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CORRESPONDENCE

Comment on a Published Paper

[1]Helen M. Dodds & *,[1,2]Laurent P. Rivory

[1]Department of Pharmacology, University of Sydney, NSW 2006, Australia and [2]Department of Medical Oncology, The Sydney Cancer Center, Sydney, NSW 2050, Australia

In a recent study, Blandizzi *et al*. (*Br. J. Pharmacol*., 132: 73–84, 2001) reported that the adverse cholinergic effects of the chemotherapeutic agent irinotecan (CPT-11) were unlikely to be mediated by the inhibition of acetylcholinesterase (AChE). This observation was based on results of *in vitro* assays performed on AChE from two sources (human erythrocyte and electric eel). They reported only modest inhibition of AChE even at the highest concentration of CPT-11 used (100 μM). As pointed out by the authors, these data are not consistent with our own results (Dodds & Rivory, 1999), and those of others using very similar systems in which values of 0.2 μM for the IC_{50} have been consistently reported (Kawato *et al*., 1993; Rivory *et al*., 1996; Morton *et al*., 1999). In our most recent study, we demonstrated that CPT-11 is a potent inhibitor of AChE at clinically relevant concentrations and revealed its mechanism of inhibition as being instantly reversible and apparently non-competitive (Dodds & Rivory, 1999). On reading the paper by Blandizzi *et al*. (2001), we could not explain the reported discrepancy. We therefore set out to replicate exactly the system used by these authors to investigate this further, given that even minor experimental differences could impact on the results obtained. In particular, Riddles *et al*. (1979) have recommended that reactions be carried out at a pH of 7.3 and at 25°C (rather than pH 8.0 and 37°C) to ensure the maximum stability of the 5,5′-dithio-bis(2-nitrobenzoic acid) during incubation. Also, CPT-11, as with all camptothecins, hydrolyses in a rapid but reversible reaction to open-ring carboxylate forms with very different pharmacological properties, including their ability to inhibit AChE (Dodds & Rivory, 1999). We have previously shown the lactone form of CPT-11 to be ~10 fold more potent at inhibiting both human and electric eel AChE (Dodds & Rivory, 1999).

We repeated the experiments of Blandizzi *et al*. (2001) using both identical conditions and those we have used in the past. The total assay volume was 3.2 ml and included ATChI as substrate (at same concentration) and 3 u ml^{-1} of AChE (electric eel). The final concentrations of CPT-11 (lactone) investigated ranged from 0–0.78 μM and these were either spiked into the incubation mix immediately prior to the reaction or pre-incubated with AChE for 20 min (as per Blandizzi *et al*., 2001). The concentration resulting in a 50% reduction of enzyme activity (IC_{50}) was estimated from the resulting plots. All assays were performed in triplicate and expressed as mean ± s.d. As summarized in Table 1, there were subtle differences in the potency of the inhibition between the conditions used. At the lower temperature, CPT-11 was a more potent inhibitor. Also, pre-incubation for 20 min resulted in less inhibition of AChE, consistent with the hydrolysis of the lactone form under these conditions (Rivory *et al*., 1994). These effects, when combined, led to an ~5 fold loss in potency at pH 8. However, they do not explain the >1000 lower inhibitory capacity of CPT-11 in the hands of Blandizzi *et al*. (2001). In contrast, the IC_{50} obtained by Blandizzi *et al*. (2001) for physostigmine in the same system is compatible with the literature.

In conclusion, we are unable to explain the results of Blandizzi *et al*. (2001). Although we show that the classical experimental variables (temperature, pH and time of incubation) can all impact on the potency of the inhibition of AChE by CPT-11, there must remain additional and unknown factors that dramatically affect the potency of CPT-11. These factors merit further elucidation.

Table 1 Interaction of CPT-11 (lactone) with AChE under different assay conditions

	IC_{50} (μM), pH 7.3		IC_{50} (μM), pH 8.0	
Temperature	*t* = 0	*t* = 20 min	*t* = 0	*t* = 20 min
25°C	0.069 ± 0.003	0.099 ± 0.003	0.088 ± 0.002	0.135 ± 0.007
37°C	0.137 ± 0.015	0.277 ± 0.019	0.148 ± 0.011	0.344 ± 0.026

*Author for correspondence at: Department of Medical Oncology, The Sydney Cancer Center, Level 6 Gloucester House, Royal Prince Alfred Hospital, Camperdown, 2050 NSW, Australia;
E-mail: lrivory@canc.rpa.cs.nsw.gov.au

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Reply to Dodds & Rivory

[1]C. Blandizzi, [1]B. De Paolis, [1]R. Colucci, [1]G. Lazzeri, [1]F. Baschiera & *,[1]M. Del Tacca

[1]Division of Pharmacology and Chemotherapy, Department of Oncology, Transplants and Advanced Technologies in Medicine, University of Pisa, Pisa, Italy

We appreciate the interest of H.M. Dodds and L.P. Rivory in our study on the mechanisms related to the acute cholinergic effects of irinotecan (Blandizzi *et al*., *Br. J. Pharmacol*., 132: 73–84, 2001). However, despite the efforts made to reproduce our experiments on the *in vitro* acetylcholinesterase (AChE) assay, we note that these authors still fail to propose any convincing explanation accounting for the discrepancy between our data (Blandizzi *et al*., 2001) and their repeated observations (Dodds & Rivory, 1999). Therefore, it is conceivable that *in vitro* assays, carried out on purified preparations of AChE extracted from various sources, do not offer sufficient levels of reliability to allow a clear assessment of the putative anti-AChE properties of irinotecan. For instance, while in their letter Dodds and Rivory emphasize the fact that the incubation time of irinotecan with AChE must be kept as close as possible to zero, at a temperature of 25°C, to avoid spontaneous degradation of the drug, Morton *et al*. (1999) allowed an incubation time of 22 h at 37°C, which is much longer than that adopted in our study (20 min).

We wish also to point out that, beyond the debate concerning the *in vitro* effects of irinotecan on AChE activity, several lines of evidence, emerging from the variety of experimental models adopted in our study (Blandizzi *et al*., 2001), as well as from previous clinical reports (Gandia *et al*., 1993; Rowinsky *et al*., 1994), argue against the hypothesis that AChE blockade plays a significant role in the acute cholinergic syndrome evoked by irinotecan. In particular, the following points are worthy to be considered:

(1) At variance with physostigmine (0.01–0.1 μM), irinotecan (tested up to 100 μM) did not significantly affect the atropine-sensitive motor responses induced by electrical stimulation of human colonic longitudinal muscle (Blandizzi *et al*., 2001).

(2) Irinotecan moderately enhanced the cholinergic twitch contractions of guinea-pig ileum longitudinal muscle strips only when applied at very high concentrations (Blandizzi *et al*., 2001). Of interest, in this model there is no need for long pre-incubation time, and changes in motor activity of ileal smooth muscle can usually be recorded immediately after spiking the test drugs into the organ bath solution.

(3) The measurement of electrically-induced acetylcholine release, from longitudinal muscle strips of guinea-pig ileum, represents a highly suitable technique in studies aiming to characterize the influence of drugs on synaptic cholinergic neurotransmission. When tested on this model, irinotecan (up to 100 μM) slightly inhibited acetylcholine release, whereas, as expected, physostigmine (0.1 μM) caused a marked decrease through an indirect activation of muscarinic autoreceptors located on cholinergic axon terminals (Blandizzi *et al*., 2001).

(4) In the clinical setting, we could not detect any significant inhibition of AChE activity in the blood of colorectal cancer patients subjected to treatment with irinotecan (Blandizzi *et al*., 2000). In this study, irinotecan was infused at the dose of 200–350 mg/m^2 over 60 min, and whole blood samples were taken immediately before (baseline) as well as 15 and 45 min after the beginning of drug infusion. Interestingly, similar data have been reported also by Gandia *et al*. (1993), who measured AChE activity in erythrocytes isolated from cancer patients under infusion with irinotecan.

*Author for correspondence at: Divisione di Farmacologia e Chemioterapia, Dipartimento di Oncologia, dei Trapianti e delle Nuove Tecnologie in Medicina, Università di Pisa, Via Roma, 55, 56126 Pisa, Italy; E-mail: m.deltacca@do.med.unipi.it

(5) When examining the features of the acute syndrome evoked by irinotecan (Gandia *et al*., 1993; Rowinsky *et al*., 1994), it can be noted that all symptoms are consistent with a picture of parasympathetic activation, leading to the recruitment of muscarinic receptors on peripheral effector organs. However, drugs causing a systemic blockade of AChE are expected also to promote a concomitant activation of nicotinic receptors, with the subsequent occurrence of symptoms, like for instance altered patterns of skeletal muscle motor activity, that have not been observed in patients treated with irinotecan (Gandia *et al*., 1993; Rowinsky *et al*., 1994).

In conclusion, much work remains to be done, at experimental level, to clarify the differences of Dodds and Rivory (1999) data on irinotecan-induced inhibition of AChE, compared with our results. However, in order to better elucidate the pathophysiological mechanisms which subserve the cholinergic side effects of irinotecan, we do not believe that *in vitro* pharmacological assays of AChE can be regarded as critical tests until they are unconfirmed on functional basis by means of more integrated methodological procedures.

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