 mg racemate kg^{-1}	6/6
T. colubriformis	Albendazole	3.8 mg kg^{-1}	0/3
Villarey	Levamisole	7.5 mg kg^{-1}	0/3
	AAD 1470	10 mg kg^{-1}	6/6
T. circumcincta	Albendazole	3.8 mg kg^{-1}	0/3
Villarey	AAD 1470	10 mg kg^{-1}	6/6

Sheep were treated orally with commercial compounds at the recommended dose. Efficacy of all compounds was tested in parallel against a susceptible *H. contortus* isolate (data not shown). An animal was considered as cured when the EPG became negative and efficacy was >98% based on nematode count reduction at necropsy.

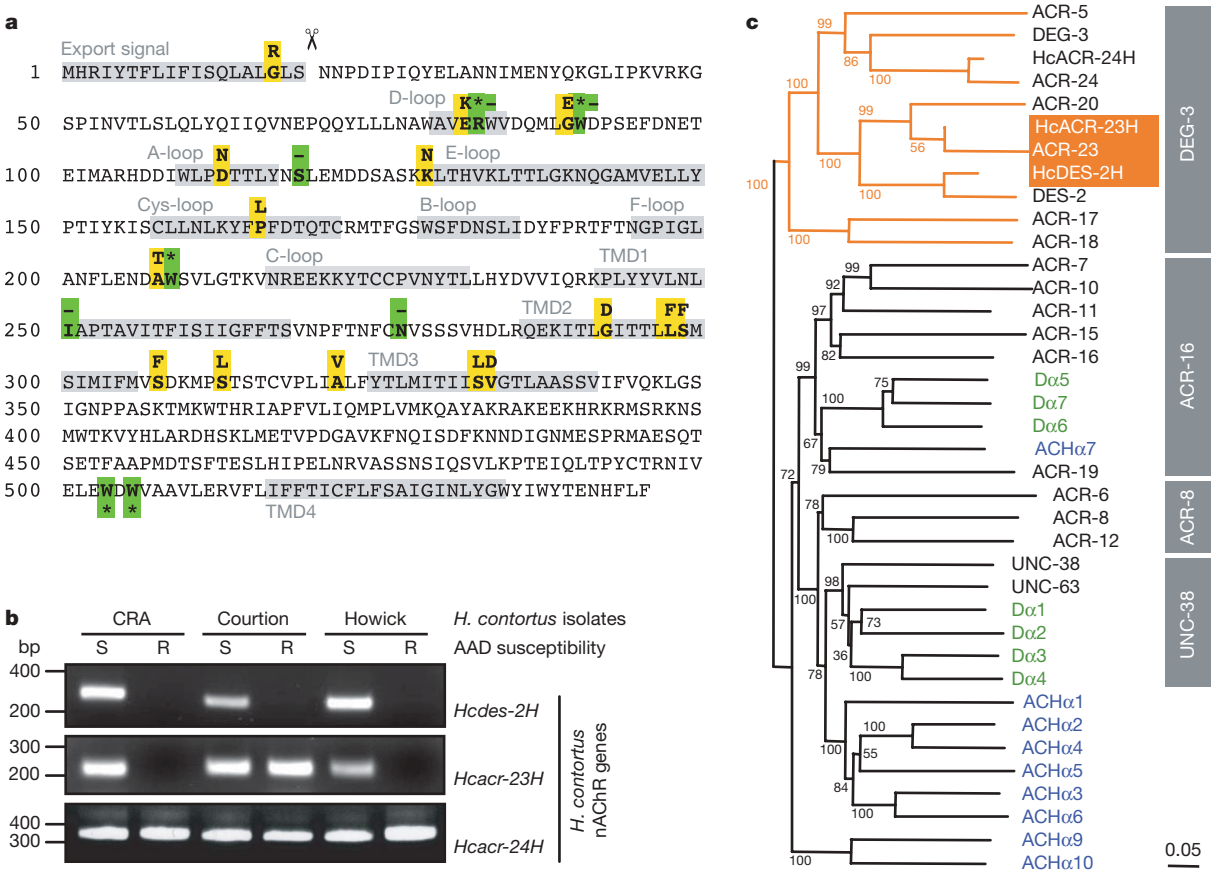

Figure 4 | Involvement of nAChRs in AAD resistance. a, *C. elegans* ACR-23 and mutations conferring AAD resistance (yellow, non-synonymous point mutations; green, nonsense mutations; asterisk, stop codon; hyphen, loss of splice acceptor). Conserved motifs in the extracellular ligand-binding domain[14] and transmembrane domains (TMD) are in grey. The cleavage site of the export signal is as predicted by SignalP[21]. **b**, nAChR-specific PCR products from AAD-sensitive parental *H. contortus* isolates (S) and their AAD-resistant progeny (R). bp, base pairs. **c**, Dendrogram[22] of nAChR α-subunits. The DEG-3 subfamily is highlighted in orange and members involved in AAD resistance are boxed (blue, *H. sapiens*; green, *Drosophila melanogaster*; Hc, *H. contortus*; grey bars, nomenclature of the *C. elegans* nAChR subfamilies[23]). Bootstrapping values are in per cent positives of 1,000 rounds. See Supplementary Fig. 2 for a multiple alignment of DEG-3 subfamily nAChRs from *C. elegans* and *H. contortus*.

project (http://www.sanger.ac.uk/Projects/H_contortus). All three AAD-resistant *H. contortus* mutants appeared to have lost at least part of a gene that we term *H. contortus des-2* homologue (*Hcdes-2H*; Fig. 4b) owing to its high degree of similarity (Fig. 4c) to *C. elegans des-2* (ref. 15). Like ACR-23, DES-2 belongs to the nematode-specific DEG-3 group of nAChRs. A *C. elegans* mutant lacking functional DES-2 did not exhibit AAD resistance (Supplementary Table 1). The two fully AAD-resistant mutants derived from *H. contortus* CRA and Howick had also lost at least part of the *H. contortus* homologue of *acr-23* (*Hcacr-23H*; Fig. 4b, c). No mutations were observed in the *H. contortus* homologue of *acr-24* (*Hcacr-24H*; Fig. 4b, c). The correlation of mutations in two nAChR genes of the *deg-3* subfamily with AAD resistance suggests that the hypothesized mode of action, activation of a nAChR signalling pathway, may be conserved between *C. elegans* and *H. contortus*.

Conclusions

The optimized AAD compounds described here meet the following requirements for an urgently needed new anthelmintic for livestock: low toxicity, favourable pharmacokinetic properties and broad-spectrum efficacy against sheep and cattle nematodes. Moreover, this efficacy includes multidrug-resistant parasites owing to a presumed activation of signalling via nematode-specific DEG-3 subtype nAChRs. However, nematodes will ultimately develop resistance to any new drug, including the AADs. To secure the maximum lifespan of the AADs as well as the current anthelmintic drugs, monitoring of drug resistance and rational exploration of combinations with current or future drugs will be necessary. If the excellent tolerability of the AADs in ruminants can be proven for humans, the class may offer an alternative anthelmintic for human medical practice.

METHODS SUMMARY

Anthelmintic efficacy tests in sheep and cattle were performed according to the guidelines of the World Association for the Advancement of Veterinary Parasitology[16]. Anthelmintic efficacy in *M. unguiculatus* was determined by nematode-count reduction after necropsy 3 days after treatment. *In vitro* activity on parasitic nematodes was assessed according to ref. 17 with minor modifications. For *C. elegans* bioassays, synchronously staged L1 hermaphrodite larvae were seeded on agar medium plus test compounds and incubated for 3 days, monitoring movement and viability. Endpoints were nematode growth and development.

Forty-three AAD-resistant *C. elegans* mutants were obtained after ethane methyl sulphonate (EMS) mutagenesis[18]; one (*cb27*) was recovered spontaneously. The *cb27* mutation is semi-dominant and mapped[19] to a genetic interval between −12 and −7 on chromosome V. Candidate genes therein were amplified by PCR and sequenced, revealing a deletion in *acr-23*. The *acr-23* genomic sequence was determined for all EMS-induced AAD-resistant mutants, and all the mutations were tested for complementation of *cb27*.

H. contortus isolates CRA (drug-sensitive reference strain from South Africa), Courtion (field isolate from Switzerland) and Howick (multidrug-resistant isolate from South Africa[20]) were maintained in sheep. *In vitro* selection for AAD resistance was performed by allowing eggs to develop to L3 larvae under drug pressure. Surviving larvae were used to re-infect sheep. AAD resistance was obtained after eight such cycles. *H. contortus* nAChR homologues were amplified by PCR from genomic DNA isolated from L3 larvae.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information *Hcdes-2H* sequences have been deposited in GenBank under accession numbers EF659746–EF659760. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details accompany the full-text HTML version of the paper at www.nature.com/nature. Correspondence and requests for materials should be addressed to R.K. (ronald.kaminsky@novartis.com).

METHODS

***In vitro* anthelmintic activity.** Freshly harvested and cleaned nematode eggs were used to seed a suitably formatted 96-well plate containing the test substances to be evaluated for anthelmintic activity. Each compound was tested by serial dilution in order to determine its minimum effective dose. The test compounds were embedded in an agar-based nutritive medium allowing the full development of eggs through to third stage larvae (L3). The plates were incubated for 6 days at 28 °C and 80% relative humidity. Egg hatching and ensuing larval development were recorded to identify a possible nematicidal activity. Efficacy was expressed as per cent reduced egg hatch, reduced development of L3, or paralysis and death of larvae of all stages.

Anthelmintic efficacy in *M. unguiculatus*. Gerbils (4 per treatment group, 6 controls) were artificially infected by gavage with approximately 2,500 sheathed L3 larvae each of *T. colubriformis* and *H. contortus*, 6 and 5 days, respectively, before treatment. Treatment was performed orally by gavage or subcutaneously by injection. Control animals received the empty formulation. Three days after treatment, gerbils were killed and dissected to recover *H. contortus* from the stomach and *T. colubriformis* from the upper midgut. Iodine-fixed nematodes were counted after de-staining. Efficacy was expressed as a percentage reduction in nematode numbers in comparison with the geometric mean of nematodes collected from the control group, using Abbot's formula.

***In vivo* anthelmintic efficacy studies.** Sheep and cattle efficacy tests were performed according to the guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine) by the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.)[16].

***Caenorhabditis elegans* assays.** *C. elegans* bioassays were carried out by adding compounds to an agar culture medium. For each experiment, the indicated strains were assayed in parallel for comparison. Some *C. elegans* strains were obtained from the *Caenorhabditis* Genetic Center. Compound stocks were in dimethyl sulphoxide (DMSO) and the final concentration of DMSO in all assays was 1%. Assays were performed in duplicate. Synchronously staged L1 hermaphrodite larvae were placed in the assay wells (~50 nematodes per assay; exact number determined by counting). Nematodes were monitored for effects on movement and viability. After 3 days, nematode growth was assessed in each well by counting the number of gravid adults, L4 larvae/non-gravid adults and larvae younger than the L4 stage. Untreated control cultures routinely contain ≥ 90% gravid adults at the time of scoring. The lowest effective concentration was defined as the lowest observed concentration at which ≤5% of nematodes survived to the L4 or adult stages.

AAD-resistant *C. elegans* mutants. The AAD-resistant mutant *cb27* was recovered from an ivermectin-resistant strain as a single spontaneous survivor of AAD exposure. AAD resistance proved heritable and was separated from the ivermectin-resistance mutations by outcrossing with the wild-type *C. elegans* strain N2. *cb27* was mapped to a genetic interval between −12 and −7 on chromosome V, using genetic recombination with a combination of single nucleotide polymorphisms[19] and visible phenotypic markers. Candidate genes were analysed by PCR amplification of genomic sequences from individual mutants, followed by direct sequencing of purified amplification products. Sequencing revealed a deletion in the gene *acr-23* in the *cb27* strain.

Additional AAD-resistant mutants were recovered by compound selection of the F_2 generation after ethane methyl sulphonate (EMS) mutagenesis by standard methods[18]. Forty-three mutants exhibiting heritable AAD resistance were recovered from $\sim 1 \times 10^6$ mutagenized genomes. Each mutant was outcrossed (usually four times) with the parental N2 strain before characterization. The *cb27* mutation is genetically semi-dominant, with heterozygotes exhibiting compound sensitivity intermediate between that of wild-type animals and *cb27* homozygotes. The greater compound resistance of *cb27* homozygotes provided a basis for testing additional resistance alleles for genetic complementation of *cb27*. Thirty-five of the EMS-induced AAD resistance alleles failed to complement *cb27*. Eight additional resistance alleles, at least six of which genetically complement *cb27*, probably represent one or more additional AAD resistance gene(s). The *acr-23* genomic sequence was determined for all 43 surviving EMS-induced AAD-resistant mutants, revealing mutations in *acr-23* protein-coding sequences from 29 of the 35 non-complementing alleles (representing at least 27 independent mutations). It remains to be determined whether the six remaining non-complementing mutations lie in *acr-23* non-coding sequences or in another gene exhibiting non-allelic non-complementation with *acr-23*.

AAD-resistant *H. contortus* mutants. *H. contortus* strains CRA, Courtion and Howick were maintained in sheep. AAD-resistant derivatives of the strains CRA and Howick were obtained after eight consecutive rounds of *in vitro* selection, allowing eggs (500,000 per assay) to develop to L3 larvae at various drug concentrations. Larvae recovered from LC_{95} AAD were used to re-infect sheep. *H. contortus* Courtion was selected *in vivo* by consecutive rounds of sub-therapeutic treatment of infected sheep. PCR was carried out on genomic DNA of the *H. contortus* strains isolated by phenol-chloroform extraction from 50 adults (males and females). Primers were HcACRa2 (GCCCGAATGCCTACACTTTA), HcACRa3 (CATTGATAACAACGTAGTAATACC) and HcACRa5 (AAAAAGGCTTCAGTTTACGTGAG) for *Hcdes-2H*; HcACR23_frw2 (TGCGAAGACGTCTGAACATC) and HcACR23_rev2 (TTCCAAGTGTAATTTTCTCCTCTC) for *Hcacr-23H*; and HcACR24_frw (GGGTGCTTTTGGAAGCAGTA) and HcACR24_rev (GGTACATAGTCACTGGTAGTTGGC) for *Hcacr-24H*. The *Hcdes-2H* PCR products from primers HcACRa2 and HcACRa3 were cloned into pCR2.1-TOPO (Invitrogen) and sequenced from both sides; GenBank accession numbers are EF659746–EF659760. Primers HcACRa2 and HcACRa5 were used for Fig. 4b.