J Chem Ecol (2011) 37:533–536
DOI 10.1007/s10886-011-9946-z

Sequestration of Camptothecin, an Anticancer Alkaloid, by Chrysomelid Beetles

B. T. Ramesha · S. Zuehlke · R. C. Vijaya · V. Priti · G. Ravikanth · K. N. Ganeshaiah · M. Spiteller · R. Uma Shaanker

Received: 2 September 2010 / Revised: 21 March 2011 / Accepted: 3 April 2011 / Published online: 16 April 2011

Abstract Camptothecin (CPT), a monoterpene indole alkaloid, is a potent inhibitor of eukaryotic toposiomerase-I. Several derivatives of CPT are in clinical use against ovarian and lung cancers. CPT has been reported from several plant species belonging to the order Asterids, with the highest concentration in *Nothapodytes nimmoniana* (family Icacinaceae). In this paper, we report an intriguing observation of chrysomelid beetles *(Kanarella unicolor* Jacobby) feeding on the leaves of *N. nimmoniana* without any apparent adverse effect. LC-MS/MS analysis of the beetles indicated that 54.9% of the ingested CPT's was recovered from the wings, followed by lesser amounts in the head and abdomen. LC-HRMS analysis revealed that most of the CPT in the insect body was in the parental form available in the plants without any major metabolizable products, including sulfated and glucuronilated forms. The mechanism by which the beetles are able to tolerate substantially high levels of CPT in their body tissue is under investigation.

Key Words Sequestration · Anti-cancer · Alkaloid · Camptothecin · Chrysomelid beetles · Topoisomerase I

Electronic supplementary material The online version of this article (doi:10.1007/s10886-011-9946-z) contains supplementary material, which is available to authorized users.

B. T. Ramesha · R. C. Vijaya · V. Priti · G. Ravikanth · K. N. Ganeshaiah · R. Uma Shaanker
School of Ecology and Conservation,
University of Agricultural Sciences, GKVK,
Bangalore 560 065, India

B. T. Ramesha · R. Uma Shaanker (✉)
Department of Crop Physiology, University of Agricultural Sciences, GKVK,
Bangalore 560 065, India
e-mail: umashaanker@gmail.com

S. Zuehlke · M. Spiteller
Institute of Environmental Research (INFU) of the Faculty of Chemistry, Dortmund University of Technology,
44221 Dortmund, Germany

K. N. Ganeshaiah
Department of Forestry and Environmental Sciences,
University of Agricultural Sciences, GKVK,
Bangalore 560 065, India

V. Priti · G. Ravikanth · K. N. Ganeshaiah · R. Uma Shaanker
Ashoka Trust for Research in Ecology and the Environment,
Royal Enclave,
Sriramapura, Jakkur,
Bangalore 560 064, India

Introduction

Camptothecin (CPT), a monoterpene indole alkaloid, is one of the most potent cytotoxic compounds (Hsiang et al., 1985; Pommier, 2006). It is toxic to most eukaryotes including insects such as termites (Li, 2002) and drosophila (Cunha et al., 2002). CPT inhibits the enzyme topoisomerase I, which is required in DNA replication and transcription (Hsiang et al., 1985). Because of this property, several derivatives of CPT are in clinical use against ovarian, small lung and refractory ovarian cancers (Pommier, 2006). CPT is naturally distributed in several plant families of the order Asterids (Larsson, 2007). Among these, leaves and stem bark of *Nothapodytes nimmoniana* (Grahm) Mabb. (Icacinaceae), a small tree in southern India, accumulate the highest content of CPT (0.3–0.5%) (Ramesha et al., 2008). In this paper, we report an intriguing observation of specialist chrysomelid beetles (*Kanarella unicolor* Jacobby) that feed on leaves of *N. nimmoniana* without any apparent adverse effect. Liquid Chromatography-Mass Spectrometry-Mass Spectrometry

(LC-MS/MS) and Liquid Chromatography-High Resolution-Mass Spectrometry (LC-HRMS) analysis was performed to evaluate the fate of the ingested CPT in the insect body. We found that most of the ingested CPT is sequestered in the wings without any major metabolizable products. We discuss these results in the light of the possible mechanisms by which the beetles are able to tolerate substantially high levels of CPT in their body tissue.

Methods and Materials

Sampling and Maintenance of Adult Beetles We collected chrysomelid beetles feeding on leaves of *N. nimmoniana* from Biligiri Rangaswamy Temple (BRT) Wildlife Sanctuary (11.40° to 12.09° N and 77.05° to 77.15° E) in the state of Karnataka, India. The beetles were brought to the laboratory, School of Ecology and Conservation, University of Agricultural Sciences, Bangalore, India and maintained in culture jars on excised leaves of *N. nimmoniana*. Leaves were replaced every day. Adults fed actively under captivity. Throughout the culture period, the pellets (excreta) were collected. Beetles also laid eggs during this period (some of these eggs failed to hatch even after several months of incubation and hence were considered 'sterile'). These also were collected. Voucher specimens of the beetle (*Kanarella unicolor* Jacobby, subfamily Galerucinae, tribe Luperini, order Coleoptera) have been deposited in the Insect Museum, Department of Entomology, University of Agricultural Sciences, Bangalore, India.

Extraction of Camptothecins At the end of 25 days of culture, beetles (N=33) were collected, pooled, and processed for analysis of CPT and related compounds. Beetles were dissected into different parts; head, abdomen (including legs), and wings (hind and fore wings). While separating the abdomen, care was taken to ensure removal of undigested leaf material in the gut to avoid contamination of CPT from the host tissue. Insect pellets and sterile eggs collected over the culture period also were used for chemical analysis. The respective body parts, pooled from the 33 beetles, were ground in a pestle and mortar, and resultant fine powder was extracted for camptothecins. A known amount of tissue powder from each of the samples was extracted in 10 ml of 80% ethanol at 60°C for 3 hr in a shaking water bath. After cooling to room temperature, the extract was centrifuged at 10,000 rpm for 10 min at 10°C (Hermle, Germany). The supernatant was passed through a 0.2 μ filter (Tarsons, India) and analyzed for camptothecins.

LC-MS/MS Quantification of Camptothecins LC-MS/MS analysis of the extracts was performed using a Luna C18 column (0.3×150 mm, 3 μm Phenomenex, Torrance, CA, USA), with a mobile phase of H_2O with 10 mM NH_4OAc (solvent A) and CH_3CN with 0.1% HCO_2H (solvent B). All separations were performed at 30°C. NH_4OAc, HCO_2H, CH_3CN (Merck, Germany) and ultra-pure H_2O (Millipore, Schwalbach, Germany) were HPLC grade. Gradient elution consisted of the following ratios of solvent A/solvent B: 95:5, 48:52 and 0:100, with a total run time of 12 min. The column was then returned to the initial condition of 95:5 over about 0.5 min. After each run the column was re-equilibrated for 2 min. The flow rate was 0.3 ml/min with an injection volume of 10 μl.

Selected reaction monitoring (SRM) was performed using a highly sensitive TSQ quantum ultra AM mass spectrometer (Thermo, Finnigan) equipped with an ESI ion source (Ion Max) operating in positive mode. Nitrogen was employed as both the drying and nebulizer gas. Each mass transition was monitored at a peak width of 1 and a dwell time of 0.3 sec. External calibration was performed with the authentic standards in the concentration range of 0.005-10 μg/ml for CPT and 10-OH-CPT, and 0.005-1 μg/ml for 9-MeO-CPT. Limits of detection (LOD) for CPT, 10-OH-CPT and 9-MeO-CPT were 10, 5, and 4 ng/ml, respectively, providing further evidence of the high sensitivity of the system.

LC-HRMS Identification of Carboxylate, Glucuronilated and Sulfated Forms of Camptothecins Carboxylate, glucuronilated, and sulfated forms of CPT were identified based on their exact mass and retention times in LC-HRMS. Exact mass of all these CPT forms were calculated based on the molecular formula of the respective derivatives. The spectrometer was equipped with a Dionex micro-HPLC system Ultimate 3000 consisting of pump, flow manager and auto sampler with an injection volume of 0.6 μl. Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. Separations were performed by using a C18 column (0.3×150 mm, 3 μm, Phenomenex Gemini Torrance, CA, USA) with mobile phase H_2O containing 0.1% HCO_2H (solvent A) and CH_3CN with 0.1% HCO_2H (solvent B) and with a flow rate of 4 μl/min. Samples were analyzed using a gradient program with the following ratios of solvent A/solvent B: 95:5, 70:30, 30:70, 0:100, with run times of 0–5, 5–12, 12–24, 24–30 min, respectively. After 100% B, the system returned to its initial condition of 95% A within 1 min. After each run, the system was re-calibrated for 6 min. Owing to the relatively high solubility of carboxylate, glucuronilated and sulfated forms of CPT compared to parental CPT, we expected the elution of these forms to be much earlier than CPT or 10-OH-CPT.

The spectrometer was operated in positive mode with nominal mass resolving power of 60000 at *m/z* 400 (1 spectrum/*s* and with a mass range of 200–800). The scan rate

Fig. 1 Chrysomelid beetle feeding on *Nothapodytes nimmoniana* leaf

was 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation. Bis-(2-ethylhexyl)-phthalate (DEHP) was used as internal lock mass of *m/z* 391.28429.

Results and Discussion

During an extensive survey of the natural populations of *Nothapodytes nimmoniana* in its distributional range (8° to 15° N) in southern India, we found a fairly consistent occurrence of chrysomelid beetles (*Kanarella unicolor* Jacobby, subfamily Galerucinae, tribe Luperini, order Coleoptera) feeding on the leaves (Fig. 1). Only adult beetles were found on the plant; we did not recover larvae and pupa. The adults feed exclusively on *N. nimmoniana* by making small perforations on the leaf lamina. Beetles

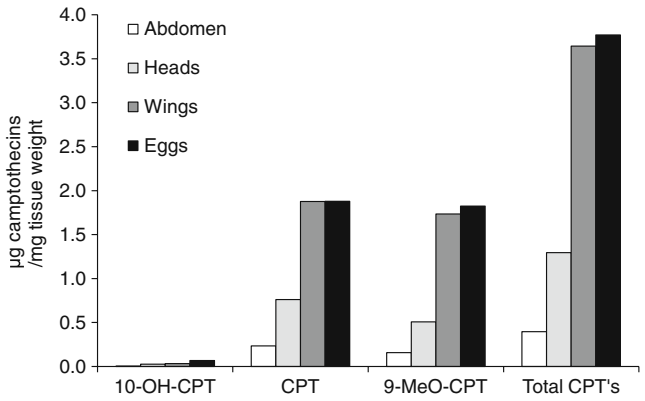

Fig. 3 Concentration of CPT and its metabolites in different body parts of beetles as quantified by LC-MS/MS. All data refer to a single analysis of all beetles (N=33) pooled

fed randomly on leaves irrespective of the leaf age and their CPT content (supplementary material Fig. S1).

For a set of laboratory maintained beetles that were fed exclusively on *N. nimmoniana* leaves for 25 days, we examined the relative proportions of CPT and related compounds in different body parts. LC-MS/MS of samples revealed the presence of CPT in head, abdomen, wings (both hind and fore wings) and also in the eggs laid by the beetles (Fig. 2). Other natural derivatives of CPT such as 10-hydroxy camptothecin (10-OH-CPT) and 9-methoxy camptothecin (9-MeO-CPT) that are known to be present in host leaves also were detected. Concentration of CPT was highest in wings (1.87 µg/mg-tissue weight), followed by head (0.76 µg/mg-tissue weight), and least in the abdomen (0.23 µg/mg-tissue weight). A similar pattern also was observed for 10-OH-CPT and 9-MeO-CPT, except the concentration of 10-OH-CPT was 10–15 fold less compared to other two compounds. In some samples, its concentration was negligible (Fig. 3).

We also examined whether or not the beetles metabolize CPT into its inactive forms, the carboxylate, glucuronilated or sulfated forms. None of the sulfated and glucuronilated forms

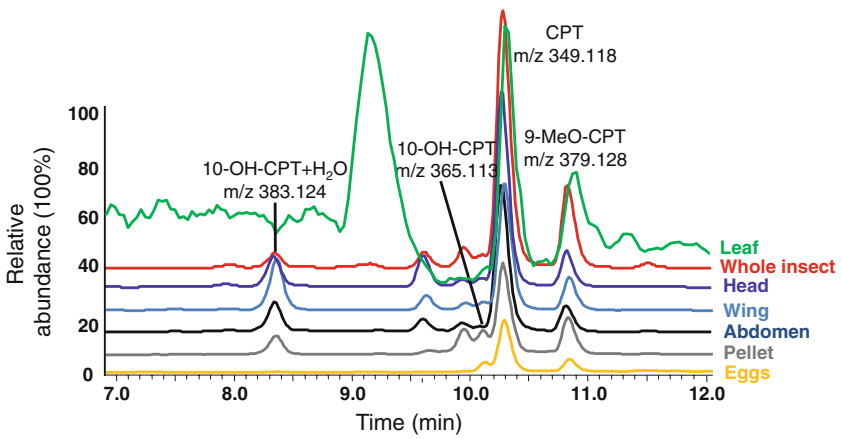

Fig. 2 Extracted total ion chromatograms (TIC) of *N. nimmoniana* leaf and different body extracts of beetle including eggs and pellets. TIC's of different samples were overlaid on each other and hence, except TIC of eggs, TIC's of other extracts does not start from 0% ionic abundance. Intensity of the peaks does not correlate with concentration of metabolites in different body parts. The large peak at ~9 min in the leaf sample is n-butyl benzenesulfoamide, plasticizer ($[M+H]^+$ 214.08963)

of CPT was detected in any of the body parts, when analyzed for their exact mass traces in LC-HRMS; sulfated CPT (cald; m/z 429.0750), sulfated 10-OH-CPT (cald; m/z 445.072), sulfated 9-MeO-CPT (cald; m/z 459.085), glucuronilated CPT (cald; m/z 525.152), glucuronilated 10-OH-CPT (cald; m/z 541.147), glucuronilated 9-MeO-CPT (cald; m/z 555.162). However, we did recover the carboxylate form of 10-OH-CPT (Rt 8.3 min, m/z 383.124) (Supplementary material Fig. S2) in all body parts including the pellets. The low concentration of carboxylate form of 10-OH-CPT (based on intensity in TIC spectra) compared to its parental form, however, suggests that the insects do not metabolize CPT to any substantial degree (Fig. 2).

In summary, the highest concentration of CPT and related compounds (10-OH-CPT and 9-MeO-CPT) was located in the wings (3.64 μg/mg-tissue weight amounting to 54% of total body CPTs), followed by abdomen (0.39 μg/mg-tissue weight, amounting to 28%), and head (1.29 μg/mg-tissue, amounting to 18%). On a single beetle body weight basis, disregarding the loss of CPT through pellets, the beetles accumulated about 3.02 μg of CPT at any given point of time, much higher than 1.7 μg/larvae that are lethal to Drosophila larvae (Cunha et al., 2002).

Sequestration of plant secondary metabolites, as predator deterrence behavior is well documented in insects, including beetles (Hartmann et al., 2001; Willinger and Dobler, 2001). A number of insects have developed adaptations to sequester pyrrolizidine alkaloids from their host plants and utilize them against their own predators (Hartmann et al., 2001). Frequently, the ingested plant secondary metabolite are either metabolized into products that are not detrimental to the insects or sequestered from the haemolymph into exocrine glands or into metabolically poor tissues such as the pronotum and elytra (Aregullin and Rodriguez, 2003); the latter serves as a "defense shield" against predators. Our results are the first to report the sequestration of CPT, a cytotoxic compounds by beetles. As indicated,, most of the CPT is sequestered in the parental chemical form in the elytra, without any major chemical conversion or metabolism. While we have not yet established the evolutionary significance of such sequestration, it is likely that the beetles use this compound to deter predators (Schroeder et al., 2006; Despres et al., 2007). It would be interesting to examine the mechanism through which the beetles tolerate high levels of CPT. Recently, Sirikantaramas et al. (2008) showed that plant species that produce CPT have specific mutations in their topoisomerase I that make these enzymes resistant to CPT. We are currently investigating if beetles have such mutations in their topoisomerase I.

Acknowledgements We thank Prof. C. A. Virakthamath and Dr. K. Chandrashekara, Department of Entomology and School of Ecology and Conservation, University of Agricultural Sciences, Bangalore for help in identification of the beetle.

References

AREGULLIN, M., and RODRIGUEZ, E. 2003. Sesquiterpene lactone sequestration by the tortoise beetle *Physonota arizonae* (Cassidinae). *J. Mex. Chem. Soc*. 47:143–145.

CUNHA, K. S., REGULY, M. L., GRAF, U., and RODRIGUES DE ANDRADE, H. H. 2002. Comparison of camptothecin derivatives presently in clinical trials: genotoxic potency and mitotic recombination. *Mutagenesis*. 17:141–147.

DESPRES, L., DAVID, J. P., and GALLET, C. 2007. The evolutionary ecology of insect resistance to plant chemicals. *Trends Ecol. Evol*. 22:298–307.

HARTMANN, T., THEURING, C., WITTE, L., and PASTEELS, J. M. 2001. Sequestration, metabolism and partial synthesis of tertiary pyrrolizidine alkaloids by the neotropical leaf-beetle *Platyphora boucardi*. *Insect Biochem. Mol. Biol*. 31:1041–1056.

HSIANG, Y. H., HERTZBERG, R., HECHT, S., and LIU, L. F. 1985. Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase–I. *J. Biol. Chem.* 260:14873–14878.

LARSSON, S. 2007. The new chemosystematics: phylogeny and phytochemistry. *Phytochemistry* 68:2903–2907.

LI, S. 2002. Utilizing *Camptotheca* products for termite control. US Patent Application No. 20020018762.

POMMIER, Y. 2006. Topoisomerase I inhibitors: camptothecins and beyond. *Nat. Rev. Cancer* 6:789–802.

RAMESHA, B. T., AMNA, T., RAVIKANTH, G., RAJESH GUNAGA, P., VASUDEVA, R., GANESHAIAH, K. N., UMA SHAANKER, R., KHAJURIA, R. K., PURI, S. C., and QAZI, G. N. 2008. Prospecting for camptothecins from *Nothapodytes nimmoniana* in the Western Ghats, south India: identification of high yielding sources of camptothecin and new families of camptothecins. *J. Chromatogr. Sci.* 46:362–368.

SCHROEDER, F. C., DEL CAMPO, M. L., GRANT, J. B., WEIBEL, D. B., SMEDLEY, S. R., BOLTON, K. L., MEINWALD, J., EISNER, T. 2006. Pinoresinol: A lignol of plant origin serving for defense in a caterpillar. *Proc. Natl. Acad. Sci. U. S. A*. 103:15497–15501.

SIRIKANTARAMAS, S., YAMAZAKI, M., and SAITO, K. 2008. Mutations in topoisomerase-I as a self-resistance mechanism coevolved with the production of the anticancer alkaloid camptothecin in plants. *Proc. Natl. Acad. Sci. U.S.A*. 105:6782–6786.

WILLINGER, G., and DOBLER, S. 2001. Selective sequestration of iridoid glycosides from their host plants in *Longitarsus* flea beetles. *Biochem. Syst. Ecol*. 29:335–346.