ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN — POLONIA
VOL. LIII, 11 SECTIO D 1998

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# *Central action of some cytostatics – methotrexat (MTX) and doxorubicin (DXR). II. The infuence on the seizure activity and the learning and memory processes in mice*

Ośrodkowe działanie niektórych cytostatyków – metotreksatu i doksorubicyny. II. Wpływ na aktywność drgawkową oraz procesy uczenia się i pamięci u myszy

All cytotoxic drugs have side effects. These commonly occur at therapeutic dosages and may be severe enough to result in reduction of the dose or even discontinuation of treatment. Side effects may occur immediately after the start of treatment or may become apparent after several days or weeks of treatment (1, 3, 6, 8). The antimetabolite of folic acid, methotrexat (MTX) and the antracycline antibiotic, doxorubicin (DXR) have been long major components of cancer treatment, as they both are highly effective chemotherapeutic agents, widely used in clinical medicine (2, 3, 5, 10, 12, 15, 18). However, the evident efficacy of MTX and DXR is seriously limited by life-threatening myelosuppression and of DXR in addition by cumulative cardiotoxicity (2, 12, 13, 20). Both drugs administered in high single doses in the way of treatment of acute leukaemia developed their long-lasting toxicity during several days, manifested by the decrease of the body weight, myelosuppression, immunosuppression and the destruction of the epithelium of the gastrointestinal tract (2, 12, 14, 18). Both drugs are able to completely inhibit DNA synthesis and induce cell death in histocultures of tumors obtained via surgery (3, 8). However, little is known about the central

action of these substances. The aim of the present studies was to investigate the influence of MTX and DXR on the central nervous system in the respect of their influence on the seizure susceptibility in chemical and electric convulsions and the modulation of the learning and memory processes in mice.

## MATERIAL AND METHODS

The experiments were carried out on male and female Albino Swiss mice (20-25g). Standard food and water were available *ad libitum*. Each experimental group consisted of 12 animals. The central action of two cytostatics: methotrexat (MTX), antimetabolite of folic acid and doxorubicin (DXR), oncostatic antibiotic belonging to antracycline group, was studied. The investigated substances were dissolved in 0.9% NaCl and injected intraperitoneally *(ip)* in single doses 5 or 10 mg/kg. The experiments were performed 14 days after a single injection of drugs, only the passive avoidance task was done after 24 h, 7 and 14 days.

The substances, the solvents used for their dilution and way of administration are listed below: Doxorubicin (Adriablastine, Pharmacia, Italy) was diluted with saline and injected *ip*. Hexobarbital (ZVEB Artzneimittel Verk, Dresden Germany) was dissolved in distilled water and injected *ip*. Methotrexat (Rhone-Poulenc Rorer, France) was dissolved in saline and administred *ip*. Pentetrazole (Sigma, St. Louis) was dissolved in saline and administered *sc*. Pilocarpine (Sigma, St. Louis) was dissolved in saline and administered *ip*. N-methylscopolamine (Sigma, St. Louis) was dissolved in saline and injected *sc*. 15 min prior to pilocarpine. All substances were administered in a constant volume of 5 ml/kg. Control animals received an equivalent volume of solvent. Exact doses of the substances are given under the description of each test.

Pentetrazole seizures in mice were induced by the *s.c.* pentetrazole injection at different doses 14 days after the MTX or DXR administration. Animals were observed during 60 min and the number of mice developing clonic and tonic seizures as well as mortality were recorded. $CD_{50}$ (a dose necessary to produce a 50% effect) of pentetrazole for clonus, tonus and mortality was calculated using the method of Litchfield and Wilcoxon (7).

Pilocarpine seizures in mice were induced by the pilocarpine administration at the dose of 380 mg/kg *ip* 14 days after the MTX or MTX treatment. 15 min before pilocarpine administration mice received N-methyl-scopolamine (1 mg/kg *s.c.*), blocker of peripheral muscarinic receptors. Animals were observed during 60 min and the latency time in min to the development of limbic and clonic seizures was recorded. Data were calculated as means ± SEM and evaluated statistically using Student's t-test.

Maximal electroshock was induced by means of alternating current (50 Hz, 25 mA, 0.2 s) with the use of ear clip electrodes according to the method of Swinyard et al. (17). The criterion of the convulsive response was the tonic extension of the hind limbs. The test was performed 14 days after the administration of MTX or DXR. The amount of mice with tonic seizures was noted.

Threshold for electroconvulsions was evaluated by using different values of alternating current (5, 6, 7, or 8 mA, 50 Hz, 0.2 s). The criterion of the convulsive response was the tonic extension of the hind limbs. The test was performed 14 days after the administration of MTX or DXR and $CS_{50}$ (in mA) was calculated using the method of Litchfield and Wilcoxon (7).

Duration of the hexobarbital sleeping time in min was measured after the *ip* administration of hexobarbital at the dose of 100 mg/kg 14 days after the MTX or DXR treatment. Animals were observed for 120 min after the hexobarbital administration. Data were calculated as means ± SEM and evaluated statistically using Student's t-test.

The step-through passive avoidance task was performed according to Venault et al. (19). Mice were placed in an illuminated box (10 x 13 x 15cm) connected to a large dark box (25x20x15cm) which was equipped with an electric grid floor. The entrance into the dark box was punished by an electric footshock (0.6 mA for 2 s; unscrambled DC current; impairment of acquisition or 0.1 mA for 2 s; facilitation of acquisition). On the next day (24 h), the same mice were placed in the illuminated box. Mice avoiding the dark compartment for over 120 s were considered as remembering the task. Retention was quantified as the number of animals avoiding the dark compartment. The results were evaluated statistically using exact Fischer's test. The experiments were performed 24 h, 7 and 14 days after the MTX or DXR administration. The step-through passive avoidance task may give information about ability to acquire the task (learning) and to recall the task (retrieval) and may be regarded as a measure involving long-term memory.

The animals were decapitated 14 days after the treatment of MTX or DXR, their brains were immediately frozen. The GABA content in brain tisssue was measured according to the method of Love et al. (9) with the modification of Sutton and Simmonds (16). Data were expressed in μg/g of tissue as means ± SEM and evaluated statistically using Student's t-test.

## RESULTS

MTX increased the susceptibility of mice to pentetrazole-induced seizures, expressed as the decrease of $CD_{50}$ of PTZ for tonus, clonus and mortality in both administered doses 14 days after the treatment. DXR showed the weak tendency of proconvulsive action for PTZ, but the results were significant only in the case of clonic seizures (Tab. 1).

Both drugs, MTX and DXR, possessed proconvulsive activity in pilocarpine-induces seizures. MTX in both administered doses shortened the latency for limbic and clonic seizures evoked by pilocarpine. DXR at the dose of 5 mg/kg shortened only the latency to clonic convulsions. In a higher dose, 10 mg/kg, had the proconvulsive activity in respect to limbic and clonic seizures (Tab. 2)

Neither MTX nor DXR had the influence on the MES convulsions (Tab. 3). But the threshold for electroconvulsions expressed as $CS_{50}$ was decreased by MTX administered at the doses 5 and 10 mg/kg. DXR did not change the threshold for electroconvulsions (Tab. 4).

MTX shortened the hexobarbital-sleeping time 14 days after single administration in both doses used. DXR did not affect this parameter (Tab.5).

In the passive avoidance task only MTX at the dose 10 mg/kg 14 days after the administration caused the impairment of fresh working memory in mice. DXR had no effect on this parameter (Tab. 6).

MTX decreased the GABA content in mice brain tissue. In the action of DXR the same tendency could be observed, but the differences were not significant (Tab. 7).

Table 1. The influence of MTX and DXR 14 days after treatment on the threshold for pentetrazole-induced seizures in mice (n=12)

| Compound | Dose mg/kg | $CD_{50}$ for pentetrazole in mg/kg | | |
|---|---|---|---|---|
| | | clonus | tonus | mortality |
| Control | solvent | 87.3 [82.0-93.0] | 98.5 [86.0-112.7] | 98.6 [92.3-105.2] |
| MTX | 5 | 77.1 [68.0-88.0]* | 84.6 (80.3-89.0]* | 86.7 [81.4-92.4)** |
| MTX | 10 | 66.8 [58.4-76.5]*** | 79.7 [70.7-89.8]* | 80.1 [73.0-88.0]*** |
| DXR | 5 | 81.8 [64.1-104.5] | 93.3 [83.4-105.8] | 95.2 [73.2-123.9] |
| DXR | 10 | 77.4 [72.0-83.3] * | 86.2 [69.4-107.1 ] | 89.4 [77.9-102.6] |

*p<0.05; **p<0.01; *** p<0.001 versus control. Litchfield and Wilcoxon.

Table 2. The influence of MTX and DXR 14 days after treatment on the latency time to pilocarpine-induced ( 380 mg/kg *ip)* seizures in mice (n=12)

| Compound | Dose mg/kg | Latency time (min) ~ SEM to appearance of seizures: | |
|---|---|---|---|
| | | limbic | clonic |
| Control | solvent | 28.4 ± 2.8 | 55.7 ± 4.6 |
| MTX | 5 | 17.1 ± 2.2** | 31.6 ± 5.7** |
| MTX | 10 | 15.4 ± 4.1** | 20.6 ± 3.5*** |
| DXR | 5 | 20.1 ± 13.9 | 36.4 ± 3.3** |
| DXR | 10 | 18.2 ± 3.4* | 31.9 ± 14.2** |

* p<0.05; ** p<0.01; *** p<0.001 vs control. Student's t-test.

Table 3. The influence of MTX or DXR 14 days after treatment on the seizure activity in maximal electroshock (MES) in mice

| Compound | Dose mg/kg | Number of animals with tonic seizures |
|---|---|---|
| Control | solvent | 12/12 |
| MTX | 5 | 12/12 |
| MTX | 10 | 12/12 |
| DXR | 5 | 12/12 |
| DXR | 10 | 12/12 |

Table 4. The influence of MTX or DXR 14 days after treatment on the seizure threshold for electroconvulsions in mice (n=12)

| Compound | Dose (mg/kg) | $CS_{50}$ (mA) |
|---|---|---|
| Control | solvent | 6.5 [5.7-7.3] |
| MTX | 5 | 5.3 [4.8-5.7)** |
| MTX | 10 | 5.2 [4.4-6.1]* |
| DXR | 5 | 5.7 [5.0-6 4] |
| DXR | 10 | 5.6 [5.0-6.4] |

*p<0.05; **p<0.01; vs control. Litchfield and Wilcoxon.

Table 5. The influence of MTX or DXR 14 days after treatment on the duration of the hexobarbital-induced ( 100 mg/kg) sleeping time in mice (n=12)

| Compound | Dose mg/kg | Sleeping time in min ± SEM |
|---|---|---|
| Control | solvent | 118.5 ± 7.6 |
| MTX | 5 | 86.7 ± 7.7* |
| MTX | 10 | 76.2 ± 9.5*** |
| DXR | 5 | 109.6 ± 10.4 |
| DXR | 10 | 100.2 ± 9.1 |

* p<0.05; *** p<0.001 vs control, Student's t-test.

Table 6. The influence of MTX or DXR 24 h, 7 and 14 days after treatment on the long-term memory in passive avoidance task (n=15)

| Compound | Dose mg/kg | Number of mice remembering the task after: | | |
|---|---|---|---|---|
| | | 24 h | 7 days | 14 days |
| Control | solvent | 15/ 15 | 15/ 15 | 15/ 15 |
| MTX | 10 | 15/15 | 9/15 | 4/15** |
| DXR | 10 | 15/15 | 12/15 | 9/15 |

** p<0.01 vs control, exact Fischer's test.

Table 7. The influence of MTX or DXR 14 days after treatment on the brain GABA content in mice (n=12)

| Compound | Dose mg/kg | GABA content in μg/ g tissue ± SEM |
|---|---|---|
| Control | solvent | 364.8 ± 7.4 |
| MTX | 5 | 329.7 ± 11.2* |
| MTX | 10 | 289.6 ± 8.4*** |
| DXR | 5 | 347.8 ± 9.5 |
| DXR | 10 | 3 36.4 ± 12.8 |

## DISCUSSION

Our results indicate that the behavioral effects induced by both investigated drugs are connected with their central action, but the influence of MTX was more pronounced. MTX strongly increased the susceptibility to seizures of mice: decreased the threshold for pentetrazole and for electroconvulsions and prolonged the latency to pilocarpine-induced limbic and clonic seizures. The influence of DXR on the seizure susceptibility is weakly pronounced only as a prolongation of the latency to pilocarpine and decrease of the threshold for pentetrazole-induced clonic seizures.

The investigated oncostatics did not affect the sensitivity of mice to the maximal electroshock.

In addition MTX shortened the hexobarbital-induced sleeping time, decreased the brain GABA content and caused the impairment of the long-term memory in the passive-avoidance task. DXR did not change these parameters.

The proconvulsive action of MTX may depend on the GABA-ergic system. Diminution of the level of this inhibitory neurotransmitter results in the spontaneous seizure activity in the case of the treatment of glutamate decarboxylase inhibitor, 3-mercaptopropionic acid (9, 16). It is well known from human treatment that MTX administered into the spinal fluid in the case of leukemic metastases into CNS can induce seizures (4, 11). Our results indicate that also after *ip* administration proconvulsive properties of MTX are observed. Moreover the action of hexobarbital is connected with the activity of GABA-ergic system (16), and the decrease of GABA content in our study can explain the reduction by MTX the time of sleep induced by hexobarbital.

DXR showed only a weak, not significant tendency to decrease the BABA concentration in the brain tissue and possessed much lower than MTX pro-convulsive activity.

MTX, but not DXR, caused the impairment of the memory examined in the passive avoidance task investigated two weeks after the treatment. It is known that the enhancement of the GABA-ergic activity induced for ex. by benzodiazepines results in the diminution of the learning and memory, and that antagonists of GABA-ergic receptors facilitated learning (19). In the present work we observed the impairment of the recall of the task and parallelly the decrease of the GABA content in the brain after MTX administration. However, the processes of learning and memory are complicated, connected with different neurotransmitters, neuromodulators, and the mechanisms which are invoved in their regulation are not clear yet. In our previous studies it was found that MTX increased the activity of the dopaminergic system but decreased the level of NA in mice brain. Also the exploratory activity of animals was diminished (14). The impairment of the recall of the task in passive avoidance test can be connected with the changes of several neurotransmitter systems and could be the result of the large central influence of MTX on different brain structures.

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Otrz.: 1998.07.12

## STRESZCZENIE

Doświadczenia przeprowadzono na białych myszach, samcach i samicach szczepu Albino-Swiss. Badano ośrodkowe działanie dwu powszechnie stosowanych klinicznie cytostatyków: metotreksatu (MTX) – antymetabolitu kwasu foliowego i doksorubicyny (DXR, adriablastyny), antybiotyku z grupy antracyklin. Obydwa leki stosowano dootrzewnowo (*ip*) w jednorazowych dawkach 5 i 10 mg/kg, które odpowiadały dawkom stosowanym w leczeniu np. ostrych białaczek w okresie indukcji remisji. Doświadczenia behawioralne wykonywano po 14 dniach, a biochemiczne po 5 godz. i 14 dniach od zastosowania leków. Wykazano, że MTX wywiera działanie prodrgawkowe: obniża $CD_{50}$ dla penetrazolu, skraca czas latencji w drgawkach wywołanych pilokarpiną i obniża próg drgawkowy w drgawkach elektrycznych. Ponadto MTX skraca czas trwania snu wywołanego heksobarbitalem oraz powoduje upośledzenie pamięci odruchu biernego unikania. Powyższe działania MTX mogą zależeć od zmniejszenia aktywności układu GABA-ergicznego, ponieważ zmniejsza on zawartość GABA w tkance mózgowej. Ośrodkowe działanie DXR jest znacznie słabiej wyrażone. Wykazuje ona niewielkie działanie prodrgawkowe w drgawkach wywołanych pentetrazolem i pilokarpiną, pozostając bez wpływu na drgawki elektryczne. Nie zmienia zawartości GABA oraz nie skraca czasu trwania snu heksobarbitalowego. Nie upośledza też pamięci.