

In vitro genotoxicity of rocuronium bromide in human peripheral lymphocytes

Umit Zan · Mehmet Topaktas · Erman Salih Istifli

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Abstract Rocuronium bromide (RB), an aminosteroid type neuromuscular blocking agent, acts by reducing or inhibiting the depolarising effect of acetylcholine on the terminal disc of the muscle cell. To our knowledge, there is no adequate information on the genotoxic effects of RB, up to now. In the present study, possible genotoxic effects of RB have been determined by means of sister chromatid exchange (SCE), chromosome aberration (CA) and micronucleus (MN) analyses in human peripheral blood lymphocytes. The human peripheral blood lymphocytes were exposed to three different concentrations of RB (60, 80 and 100 µg/mL) for 24- and 48-h. In this study, RB increased the frequency of CAs, however, did not increase the frequency of SCEs. RB did not decrease the proliferation index (PI) and mitotic index (MI). Accordingly, RB increased the frequency of micronucleus (MN) but did not decrease the nuclear division index (NDI). Findings from this study suggest that rocuronium bromide is clastogenic but not cytotoxic to cultured human peripheral blood lymphocytes.

Keywords Rocuronium bromide · Human peripheral blood lymphocytes · SCE · CA · MN

Introduction

Rocuronium bromide (RB), a derivative of 3-hydroxy metabolite of vecuronium, is a steroidal non-depolarising aminosteroid neuromuscular blocking agent, which couples with the acetylcholine receptors to reduce or inhibit the depolarising effect of acetylcholine on the terminal disc of the muscle cell. A comprehensive literature search showed that RB has never been investigated based on its genotoxic-carcinogenic properties except the data from Physician's Desk Reference which demonstrates negative results for the genotoxicity and mutagenicity of RB (Synder and Green 2001; Synder et al. 2004; Synder 2009). The Norwegian Medicines Agency proposed that RB be withdrawn from routine practice due to frequent reports of anaphylaxis (Laake and Røttingen 2001), and 41 RB-related anaphylactic incidents were reported in France in a survey between 1994 and 1996 (Heier and Guttormsen 2000). Similar anaphylactic and bronchospasm incidents were described in the literature related to the use of other aminosteroid type muscle relaxants (Mishima and Yamamura 1984; O'Callaghan et al. 1986; Farrell et al. 1988; Treuren and Buckley 1990; Kumar et al. 1993; Baird and Futter 1996; Clendenen

U. Zan · E. S. Istifli
Department of Biology, Institute of Basic and Applied
Sciences, Çukurova University, Adana, Turkey

M. Topaktas (✉)
Department of Biology, Faculty of Science and Letters,
Çukurova University, Adana, Turkey
e-mail: mtopaktas@cu.edu.tr

et al. 1997; Toh et al. 1999). On the other hand, RB has been demonstrated to have low developmental toxicity in cultured rat embryos (Karabulut et al. 2004). Among the various adverse reactions that a drug may cause, the occurrence of genotoxic-carcinogenic effects can not be excluded (Brambilla and Martelli 2009). Chromosome aberrations, both structural and numerical, have been associated with human health (Natarajan 2002). Therefore, standard genotoxicity testing should be performed to identify the potential genotoxic/carcinogenic properties of chemical agents. For this purpose, human peripheral blood lymphocyte is an extremely sensitive indicator of both in vivo (Evans et al. 1979; Evans 1982) and in vitro (Evans 1970; Buckton and Evans 1973) induced chromosome structural and numerical change.

In this study, genotoxic effects of RB have been investigated in order to extend the genotoxicity information on this widely used muscle relaxant by means of sister chromatid exchange (SCE), chromosome aberration (CA) and micronucleus (MN) test systems in human peripheral blood lymphocytes.

Materials and methods

RB (Organon, USA) was used as the test substance for the in vitro assays. The properties of RB are shown as follows:

CAS number: 119302-91-9

Linear Formula: $C_{30}H_{49}BrN_2O_4$

Molecular Weight: 609.69

Rocuronium bromide (RB), in its liquid formulation, was used as the test substance. Test concentrations (60, 80 and 100 $\mu\text{g}/\text{mL}$) were roughly 25–40 times higher than plasma levels achieved in patients under surgery.

The methods of Evans (1984) and Perry and Thomson (1984) were followed for preparation of the CA and the SCE tests with minor modifications. This study was conducted according to the IPCS guidelines (Albertini et al. 2000). Whole blood (0.2 mL) from four healthy donors (two male, age: 18, 20 and two female, age: 19, 20, non-smokers, blood samples not pooled), was added to 2.5 mL chromosome medium B (Biochrom AG, F 5023) supplemented with 10 $\mu\text{g}/\text{mL}$ bromodeoxyuridine (Sigma, St. Louis, MO, B5002). Cultures were incubated at

37 °C for 72 h. The cells were treated with 60, 80 and 100 $\mu\text{g}/\text{mL}$ concentrations of RB for 24 h (RB was added 48 h after initiating the culture) and 48 h (RB was added 24 h after initiating the culture). A negative control and a positive control (Mitomycin-C (MMC), 0.25 $\mu\text{g}/\text{mL}$, Sigma M-05030) were also used. The cells were exposed to colchicine (0.06 $\mu\text{g}/\text{mL}$, Sigma C9754) 2 h before harvesting. The cells were treated with 0.4% KCl (37 °C) as the hypotonic solution and methanol: glacial acetic acid (3:1) as the fixative (at room temperature 22 ± 1 °C, fixative treatments were repeated three times). The staining of the air-dried slides was performed following the standard methods using 5% Giemsa stain for CA and the modified fluorescence plus Giemsa method for SCE (Speit and Haupter 1985). The slides were irradiated with 30 W, 254 nm UV lamp at 15 cm distance in Sorensen buffer for 30 min, then incubated with $1 \times \text{SSC}$ (standard saline citrate) at 60 °C for 50 min and stained with 5% Giemsa prepared with Sorensen buffer. The number of CAs was obtained by calculating the percentage of the metaphases from each concentration and treatment period that showed structural and/or numerical alterations. The CA was classified according to the ISCN (International System for Human Cytogenetic Nomenclature) (Paz-y-Miño et al. 2002). CAs were evaluated in 100 well spread metaphases per donor (totally 400 metaphases per concentration). Gaps were not evaluated as CA according to Mace et al. (1978). CAs were classified as structural aberrations. Structural CAs consisted of the chromatid type (breaks and exchanges) and the chromosome type (breaks, fragments, sister unions, dicentrics) abnormalities. Only the structural CAs were taken into consideration in order to determine the genotoxicity. The scoring of SCE was carried out according to the IPCS guidelines (Albertini et al. 2000). To score SCE, a total of 100 s division metaphases (25 cells per sample) were analyzed. The results were used to determine the mean number of SCE (SCE/cell). In addition, a total of 400 cells (100 cells from each donor) were scored for the proliferation index (PI). The PI was calculated according to the formula as follows: $\text{PI} = (\text{M1} \times 1) + (\text{M2} \times 2) + (\text{M3} \times 3) / \text{total scored cells}$. M1, M2, and M3 are the fraction of cells undergoing the first, second, and third mitosis during 72-h cell culture times. The MI was also determined by scoring 3,000 cells per concentration.

For the analysis of MN in binucleated lymphocytes, 0.2 mL of fresh blood was used to establish the cultures and the cultures were incubated for 68 h. The cells were treated with 60, 80 and 100 µg/mL concentrations of RB for 24- and 48-h treatment periods. Cytochalasin B (Sigma, C6762) was added at 44 h of the incubation to a final concentration of 6 µg/mL to block cytokinesis. After an additional 24-h incubation at 37 °C, cells were harvested by centrifugation and processed for the MN test in peripheral blood lymphocytes (Rothfuss et al. 2000; Kirsch-Volders et al. 2003). In all subjects, 2,000 binucleated lymphocytes were scored from each donor (8,000 binucleated cells were scored per concentration). A total of 1,000 cells were scored to determine the frequency of the cells with 1, 2, 3, or 4 nuclei and to calculate the nuclear division index (NDI) for the cytotoxicity of agents using the formula: $NDI = (M1 + (2 \times M2) + (3 \times M3) + (4 \times M4))/N$, where M1–M4 represent the number of cells with one to four nuclei and N is the total number of the cells scored (Fenech 2000).

T-test was utilized for establishing the statistical significance of all parameters. SCE, CA, PI, MI, micronucleated binuclear (MNBN) cell and NDI data obtained from microscopic analyses were compared to the corresponding negative and positive control groups. Concentration–response relationships were determined from the correlation and regression coefficients for the percentage of cells with CA, as

well as for the mean SCE, PI, MI, NDI and micronucleated binuclear (MNBN) cell.

Results

The human peripheral lymphocytes were treated with 60, 80 and 100 µg/mL of RB dissolved in sterile bidistilled water, for 24- and 48-h treatment periods to investigate the effects of RB on SCEs and CAs. Data of structural chromosomal aberrations induced by RB are presented in Table 1. Following treatment with RB, a statistically significant increase in CA frequency was observed in both 24- and 48-h treated lymphocyte cultures (Table 1). Chromatid type breaks were the most common abnormalities.

No statistically significant increases were obtained in the SCE frequency after 24- and 48-h treatment with RB (Table 2).

RB induced statistically significant MNBN cell formation in comparison to the negative control for 24- and 48-h treatment periods. However, the increase was not dose dependent ($p > 0.05$) (Table 3).

Cytotoxic effect of RB was analyzed by investigating the NDI, MI and PI parameters. Treatment of peripheral blood lymphocytes with various concentrations of RB did not lead to a significant alteration in the NDI, for both 24- and 48-h treatment periods (Table 3). Furthermore, there were no significant

Table 1 Effect of rocuronium treatment on CA in human peripheral blood lymphocytes for 24- and 48-h treatment periods

Test substance	Treatment		Structural CA		Percentage of cells with structural aberrations ±SE
	Time (h)	Conc. (µg/mL)	Chromatid type	Chromosome type	
Neg. Control	–	–	19	3	4.50 ± 0.86
MMC	24	0.25	63	42	26.25 ± 1.25
Rocuronium	24	60	19	6	6.25 ± 0.75 _{a1b3}
Rocuronium	24	80	25	6	7.75 ± 1.43 _{a1b3}
Rocuronium	24	100	43	4	11.75 ± 2.12 _{a1b2}
MMC	48	0.25	91	63	38.50 ± 6.73
Rocuronium	48	60	38	6	11.00 ± 2.27 _{a1b3}
Rocuronium	48	80	30	3	8.25 ± 2.46 _{b3}
Rocuronium	48	100	36	7	10.75 ± 2.56 _{a1b2}

Data are expressed as the mean values (±S.E) obtained from four donors; $n = 4$

a significant from negative control; *b* significant from positive control

$a_1b_1 p \leq 0.05$, $a_2b_2 p \leq 0.01$, $a_3b_3 p \leq 0.001$

Table 2 Mean SCE values in human peripheral blood lymphocytes treated with rocuronium for 24- and 48-h

Test substance	Treatment (h)	Concentration ($\mu\text{g/mL}$)	Min–Max SCE	SCE/Cell \pm SE
Neg. Control	–	–	1–17	8.58 \pm 0.51
MMC	24	0.25	6–64	30.5 \pm 3.30
Rocuronium	24	60	4–27	9.25 \pm 1.09b ₃
Rocuronium	24	80	2–26	8.77 \pm 0.09b ₃
Rocuronium	24	100	4–16	9.18 \pm 0.29b ₃
MMC	48	0.25	4–104	48.43 \pm 7.34
Rocuronium	48	60	4–18	8.73 \pm 0.58b ₃
Rocuronium	48	80	3–18	8.42 \pm 0.43b ₃
Rocuronium	48	100	4–20	8.60 \pm 0.08b ₃

Data are expressed as the mean values (\pm S.E) obtained from four donors; $n = 4$

a significant from negative control, *b* significant from positive control

$a_1b_1 p \leq 0.05$, $a_2b_2 p \leq 0.01$, $a_3b_3 p \leq 0.001$

alterations in both the MI and PI after 24- and 48-h treatment periods (Table 4).

Discussion

In this study, the treatment of peripheral blood lymphocytes with various concentrations of RB led to a statistically significant increase in the CA frequency. In contrast, treatment of peripheral blood lymphocytes with diazepam and propofol did not lead to a marked increase in the CA frequency as reported by Karahalil et al. (2005). One reason for the different results can be attributed to the difference of the classes of the anesthetics used in those studies, and the other one can be the test platform which is in vivo in the study of Karahalil et al. (2005). However, an in vitro study demonstrated that sodium thiopental could increase the CA frequency by two-fold when compared to the negative control (Klimova and Paskevich 1984). Similar in vitro increments of the CA frequency in case of diazepam and hexenal were reported by various researchers (Klimova and Paskevich 1984; Huong et al. 1988). SCE frequency after RB treatment was not found to be statistically significant, leading to the conclusion that the SCE-inducing lesions generated by RB in DNA are efficiently repaired before replication. Ishii and Bender (1980) have previously argued that various inhibitors of replication induce SCEs. Thus, it could be proposed that RB is not an inhibitor of replication. Statistically insignificant increase of SCE frequency

in human peripheral blood lymphocytes has been also shown for diazepam at a concentration of 0.2 mg/kg (Husum et al. 1985).

In this study, increased MNBN cell frequencies were observed after RB treatment for 24- and 48-h. Furthermore, induced formation of structural CAs was also detected after treatment with RB for 24- and 48-h treatment periods. Thus, it could be inferred that the resulting structural CAs are playing a major role in the formation of micronuclei after RB treatment. Diazepam-induced MN formation was also shown in mice bone-marrow cells in vivo (Leal Garza et al. 1998). In addition, multipolar anaphase-inducing capability (Topaktaş and Rencüzoğulları 1995) of RB should be responsible for the relatively large micronuclei formation that we encountered in this study.

24- and 48-h exposure to RB did not significantly decrease the PI, MI, NDI, as evidenced by the non-toxic behaviour of the employed concentrations. Interestingly, RB increased the CA frequency without decreasing the MI, PI and NDI. We further performed a thorough analysis of the literature in order to find similar reports which may support our finding on the induction of CA without cytotoxicity. Olivier et al. (1998) noted that *p53* wild type tumor cells tolerated unhealed chromosomal breaks and continued cycling after genotoxic insult, suggesting a relaxed/impaired cell-cycle control. In addition to this, etoposide has been shown to induce significant increases in %MN at concentrations >0.01 mg/mL without cytotoxicity (Lynch et al. 2003). Researchers concluded that there was a threshold point between 0.005 and 0.001 mg/

Table 3 The percentage of micronucleated binuclear cell and nuclear division index (NDI) in cultured human peripheral blood lymphocytes treated with rocuronium for 24- and 48-h

Test substance	Treatment		Percentage of micronucleated binuclear cell \pm SE	NDI \pm SE
	Time (h)	Concentration (μ g/mL)		
Neg. Control	–	–	0.35 \pm 0.06	1.37 \pm 0.12
MMC	24	0.25	1.45 \pm 0.19	1.43 \pm 0.08
Rocuronium	24	60	0.60 \pm 0.04 _{a2b3}	1.39 \pm 0.09
Rocuronium	24	80	0.60 \pm 0.04 _{a2b3}	1.29 \pm 0.07
Rocuronium	24	100	0.72 \pm 0.09 _{a1b2}	1.30 \pm 0.09
MMC	48	0.25	2.22 \pm 0.13	1.30 \pm 0.04
Rocuronium	48	60	0.62 \pm 0.04 _{a2b3}	1.42 \pm 0.04
Rocuronium	48	80	0.75 \pm 0.02 _{a3b3}	1.46 \pm 0.06
Rocuronium	48	100	0.82 \pm 0.06 _{a2b3}	1.46 \pm 0.05

Data are expressed as the mean values (\pm S.E) obtained from four donors; $n = 4$

a significant from negative control, *b* significant from positive control

$a_1b_1 p \leq 0.05$, $a_2b_2 p \leq 0.01$, $a_3b_3 p \leq 0.001$

Table 4 Proliferation index (PI) and mitotic index (MI) in human peripheral blood lymphocytes treated with rocuronium for 24- and 48-h

Test substance	Treatment		M1	M2	M3	PI \pm SE	MI \pm SE
	Time (h)	Concentration (μ g/mL)					
Neg. Control	–	–	62	116	222	2.40 \pm 0.12	4.06 \pm 0.53
MMC	24	0.25	152	116	132	1.95 \pm 0.14	2.70 \pm 0.35
Rocuronium	24	60	106	115	179	2.18 \pm 0.21	4.60 \pm 1.08
Rocuronium	24	80	80	114	206	2.31 \pm 0.15	4.32 \pm 1.25
Rocuronium	24	100	103	104	193	2.22 \pm 0.26	4.33 \pm 0.95
MMC	48	0.25	217	110	73	1.64 \pm 0.03	2.11 \pm 0.27
Rocuronium	48	60	50	113	237	2.46 \pm 0.07 _{b3}	5.03 \pm 0.97 _{b1}
Rocuronium	48	80	55	112	233	2.44 \pm 0.12 _{b2}	4.94 \pm 0.50 _{b1}
Rocuronium	48	100	66	104	230	2.41 \pm 0.09 _{b2}	4.69 \pm 0.59 _{b1}

Data are expressed as the mean values (\pm S.E) obtained from four donors; $n = 4$

a significant from negative control, *b* significant from positive control

M1 first mitosis, *M2* mitosis, *M3* third mitosis

$a_1b_1 p \leq 0.05$, $a_2b_2 p \leq 0.01$, $a_3b_3 p \leq 0.001$

mL, where a clear cytotoxicity was gained at concentrations >0.00236 mg/mL. Based on these two findings, it may be proposed that RB may interfere with the normal cell cycle of human lymphocytes, or may have a threshold-like cytotoxicity which could have been detected if higher concentrations were employed.

The top concentration used in this study did not lead to a cytotoxic manifestation. It has been pointed out that the chromosome-damaging properties of various compounds are often associated with high toxicity (Müller and Sofuni 2000; Galloway 2000). Thus, cytotoxicity of RB should not be responsible for the genotoxic effects detected in this study.

There is no precise information on the genotoxic features of RB, however, the present study indicates that RB is capable of causing genotoxicity via clastogenic effects at concentrations at which a significant cytotoxic effect does not occur. Further analysis need to be performed to clarify whether this agent has a threshold-like cytotoxicity *in vitro* and *in vivo*.

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