

Selective Action of Acetogenin Mitochondrial Complex I Inhibitors

Azucena González-Coloma,^{a,*} Ana Guadaño^a, Concepción de Inés^a,
Rafael Martínez-Díaz^b and Diego Cortes^c

^a Centro de Ciencias Medioambientales, CSIC, Serrano 115-dpdo., 28006 Madrid, Spain.
Fax: 34-91-5640800. E-mail: azu@ccma.csic.es

^b Departamento de Medicina Preventiva (Parasitología), Facultad de Medicina,
Universidad Autónoma de Madrid, Spain

^c Departamento de Farmacología, Facultad de Farmacia, Universidad de Valencia, Spain

* Author for correspondence and reprint requests

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Five annonaceous acetogenins, rolliniastatin-1 (**1**), rolliniastatin-2 (**2**), laherradurin (**3**), squamocin (**4**), annonacin (**5**), and rotenone as a reference, differing in their NADH oxidase inhibition activity, have been evaluated for antifeedant, insecticidal, trypanocidal and cytotoxic effects on insect, mammalian and tumor cells. All the test compounds were toxic to *Leptinotarsa decemlineata*, demonstrated selective cytotoxicity to insect Sf9 cells and a panel of tumor cell lines with the multidrug-resistant SW480 (P-glycoprotein⁺, Pgp⁺) being the most sensitive one. Compounds **1**, **2**, **4**, and rotenone had post-ingestive effects against *Spodoptera littoralis* larvae while **1**, **4**, **5**, and rotenone were active against *Trypanosoma cruzi*. Based on their biochemical properties (inhibition of the mitochondrial NADH oxidase activity), the *in vivo* effects of these compounds on *S. littoralis* and their cytotoxic effects on Sf9 and tumor cells were more predictable than their effect on *T. cruzi* and mammalian cells.

Introduction

Annonaceous acetogenins (ACGs) are a family of secondary metabolites isolated from the plant family Annonaceae that are characterized by a terminal γ -lactone subunit (either saturated or unsaturated), with one to three tetrahydrofuran rings (THF) and a long aliphatic region with other functional groups (Alali *et al.*, 1999; Tormo *et al.*, 1999; Zafra-Polo *et al.*, 1998).

The sources, biogenesis, isolation, chemistry, synthesis and bioactivity of these compounds have been extensively reviewed (Alali *et al.*, 1999; Tormo *et al.*, 1999; Zafra-Polo *et al.*, 1998). They are potent cytotoxics with antitumoral, insecticidal, acaricidal, fungicidal, antiparasitic, and bactericidal activities (Alali *et al.*, 1999; Guadaño *et al.*, 2000). Their mode of action targets mitochondrial electron transport with a specific action at NADH: ubiquinone oxidoreductase (NADH-dehydrogenase, also known as complex I) (Lewis *et al.*, 1993; Londerhausen *et al.*, 1991). Furthermore, the inhibitory effects of ACGs have been shown to be more potent than those of classical respiratory inhibitors such as rotenone or piericidin A (Degli Esposti *et al.*, 1994).

In this work, we have tested several head-series acetogenins for a functional type based on their complex I inhibition kinetics (Tormo *et al.*, 1999): the rolliniastatin-2 type that binds a different site from rotenone (ACG **2**); the general type showing the same behavior as rotenone (ACGs **3–5**) and the dual rolliniastatin-1 type (ACG **1**) with overlapping binding with rotenone and rolliniastatin-2 (Tormo *et al.*, 1999), plus the classical complex I inhibitor rotenone for comparative antifeedant and toxic effects on several insect species (*Spodoptera littoralis*, *Leptinotarsa decemlineata*, and *Myzus persicae*) and their trypanocidal effects on epimastigote forms of the Chagas disease agent *Trypanosoma cruzi*. The cytotoxicity of these compounds has been tested on *Spodoptera frugiperda* Sf9 cells, mammalian CHO and several tumoral cell lines, and these effects have been compared with their reported mitochondrial NADH oxidase inhibition activity.

Experimental Section

Compounds. Rolliniastatin1 (**1**) was isolated from *Rollinia membranacea* (Saez *et al.*, 1993). Rolliniastatin 2 (**2**), laherradurin (**3**) and squamo-

cin (**4**) were isolated from *Annona cherimolia* seeds (Cortes *et al.*, 1993). Annonacin (**5**), was isolated from *Annona glabra* seeds (Gallardo *et al.*, 1998). All the samples of acetogenins were more than 95% pure as judged by chromatographic criteria. Rotenone and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich. Parasite and cell viability were measured in a SLT Lab instruments, Vienna/Austria, microplate reader.

Insect Bioassays. *L. decemlineata*, *S. littoralis* and *M. persicae* colonies were reared on potato foliage, artificial diet and bell pepper (*Capsicum annuum*) plants, respectively, and maintained at 22 ± 1 °C, > 70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber.

Choice Feeding Assays. These experiments were conducted with adult *L. decemlineata*, newly emerged fifth-instar *S. littoralis* larvae and *M. persicae* apterous adults. Percent feeding inhibition (%FI) and percent settling inhibition (%SI) were calculated as described by Reina *et al.* (2001). Compounds with an FI/SI > 50% were tested in a dose-response experiment to calculate their relative potency (EC₅₀ values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FI or %SI on log dose).

Oral Cannulation. This experiment was performed with pre-weighed newly molted *S. littoralis* L6-larvae as previously described (Reina *et al.*, 2001). The possible effect of variations in initial larval weight was analyzed by an analysis of covariance (ANCOVA) performed on biomass gains with initial biomass as covariate. The covariate effect was not significant ($p > 0.05$), showing that changes in insect biomass were similar among all treatments. A second ANCOVA analysis was performed on biomass gains with food consumption as covariate to test for post-ingestive effects (Horton and Redak, 1993; Raubenheimer and Simpson, 1992).

Hemolymph Injection. DMSO solutions of the test compounds (10 µg/insect) were injected in twenty adult *L. decemlineata* beetles as described in Reina *et al.* (2001). Beetle mortality was recorded up to 3 days after injection. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

Trypanocidal activity. This activity was assayed on epimastigote forms of *T. cruzi*, Y strain, cul-

tured at 28 °C in liver infusion tryptose medium (LIT), supplemented with 10% heat inactivated (56°C for 30 min) fetal calf serum (Herrero *et al.*, 1992).

Parasites in logarithmic growth phase (from an initial culture with 2×10^6 epimastigotes/ml) were distributed in 96-well flat bottom plates. Each well was treated with increasing concentrations of the test compounds in DMSO (< 1% final concentration) for 96 h. Parasite viability was analyzed by a modified MTT colorimetric assay method (Muelas-Serrano *et al.*, 2000). All assays were carried out in triplicate and the activity was calculated as % growth inhibition. ED₅₀ values were determined for each compound as the effective dose to produce 50% growth inhibition which was determined from linear regression analysis (% growth inhibition on log dose).

Cytotoxicity. Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures, ECCC) were maintained in TC-100 insect cell medium supplemented with 10% fetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin at 26 °C. Mammalian Chinese hamster ovary cells (CHO, a gift from Dr. Pajares, C. Biomédicas, CSIC), SW480 (MDR, human colon carcinoma, a gift from Deutsches Krebsforschungszentrum, DKFZ), HeLa (human cervical carcinoma, DKFZ) and SkMel28 (human malignant melanoma, DKFZ) were grown in RPMI 1640 medium supplemented as above at 37 °C under a humidified atmosphere of 5% CO₂/95% air.

Cells seeded in 96-well flat-bottom microplates with 100 µl medium per well (initial densities of 5×10^4 and 10^4 cells per well for the insect and mammalian cultures respectively), were exposed for 48 h to serial dilutions of the test compounds in DMSO (< 1% final concentration). Cell viability was analyzed by the MTT colorimetric assay method (Mossman, 1983). The purple-colored formazan precipitate was dissolved with 100 µl of DMSO. Cell viability was calculated as the percent absorbance of the control (untreated cells). The active compounds were tested in a dose-response experiment to calculate their relative potency (ED₅₀ values, the effective dose to give 50% cell viability) which was determined from linear regression analysis (% cell viability on log dose).

Results and Discussion

None of the test compounds had antifeedant effects against *S. littoralis* or *M. persicae* when tested at a dose of 50 $\mu\text{g}/\text{cm}^2$ (%FI < 40%). Rolliniastatin 2 (**2**) did have an antifeedant effect on *L. decemlineata* with an effective dose (EC_{50}) of 2.3 $\mu\text{g}/\text{cm}^2$ (0.7–7.4, 95% confidence limits), 4-times stronger than compound **5** (Guadaño *et al.*, 2000). Compounds **1–3** significantly increased *L. decemlineata* mortality (97–100% mortality in 24 h). Similarly, compound **4** and rotenone have been reported as being toxic to this insect (Guadaño *et al.*, 2000). The selective antifeedant effect of **2** suggests a mode of action different from the toxicity-related inhibition of Complex I, as suggested for the antifeedant effect of **5** (Guadaño *et al.*, 2000).

An ANCOVA analysis of food consumption and biomass gains of orally injected *S. littoralis* larvae (20 $\mu\text{g}/\text{insect}$) with initial larval weight as covariate (covariate $p > 0.05$), revealed that consumption rates and weight gains significantly decreased with compounds **1**, **2**, **4** and rotenone (treatment $p < 0.0001$, Table 1). Additional ANOVA and ANCOVA analysis performed on larval biomass gains with food consumption as covariate showed that the treatment effects of these compounds disappeared following covariance adjustment (ANOVA $p < 0.00001$ for consumption and weight gains and ANCOVA $p > 0.05$ for weight gains), indicating that the decreased consumption caused by the treatment accounted for the decrease in body mass with no further post-ingestive toxicity (Horton and Redak, 1993; Raubenheimer and Simpson, 1992). Compounds **4**, **5** and rotenone did not have any

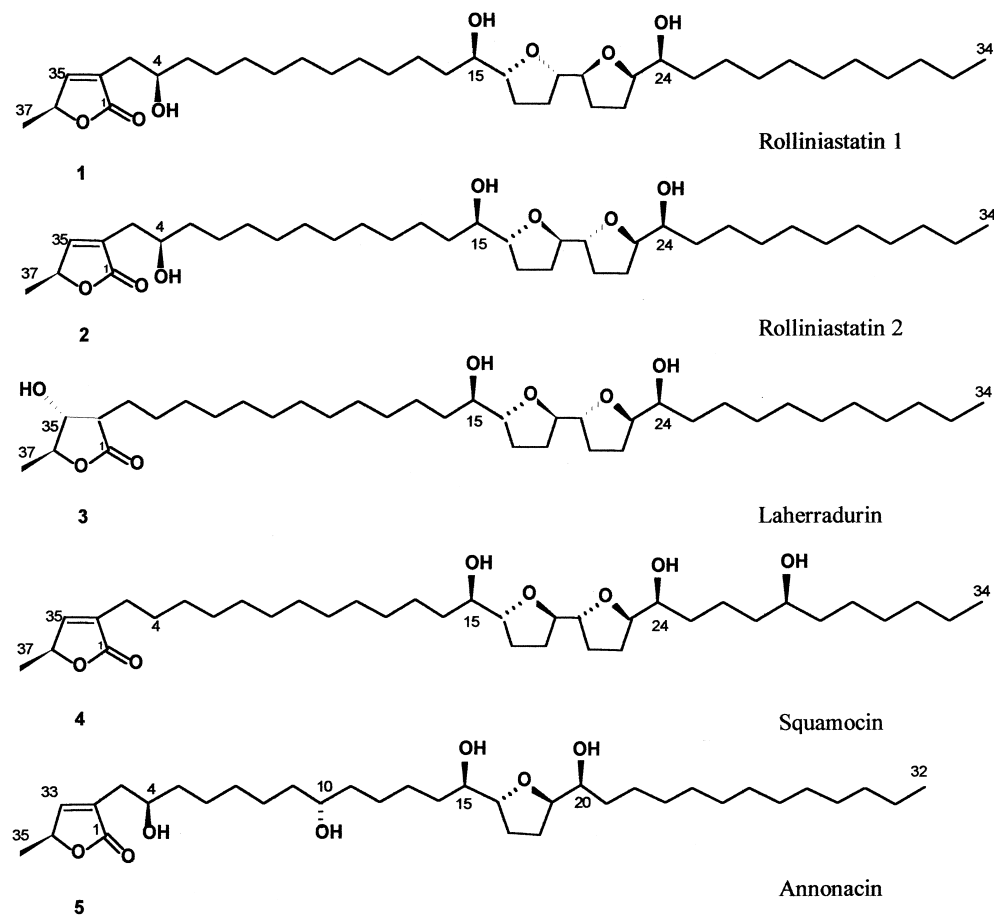


Fig. 1. Molecular structures of the test compounds.

Table I. Consumption (I) and biomass gain (ΔB) of orally injected *Spodoptera littoralis* larvae, cytotoxic effects on Sf9 and CHO cells and trypanocidal action on *Trypanosoma cruzi* epimastigotes of the test compounds. NADH oxidase values are included for comparison purposes.

| Compound | <i>S. littoralis</i> (% of control) | | | ED ₅₀ [μ M] ^a | | NADH oxidase ^c |
|----------|--|------------|---|--|------------------------|--|
| | I | ΔB | Sf9 | CHO | <i>T. cruzi</i> | IC ₅₀ [nM] |
| 1 | 40.3* | 25.3* | 0.32×10^{-2} (0.14×10^{-3} , 0.96×10^{-2}) ^b | > 90 | 32.75 (22.20, 48.21) | 0.3–0.5 (Degli-Esposti <i>et al.</i> , 1994; Estornell <i>et al.</i> , 1997) |
| 2 | 29.7* | 56.2* | 0.03 (0.01, 0.08) | 1.51 (0.69, 3.37) | 163.72 (91.30, 293.87) | 0.6 (Gallardo <i>et al.</i> , 2000) |
| 3 | 86 | 78 | 0.57 (0.32, 0.99) | 8.42 (3.36, 21.08) | > 100 | 0.2 (Tormo <i>et al.</i> , 2001) |
| 4 | 64* | 53* | 0.51 (0.32, 0.83) | > 100 | 40.16 (26.02, 61.88) | 0.6 (Gallardo <i>et al.</i> , 2000) |
| 5 | 86 | 97 | 1.19 (0.97, 1.46) | 61.24 (35.15, 10.66) | 69.07 (32.85, 145.25) | 2.3 (Gallardo <i>et al.</i> , 1998) |
| Rotenone | 56* | 62* | 0.23 (0.15, 0.30) | 38.14 (14.36, 101.16) | 34.89 (24.67, 49.36) | 5.1 (Estornell <i>et al.</i> , 1997) |

^a ED₅₀ = Concentration needed to produce 50% cell viability or epimastigote mortality.

^b 95% Confidence limits.

^c The reported data has been produced under similar experimental conditions.

* Significantly different from the control, * $p < 0.05$, LSD test.

negative effects on *S. littoralis* larvae when orally injected at a dose of 10 μ g/insect (Guadaño *et al.*, 2000), suggesting that this insect can tolerate lower doses of these compounds through metabolic inactivation.

All the test compounds (**1–5**) and rotenone were cytotoxic against insect-derived Sf9 cells (Table I) with **1** being the most potent, followed by **2** (10-times less toxic than **1**), rotenone (45-times less active than **1**), **3**, **4** (> 150-times less active than **1**), and **5** (350-times less active than **1**). Previous studies have shown that rolliniastatin 2 (**2**) affected cellular respiration in *S. frugiperda* Sf9 cells and mitochondrial preparations from *Manduca sexta* (Ahammadsahib *et al.*, 1993; Hollingworth *et al.*, 1994), suggesting that respiratory inhibition could cause the cytotoxic effects of the test compounds on Sf9 cells. There was a positive correlation between the “*in vivo*” effects on *S. littoralis* and the Sf9 cytotoxicity for compounds **1**, **2** and rotenone. ACGs **3** and **4** had the same cytotoxic potency but only **4** affected *S. littoralis* larval performance.

The cytotoxicity of these compounds on mammalian CHO cells showed a different pattern, **2** being the most toxic followed by **3** (5-times less active than **2**), rotenone (15-times less active than **2**), and **5** (36-times less active than **2**) (Table I). In all cases, mammalian CHO cells were less sensitive to the test compounds than Sf9 cells (450–100 times less sensitive), indicating an insect-selective action for these compounds.

Compounds **1**, **4**, **5** and rotenone were active against *T. cruzi* with rotenone, **1**, and **4** showing the strongest effects (Table I). Compounds **1** and **4** were more trypanocidal than cytotoxic to CHO cells (3 and 4 times more respectively), while **5** and rotenone had similar cytotoxic and trypanocidal effects.

Rolliniastatin 1 (**1**) and squamocin (**4**) have been found effective in reducing the number of bloodstream forms of *T. cruzi*, but a structure-relationship was not found (Fevrier *et al.*, 1999). Squamocin (**4**) was also effective against *Leishmania* spp. promastigotes and *T. brucei* trypomastigotes, with rolliniastatin 2 (**2**) being less active (Sahpaz *et al.*, 1994).

Table II shows the cytotoxic effects of the test compounds on a panel of cancer cells. Overall, the multidrug resistant (MDR) SW480 (Pgp⁺) was the most sensitive line to the test compounds. SW480 cells were most sensitive to **2**, **1** and **3**, and HeLa to **2**, **3**, and **4**. SkMel28 cells showed overall lower sensitivity. Rotenone demonstrated cytotoxic effects against these cell lines with SW480 and HeLa being the most sensitive ones. Compounds **1**, **2**, **4** and **5** have been reported as cytotoxic against several tumor cells (Tormo *et al.*, 1999) but this is the first report on the antitumoral effects of compound **3**.

Recent studies have shown that MDR cell lines expressing higher levels of ATP-dependent transporter proteins (Pgp⁺) are more susceptible to ACGs with rolliniastatin 2 (**2**) being the most po-

Table II. Cytotoxic effects of the test compounds on tumoral cell lines SW480 (Pgp⁺), SkMel28 and HeLa.

| Compound | ED ₅₀ [μM] ^a | | |
|----------|--|---------------------------------------|--|
| | SW480 | SkMel28 | HeLa |
| 1 | 0.70×10 ⁻⁷ (0.13 × 10 ⁻⁹ , 0.5 × 10 ⁻⁶) ^b | 0.03 (0.02 × 10 ⁻¹ , 0.42) | 0.43 (0.02, 8.80) |
| 2 | 0.1 × 10 ⁻⁸ (0.32 × 10 ⁻¹² , 0.37×10 ⁻⁴) | 0.09 (0.01, 0.57) | 4.74 (0.15 × 10 ⁻³ , 0.14) |
| 3 | 0.64 × 10 ⁻⁶ (0.23 × 10 ⁻⁷ , 0.17 × 10 ⁻⁴) | 0.14 (0.47 × 10 ⁻² , 4.50) | 0.74 × 10 ⁻² (0.20 × 10 ⁻² , 0.42) |
| 4 | 0.50 × 10 ⁻³ (0.15 × 10 ⁻³ , 0.16 × 10 ⁻²) | 2.81 (0.38, 20.86) | 0.04 (0.37 × 10 ⁻³ , 0.03) |
| 5 | 2.72 (1.76, 4.21) | 24.24 (8.28, 70.94) | 1.63 (0.90, 2.88) |
| Rotenone | 0.17 × 10 ⁻⁵ (0.25 × 10 ⁻⁶ , 0.12 × 10 ⁻⁴) | 0.29 (0.06, 1.37) | 0.15 × 10 ⁻³ (0.35 × 10 ⁻⁴ , 0.69 × 10 ⁻³) |

^a ED₅₀ = Concentration needed to produce 50% cell viability.

^b 95% Confidence limits.

tent one (Oberlies *et al.*, 1995; 1997a; 1997b). Furthermore, recent studies have reported that highly specific inhibitors of mitochondrial respiratory chain and ATP-synthase, including rolinsatatin 2 (**2**), induce apoptosis (Chih *et al.*, 2001; Wolvetang *et al.*, 1994). We have observed morphological cellular changes suggesting that the cytotoxic effects of the ACGs and rotenone could have been the result of induced apoptosis, but further research is needed to proof this hypothesis.

Indirect evidence suggests that the insecticidal effects of ACGs are due to their inhibition of mitochondrial complex I (Ahammadsahib *et al.*, 1993; Hollingworth *et al.*, 1994; Lewis *et al.*, 1993; Londerhausen *et al.*, 1991). Similarly, respiratory chain inhibition appears to be a target for trypanosomicidal activity (Maya *et al.*, 2001) and the anti-tumoral effect of ACGs has been attributed to their ability to lower ATP levels via inhibition of the mitochondrial complex I (Lewis *et al.*, 1993; Londerhausen *et al.*, 1991) and inhibition of NADH-oxidase which is constitutively expressed in the plasma membrane of cancer cells (Morré *et al.*, 1991). Furthermore, enzymatic and immunochemical evidence indicated a high degree of similarity between insect, mammalian and fungal NADH: ubiquinone oxidoreductases (Lümmen, 1998). Therefore, Complex I inhibition activity of ACGs has conducted most of the studies attempting to characterize relevant structural factors to design more potent derivatives with potential applications (Gallardo *et al.*, 2000; Tormo *et al.*, 2001).

Our results indicate that among the biological systems tested here, the cytotoxicity of ACGs on tumoral cell lines showed a moderate correlation

with their NADH-oxidase inhibition activity, and increased with the cellular ATP requirements. Their effects on *S. littoralis* larvae and Sf9 cells followed the NADH-oxidase inhibition pattern except for compound **3**, the most potent inhibitor, with a β-hydroxylated saturated γ-lactone that contributes to tight binding to the enzyme (Tormo *et al.*, 2001) but might be more accessible to metabolic inactivation by *S. littoralis* and/or less bioavailable to the insect cells. Their CHO cytotoxicity did not correlate with the enzymatic inhibition activity of **1** and **4** probably due to membrane transport factors. This hypothesis is further supported by the selective cytotoxicity observed between CHO and Sf9 cells given the slight difference in sensitivity to inhibition among mitochondrial Complex I preparations between insects and vertebrates (Hollingworth *et al.*, 1994). The trypanocidal effect of these compounds did not correlate with the enzymatic inhibition activity of **2** and **3** probably due to metabolic inactivation and/or membrane-related factors. Rotenone was active against all the biological models tested.

In summary, in this work we have shown that the pesticidal, antiparasitic and cytotoxic effects of ACGs **1–5** were species/cell and structure dependent and only followed a general trend of activity based on their *in vitro* NADH oxidase inhibition activity for the insect system and tumor cells. On the contrary, the reference compound rotenone, with a similar mode of action, did not show such selectivity indicating that structure-dependent membrane factors and metabolic inactivation play an important role in the biological activity of these compounds. Therefore, structure-activity studies of ACGs should be target oriented.

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- Abbott W. S. (1925), A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* **18**, 265–267.
- Ahamedsahib K. I., Hollingworth R. M., McGovern J. P., Hui Y.-H. and McLaughlin J. L. (1993), Mode of action of bullatacin: a potent antitumor and pesticidal annonaceous acetogenin. *Life Sci.* **53**, 1113–1120.
- Alali F. Q., Liu X.-X. and McLaughlin J. L. (1999), Annonaceous acetogenins: Recent progress. *J. Nat. Prod.* **62**, 504–540.
- Chih H.-W., Chiu H.-F., Tang K.-S., Chang F.-R. and Wu Y.-C. (2001), Bullatacin, a potent antitumor annonaceous acetogenin inhibits proliferation of human hepatocarcinoma cell line 2.2.15 by apoptosis induction. *Life Sci.* **69**, 1321–1331.
- Cortes D., Myint S. H., Dupont B. and Davoust D. (1993), Bioactive acetogenins from seeds of *Annona cherimolia*. *Phytochemistry* **32**, 1475–1482.
- Degli Esposti M., Ghelli A., Ratta M., Cortes D. and Estornell E. (1994), Natural substances (acetogenins) from the family Annonaceae are powerful inhibitors of mitochondrial NADH dehydrogenase (complex I). *Biochem. J.* **301**, 161–167.
- Estornell E., Tormo J. R. and Cortes D. (1997), Chermolin-1, new selective inhibitor of the first energy-coupling site of the NADH: ubiquinone oxidoreductase (complex I). *Biophys. Res. Commun.* **240**, 234–238.
- Février A., Rojas de Arias A., Hocquemiller R. and Waechter A. I. (1999), Acetogenins and other compounds from *Rollinia emarginata* and their antiprotozoal activities. *Planta Med.* **65**, 47–49.
- Gallardo T., Aragón R., Tormo J. R., Blazquez M. A., Zafra-Polo M. C. and Cortes D. (1998), Acetogenins from *Annona glabra* seeds. *Phytochemistry* **47**, 811–816.
- Gallardo M. T., Zafra-Polo C., Tormo J. R., González M. C., Franck X., Estornell E. and Cortes D. (2000), Semisynthesis of antitumoral acetogenins: SAR of functionalized alkyl-chain bis-tetrahydrofuranic acetogenins, specific inhibitors of mitochondrial complex I. *J. Med. Chem.* **43**, 4793–4800.
- Guadaño A., Gutiérrez C., De la Peña E., Cortes D. and González-Coloma A. (2000), Insecticidal and mutagenic evaluation of two annonaceous acetogenins. *J. Nat. Prod.* **63**, 773–776.
- Herrero A., Ochoa C., Atienza J., Escario J. A., Gómez-Barrio A. and Martínez-Fernández A. R. (1992), Synthesis and antiprotozoal properties of 1,2,6-thiadiazine 1,1-dioxide derivatives. *Arch. Pharm.* **325**, 509–514.
- Hollingworth R. M., Ahamedsahib K. I., Gadelhak G. and McLaughlin J. L. (1994), New inhibitors of complex I of the mitochondrial electron transport chain with activity as pesticides. *Biochem. Soc. Trans.* **22**, 230–233.
- Horton R. D. and Redak R. A. (1993), Further comments on analysis of covariance in insect dietary studies. *Entomol. Exp. Appl.* **69**, 263–275.
- Lewis M. A., Arnason J. T., Philogene B. J. R., Rupprecht J. K. and McLaughlin J. L. (1993), Inhibition of respiration at site I by asimicin, an insecticidal acetogenin of the Pawpaw, *Asimina triloba* (Annonaceae). *Pest. Biochem. Physiol.* **45**, 15–23.
- Londerhausen M., Leicht W., Lieb F., Moeschler H. and Weiss H. (1991), Molecular mode of action of annonins. *Pestic. Sci.* **33**, 427–438.
- Lümmen P. (1998), Complex I inhibitors as insecticides and acaricides. *Biochim. Biophys. Acta* **1364**, 287–296.
- Maya J. D., Morello A., Repetto Y., Rodríguez A., Puebla P., Caballero E., Medarve M., Núñez-Vergara L. J., Squella J. A., Ortiz M. E., Fuentealba J. and San Feliciano A. (2001), *Trypanosoma cruzi* inhibition of parasite growth and respiration by oxazolo (thiazolo) pyridine derivatives and its relationship to redox potential and lipophilicity. *Exp. Parasitol.* **99**, 1–6.
- Morré J. D., De Cabo R., Farley C., Oberlies N. H. and McLaughlin J. L. (1995), Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity HeLa and HL-60 but not liver, plasma membranes. *Life Sci.* **56**, 343–348.
- Mossman T. (1983), Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods.* **65**, 55–63.
- Muelas-Serrano S., Nogal-Ruiz J. J., Gómez-Barrio A. (2000), Setting of a colorimetric method to determine the viability of *Trypanosoma cruzi* epimastigotes. *Parasitol. Res.* **86**, 999–1002.
- Oberlies N. H., Jones J. L., Corbett T. H., Fotopoulos S. S. and McLaughlin J. L. (1995), Tumor cell growth inhibition of annonaceous acetogenins in an “*in vitro*” disk diffusion assay. *Cancer Lett.* **96**, 55–62.
- Oberlies N. H., Chang C. C. and McLaughlin J. L. (1997a), Structure-activity relationships of diverse annonaceous acetogenins against multidrug resistant human mammary adenocarcinoma (MCF-7/Adr) cells. *J. Med. Chem.* **40**, 2102–2106.

- Oberlies N. H., Croy V. L., Harrison M. L. and McLaughlin J. L. (1997b), The annonaceous acetogenin bullatacin is cytotoxic against multidrug-resistant human mammary adenocarcinoma cells. *Cancer Lett.* **115**, 73–79.
- Raubenheimer D. and Simpson S. J. (1992), Analysis of covariance: an alternative to nutritional indices. *Entomol. Exp. Appl.* **62**, 221–231.
- Reina M., González-Coloma A., Gutiérrez C., Cabrera R., Rodríguez M. L., Fajardo V. and Villarreal L. (2001), Defensive chemistry of *Senecio miser* Hook. *J. Nat. Prod.* **64**, 6–11.
- Saez J., Sarpaz S., Villaescusa R., Hocquemiller R., Cavé A. and Cortes D. (1993), Rioclarine et membracine deux nouvelles acétogénines bis-tétrahydrofuraniques des graines de *Rollinia membranacea*. *J. Nat. Prod.* **56**, 351–356.
- Sarpaz S., Bories Ch., Leiseau P. M., Cortes D., Hocquemiller R., Laurens A. and Cavé A. (1994), Cytotoxic and antiparasitic activity from *Annona senegalensis* seeds. *Planta Med.* **60**, 538–540.
- Tormo J. R., Gallardo T., González M. C., Bermejo A., Cabedo N., Andreu I. and Estornell E. (1999), Annonaceous acetogenins as inhibitors of mitochondrial complex I. *Current Topics in Phytochemistry* **2**, 69–90.
- Tormo J. R., Estornell E., Gallardo T., González M. C., Cavé A., Granell S., Cortes D. and Zafra-Polo M. C. (2001), γ -Lactone-functionalized antitumoral acetogenins are the most potent inhibitors of mitochondrial complex I. *Bioorg. Med. Chem. Lett.* **11**, 681–684.
- Wolvetang E. J., Johnson K. L., Krauer K., Ralph S. J. and Linnane A. W. (1994), Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Lett.* **339**, 40–44.
- Zafra-Polo M. C., Figadère B., Gallardo T., Tormo J. R. and Cortes D. (1998), Natural acetogenins from annonaceae, synthesis and mechanisms of action. *Phytochemistry* **48**, 1087–1117.