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Biological Activity of Twenty-three Hydantoin Derivatives on Intrinsic Efflux Pump System of *Salmonella enterica* serovar Enteritidis NCTC 13349

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Abstract. *Background: Hydantoin derivatives have important biochemical and pharmacological properties. In the present study, 23 hydantoin compounds were evaluated for their efflux-modulating effects in Salmonella enterica serovar Enteritidis NCTC 13349 using real-time fluorimetry based on the intracellular accumulation of ethidium bromide (EB), a universal substrate of efflux pumps. Materials and Methods: Twenty-three hydantoin derivatives were tested for intrinsic efflux pump modulation in S. enterica Enteritidis NCTC 13349 by an automated real-time fluorimetric method that monitors the intracellular accumulation of ethidium bromide. Results: None of the compounds were found to have antibacterial activities at concentrations as high as 200 mg/l. Among the hydantoin derivatives tested in our study, only compound SZ7 showed efflux modulating activity on S. enterica Enteritidis NCTC 13349 in the assays that contained or omitted glucose as the singular source of metabolic energy. Conclusion: SZ7 is shown to be non-toxic and it could be evaluated to cure an efflux mediated MDR Salmonella infection, furthermore a quantitative activity-structural relationship is also planned in order to determine the site of the SZ7 molecule responsible for its efflux pump inhibitory activity.*

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Hydantoins play an important role in the purine catabolic pathway that regulates the purine pool in the cell to provide precursors for nucleic acid synthesis (1). In addition, hydantoinases have essential metabolic function because they hydrolyse hydantoin and 5'-monosubstituted hydantoin derivatives, and for this reason their biotechnological application is valuable in the production of optically pure amino acids (2). The nucleobase cation symport-1 (NCS1) transporters are essential components of salvage pathways for nucleobases and related metabolites, *e.g.* NCS1 benzyl-hydantoin transporter, Mhp1, from *Microbacterium liquefaciens* (3).

Besides these biochemical processes, hydantoins have pharmacological properties and are used to treat many human diseases. A well known example of a drug featuring a hydantoin is phenytoin (5,5-diphenylhydantoin, Dilantin), which has been used for decades to treat epilepsy (4). Hydantoins have different pharmacological properties depending on the nature of substitution on the hydantoin ring, *e.g.* fungicidal, herbicidal, antitumor, anti-inflammatory, anti-HIV, hypolipidemic, antiarrhythmic and antihypertensive activities, have also been identified (5-8). Furthermore, it has been demonstrated that 5-arylidene-2-thiohydantoins have *in vitro* antimycobacterial activity (9).

Overexpression of efflux pumps that extrude a variety of structurally unrelated antibiotics are associated with multidrug resistant (MDR) phenotypes in Gram-negative bacteria. The characterization of efflux activity has been essentially restricted to bacteria that overexpress efflux components (10). However, the intrinsic efflux pump apparatus of the wild-type Gram-negative strains that colonise the colon and produce infection, provides the means by which the bacterium survives its journey through the

gastrointestinal system of the host, a system that threatens the existence of the bacterium exposing it to pH as low as 2, and to toxic agents such as bile salts (11). The bacterium responds to the presence of antibiotics by overexpressing its efflux pump system (12). Therefore, it is the intrinsic efflux pump system of Gram-negative wild-type bacteria that merits full characterization.

Salmonella strains have very well developed mechanisms of resistance to antibiotics and hence an infection of the colon is very difficult to treat with conventional antibiotics. Because these infections are usually self-limiting due to the diarrhea that flushes out the organism, they are not treated unless the infection takes place in an infant, a geriatric patient, or a patient that has has a resection of the colon (13). The mechanisms that render the organism resistant to antibiotics when the organism infects the human are site specific. In the colon, down-regulation of porins of the bacterium, thereby reducing the amount of hydrophilic antibiotic that enters the cell (14) and up-regulation of the main efflux pump AcrAB that extrudes noxious agents and antibiotics prior to their reaching their intended targets (14), take place. When the organism penetrates the epithelial barrier of the colon, it is rapidly phagocytosed by neutrophils. However, the organism survives because the very low pH of the phagolysosomal vacuole activates the PmrA/B two component regulon of the organism and the synthesis of lipid A and its subsequent introduction into the nascent lipopolysaccharide layer prevent the degradation of the organism and make the organism resistant to practically everything (15).

We have conducted a search for agents that may inhibit the efflux pump apparatus of *Salmonella enterica* serovar Enteritidis NCTC 13349 which, if effective and proven non-toxic, may progress to clinical consideration for therapy of intestinal salmonellosis. Because some members of a series of hydantoin compounds had significant activity against the ABCB1 ABC transporter of cancer cells (16), and many such inhibitors also inhibit proton motive force-dependent efflux pumps of Gram-negative bacteria (17), in the present study, we evaluated 23 hydantoin compounds for their modulating effects on the intrinsic efflux system of Gram-negative bacteria *via* the use of an automated real-time fluorimetric method that monitors the intracellular accumulation of ethidium bromide (10, 18).

Materials and Methods

Compounds and materials. Twenty-three hydantoin derivatives (SZ2, SZ7, LL-9, BS-1, JH-63, MN-3, TD-7k, GG-5k, P3, P7, RW-15b, AD-26, RW-13, AD-29, KF-2, PDPH-3, Mor-1, KK-XV, Thioam-1, JHF-1, JHC-2, JHP-1, Fur-2), kindly provided by Dr. Jadwiga Handzlik and Professor Katarzyna Kiec-Kononowicz (Cracow, Poland) were evaluated for effects on the efflux pump system of a selected Gram-negative bacterium. The compounds were dissolved in DMSO. Ethidium bromide (EB), thioridazine (TZ), CCCP (carbonyl cyanide m-chlorophenylhydrazone) and palmitic acid purchased from Sigma (Madrid, Spain).

Mueller-Hinton Broth (MHB) in powder form was purchased from Difco (Madrid, Spain) for the preparation of broth and agar; tryptic soy broth (TSB) was purchased from Scharlau Chemie S.A. (Barcelona, Spain).

Bacterial strain. *Salmonella enterica* serovar Enteritidis NCTC 13349 was kindly provided by Professor Seamus Fanning (School of Agriculture, Food Science, and Veterinary Medicine, UCD Veterinary Sciences Centre, University College Dublin, Dublin, Ireland)

Determination of minimum inhibitory concentrations (MICs). The MICs of compounds, including EB, were determined by using broth microdilution method in Mueller-Hinton Broth (MHB), according to Clinical and Laboratory Standards Institute (CLSI) recommendations (19).

Real-time EB accumulation assay. The activity of compounds on the real-time accumulation of EB was assessed by the automated EB method, previously described in detail elsewhere (20), using a Rotor-Gene 3000™ thermocycler with real-time analysis software (Corbett Research, Australia). Briefly, strains were cultured in MHB medium until they reached an optical density (OD) of 0.6 at 600 nm; they were then centrifuged at 13,000 rpm for 3 min, the pellets were re-suspended in phosphate-buffered saline (PBS) (pH 7.4) with and without a final concentration of glucose of 0.4% and the OD adjusted to 0.6 at 600 nm. Aliquots of 45 μl of the cell suspension were distributed to 0.2 ml tubes. The compounds were individually added at different concentrations (40, 80 and 120 mg/l final concentration) in 5 μl volumes of their stock solutions, and finally 45 μl of EB was added to yield a final concentration of 1 mg/l in PBS with and without glucose. It is important to note that prior to the experiments described, the maximum concentration of EB which was within the capacity of the bacterium to extrude was determined at least three times. For the wild-type reference bacterial strain employed in the study, this concentration of EB was determined to be 1 mg/l (10, 18). The tubes were placed into a Rotor-Gene 3000™ thermocycler and the fluorescence monitored on a real-time basis.

From the real-time data, the activity of the compound, namely the relative final fluorescence (RFF) of the last time point (minute 60) of the EB accumulation assay was calculated according to the formula:

$$RFF = \frac{RF_{treated} - RF_{untreated}}{RF_{untreated}}$$

$RF_{treated}$=relative fluorescence (RF) at the last time point of EB retention curve in the presence of an inhibitor.
$RF_{untreated}$=relative fluorescence at the last time point of the EB retention curve of the untreated control having the solvent control (DMSO). The greater the difference between $RF_{treated}$ and $RF_{untreated}$ the greater the degree of the EB accumulated and, therefore, the greater the degree of inhibition of the efflux pump system of the bacterium by the agent. Thioridazine, a compound that inhibits the efflux pump system of *Salmonella* (21) served as a positive control for the inhibition of efflux of EB.

The experiments were repeated three times and the RFF values presented are the average of three independent assays.

Results

Twenty-three hydantoin compounds were tested for their efflux modulating effects on *S. enterica* Enteritidis NCTC 13349 by using real-time fluorometry (20). The use of fluorometry provides a powerful tool to continuously monitor the transport of fluorescent substrates *e.g.* EB, through the cell envelope of living bacterial cells, because they penetrate the bacterial cell wall and accumulate in the periplasmic space of Gram-negative bacteria when the concentration exceeds the capacity of the efflux pump system (10) or its extrusion by the efflux pump system is reduced by an agent (20, 22).

If an agent is to be studied for activity against an efflux pump system it must be added at concentrations that do not affect the replication or viability of the cell (10). Because the MICs of the 23 hydantoin derivatives exceeded 500 mg/l (data not shown), the sub-inhibitory concentrations of the hydantoins employed were 40, 80 and 120 mg/ml. Because of the largesse of the number of figures depicting the effects of each compound at three concentrations for each of the 23 hydantoin compounds in an assay that omitted and contained metabolic energy (glucose), the results are presented in tabular form. As evident from Table I, in the evaluation of the activity of the twenty three hydantoin compounds on the efflux pump of the *S. enterica* Enteritidis NCTC 13349, only compound SZ7 was effective at the three concentrations in the assays that lacked and contained glucose as the metabolic source of energy.

Table I. *Relative final fluorescence (RFF) based on the accumulation of ethidium bromide (1mg/l) with and without glucose by Salmonella enterica serovar Enteritidis NCTC 13349 in the presence of three concentrations of the hydantoin derivatives.*

	Concentrations used					
	No glucose			Glucose		
Agent	40 mg/l	80 mg/l	120 mg/l	40 mg/l	80 mg/l	120 mg/l
SZ2	0.7	2.1	2.8	0.05	–0.09	0.24
SZ7	4.4	5.2	7.4	2.40	3.46	2.62
LL-9	–0.1	0.1	–0.1	–0.35	–0.20	0.02
BS-1	–0.02	0.1	0.1	–0.32	0.06	–0.01
JH-63	0	0.3	0.5	–0.26	0.00	0.00
MN-3	0.1	0.2	0.4	–0.28	–0.21	0.58
TD-7k	–0.1	–0.1	–0.1	–0.30	0.14	–0.07
GG-5K	–0.04	–0.2	–0.2	–0.27	–0.54	–0.22
P3	0.5	1.1	1.4	–0.32	–0.18	–0.12
P7	0.2	0.3	0.3	–0.69	–0.23	–0.06
RW-15b	0.4	1.0	0.9	–0.41	–0.09	0.21
AD-26	0.4	0.8	0.4	–0.22	–0.07	–0.13
RW-13	–0.1	–0.01	–0.1	–0.05	0.14	0.04
AD-29	0.3	0.5	0.3	–0.13	–0.20	–0.18
KF-2	0.01	0.02	0.1	–0.41	–0.44	–0.04
PDPH-3	–0.1	0.1	–0.1	–0.46	–0.36	–0.31
Mor-1	0.1	0.2	0.1	–0.59	–0.35	–0.10
KK-XV	0.03	0.04	–0.1	–0.42	–0.15	–0.23
Thioam-1	1.1	0.8	1.0	0.50	1.49	1.83
JHF-1	0.1	0.1	0.04	–0.20	–0.14	–0.36
JHC-2	0.1	0.2	0.3	–0.40	–0.27	–0.14
JHP-1	0.1	–0.1	–0.1	–0.49	–0.21	0.02
Fur-2	0.2	–0.1	–0.1	–0.38	–0.17	0.04

Assay of ethidium bromide accumulation conducted in PBS with and without glucose. The index of activity of three concentrations of each of the twenty three hydantoin compounds against the efflux pump system of the *Salmonella enterica* serovar Enteritidis NCTC 13349 reference strain was calculated with the aid of the formula described in the Materials and Methods. Thioridazine at a concentration of 50 mg/l in the assay containing glucose served as a positive control and yielded an index of activity of 5.55.

Discussion

The design of potent and selective efflux pump inhibitors requires extensive screening of compounds of natural or synthetic origin. Real-time fluorimetry is a very useful tool to screen a great number of compounds, *e.g.* a series of hydantoin derivatives, because it provides information about transport kinetics and thereby offers a rapid, high-throughput, reproducible, accurate and inexpensive screening of efflux pump inhibitors. However, it must be stated quite strongly that if the automated EB assay is to be employed for the evaluation of compounds on the efflux system of a bacterium, the assay must be conducted with glucose if a correct interpretation of an effect is to be made. In addition, the selection of the pH of the medium should approximate that of interstitial fluids, namely, a pH of 7.4, if the *in vitro* activity of the agent against the efflux pump system of a given bacterium is to be meaningful *in vivo*. It was for this reason that the assays described were conducted at pH 7.4.

In the described study, with the exception of one compound SZ7, none of the other hydantoin compounds had any activity against the efflux pump system of this species of *Salmonella*. Because of the efflux pump-mediated MDR of *Salmonella*, a compound that targets the efflux pump responsible for the MDR phenotype may have potential as an adjunct to antibiotic therapy. Nevertheless, hydantoin compounds are usually non-toxic and if SZ7 is shown to be non-toxic, we will be evaluating its potential to cure infection of a mouse with efflux-mediated MDR *Salmonella*. Lastly, a quantitative activity-structural relationship is also planned in order to determine the site of the SZ7 molecule responsible for its efflux pump inhibitory activity.

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