Exp Oncol 2014
36, 2, 90–93

Experimental ONCOLOGY

PRECLINICAL ANTITUMOR ACTIVITY OF THE DIINDOLYLMETHANE FORMULATION IN XENOGRAFT MOUSE MODEL OF PROSTATE CANCER

V.I. Kiselev[1], V.M. Drukh[1], E.L. Muyzhnek[2], I.N. Kuznetsov[3], O.I. Pchelintseva[1,], M.A. Paltsev[4]*
[1]*Peoples' Friendship University of Russia, Moscow 117198, Russia*
[2]*ZAO "MiraxBioPharma", Moscow 121248, Russia*
[3]*Moscow State Medical Stomatological University (MGMSU), Moscow 127473, Russia*
[4]*National Research Centre (NRC "Kurchatov Institute"), Moscow 123182, Russia*

***Aim*: Preclinical study of the specific anticancer pharmacological activity of the formulation containing active substance 3,3′-diindolylmethane (DIM), cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E), *in vivo* in a xenograft animal model of LNCaP. *Materials and Methods*: The DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) formulation was intragastrically administered to BALB/c-nude (nu/nu) mice during 33 days post inoculation at the dose of 133 mg/kg/day. Antitumor activity of the test drug was estimated by the rate of tumor growth inhibition (T/C% — treated versus control), dividing the tumor volumes from treatment groups with the control groups. *Results*: Statistically significant tumor xenograft regressions have been shown in group which received the DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) on the 37th day of observation post inoculation. The highest antitumor activity was achieved on the 39th day (T/C = 16,8%). Therapeutic effect lasts for 6 days after the end of therapy period. *Conclusion*: Our findings demonstrate inhibitory effect of the formulation on tumor development in the xenograft animal model due to the tumor growth rate reduction.**

***Key Words*: 3,3′-diindolylmethane, bioavailability, anticancer activity, xenograft model, LNCaP cell line, preclinical studies.**

3,3′-Diindolylmethane (DIM) — one of the most prospective compound with antitumor and immunomodulatory properties. A great number of studies have revealed that DIM is able to block the multiple molecular mechanisms which cause cancer in different organs and tissues [1, 2]. In addition to the suppression of proliferation of transformed (tumor) cells and stimulation of their apoptosis, DIM inhibits pathologic angiogenesis and reduces the metastatic potential of cancer cells by affecting targets which mediate processes of cell migration and invasion [3, 4]. It has been recently found that DIM is a selective inhibitor of certain tumorigenic minor population of non-differentiated cancer cells — the so-termed "cancer stem cells" [5], which are the main source of recurrence and metastasis according to modern ideas. DIM may provide some protection against hormone-dependent cancers by altering hormone levels, particularly due to down-regulation of androgen receptors [2].

The only significant problem in creating DIM-based anticancer drug is its low bioavailability and, as a result, the inability to achieve the therapeutic concentrations of active substance in target tissues. Generally, DIM exhibits low solubility in physiological fluids and has limited ability to permeate through membrane barriers [6, 7]. Based on this, and taking into account the uniqueness of the candidate substance DIM, the drug DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) was developed. Drug formulation is enclosed in a capsule as solution containing the active substance — DIM (150 mg), as well as organic auxiliary components — cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) providing high bioavailability and storage stability of the drug [8]. It is known that cod liver oil in combination with polysorbate significantly improves absorption in the gastrointestinal tract and distribution in the body, and vitamin E increases storage stability [8].

The new drug formulation, created by us on the basis of modern technological solution, contains DIM in a dissolved state, whereby DIM quickly enters the blood and the target organs, and reaches concentrations which manyfold exceed the concentrations of crystalline forms [9]. It was revealed that 5-fold higher concentration of DIM was observed in blood plasma of rats who received the 2,000-fold lower dose of liquid DIM formulation (the DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E)) compared to crystalline form and non-formulated crystalline DIM. We decided to carry out preclinical *in vivo* study of pharmacological activity of the formulation as a therapeutic antitumor agent.

Prostate cancer (PC) is one of the most common cancers in the world affecting men. PC in Europe is 2nd/3rd cause of cancer deaths, in the USA — 1st. PC leads to 29% of fatal causes among patients with malignant tumors [10]. In the absence of organized mass screening programs of early detection of PC disease in many cases becomes metastatic and incurable.

Modern methods of treating PC — hormone-dependent cancer — are primarily directed to total androgen blockade (androgen deprivation therapy), and may involve surgery (radical prostatectomy,

Submitted: March 27, 2014.
*Correspondence: E-mail: pchelintseva87@mail.ru
Abbreviations used: BR-DIM – bioresponse 3,3′-diindolylmethane; DIM – 3,3′-diindolylmethane; PC – prostate cancer.

conservative surgery, orchiectomy (rarely)) or hormone therapy using medications.

According to statistics, about 60% of newly diagnosed cases of PC aren't localized, and, consequently, most of patients first-time diagnosed with PC, should not undergo surgical procedures. Hormone therapy is considered the most effective treatment for non-localized forms of PC, however it allows to control the disease only for limited periods of time because hormonal resistance inevitably develops [11]. Hormone-refractory prostate tumors are resistant to treatment, highly aggressive and have a poor prognosis [12].

Future prospects of PC treatment involve chemotherapy combining anti-androgenic effects with the targeted inhibition of multiple signaling pathways that activate procarcinogens in the prostate gland.

The purpose of this investigation was to study specific pharmacological preclinical antitumor activity of the new drug — DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) *in vivo* in a xenograft mouse model of PC.

MATERIALS AND METHODS

Reagents. Drug formulation containing DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) (ZAO "MiraxBioPharma", Russia) was prepared according to special patented technology [8]. The crystalline DIM (3,3′-methandiilbis(1H-indol)) (Alexis Corporations, Switzerland), cod liver oil and polysorbate 80 (Sigma-Aldrich, USA) were used. The solvent used in the experiment was a mixture of cod liver oil and polysorbate 80 in the same proportions as developed drug formulation.

Cell culture. The LNCaP (androgen-sensitive human PC cells) was obtained from a collection of tumor strains from the N.N. Blokhin Cancer Research Center. All manipulations with cells were performed under sterile conditions under a laminar flow hood (NuAire, USA). LNCaP cells were maintained in RPMI 1640 (Gibco, USA) supplemented with 10% FBS (HyClone, USA), 2 mM L-glutamine and penicillin/streptomycin (100 MU/mL) (Pan Eco, Russia) in an incubator at 37 °C and 5% CO_2 (Shel Lab, USA). On reaching 80–90% confluence, the cells were passaged. Cells were washed twice with Versen solution, 0.025% trypsin (Pan Eco, Russia) was added to the cells and incubated for 10 min at 37 °C. After full cell separation 10 ml of growth medium were added and aggregated cells were resuspended. Suspended cells was transferred for the following cells inoculation.

Animals. LNCaP cell inoculation. Study was carried out using 60 immunodeficient male Balb/c-nude (nu/nu) mice (4–5 weeks) which were purchased from Charles River GmbH (Germany). The length of quarantine was 14 days. During this time daily inspections of behavior and general condition of each animal were undertaken.

The animals were housed in standard cages (Techniplast, Italy), connected to the air conditioning unit TouchSLIM Plus® (Techniplast, Italy), 4–8 mice/cage. The following microclimate parameters were maintained: light conditions: 12 h — light, 12 h — dark; air temperature 24–26 °C; relative humidity 30±70%; air exchange 8–10 room volumes per hour. The animals had free access to distilled water and food (PMI LabDiet® 5K67). Food and water were pre-sterilized (autoclaving).

All animals were inoculated with LNCaP cells subcutaneously along the spine (in left scapular region) after adaptation period. LNCaP cell line inoculation was carried out by injection of 0.2 ml of tumor suspension in sterile solution of PBS (Sigma, USA), containing 5 mln cells ($25 \cdot 10^6$ cells/ml). All manipulations with animals were approved by the local Animal Care and Use Committee.

Treatment protocols. At 3 days after LNCaP cell inoculation, animals which met criteria for inclusion were randomized and individually labeled. Two groups of animals were formed: control and experimental (animals treated with the formulation) — 30 mice each. In experimental group animals were administered with the formulation twice daily (in the morning and evening) with an interval of 8–9 h intragastrically at a dose of 133 mg/kg/day (per DIM) by atraumatic gavage for 33 days. The control group treated with the same amount of solvent (cod liver oil + polysorbate) on the same schedule. Tumor growth was monitored for a further 6 days after the end treatment period. In the days of administration of drug/solvent animals were inspected at a specified time prior to and two hours after administration.

Euthanasia was carried out by CO_2-inhalation.

Assessment of antitumor activity. Tumor volume measurements began at the initiation of tumor growth and continued twice a week. Tumor volumes were measured with caliper and were calculated by the following formula (V_t):

$$V_t\,(mm^3) = L \cdot W^2/2,$$

where L — the longest tumor diameter, W — the shortest tumor diameter.

Antitumor activity of the DIM formulation was estimated by tumor growth inhibition ratio (T/C%), where T and C represent the means of the tumor volumes of the control (V_C) and treatment mice (V_I) in each experiment day ($V_I/V_C \cdot 100\%$). Another value of inhibition ratio (D%) was reckoned by the formula:

$$D\% = (V_C - V_I)/V_C \cdot 100\%.$$

Behavior and general condition of animals have been registered both in experimental and control groups. Animals were weighted 2–3 times a week.

Statistical analysis. Statistical differences in tumor growth inhibition ratio (T/C%) between treated and untreated groups were determined using Student's *t*-test by means of GraphPad Prism 5 software. Differences were statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Study of the antitumor activity of the formulation in subcutaneous LNCaP xenografts. The effect of the DIM formulation on tumor growth dynamics was evaluated by measuring changes in tumor size

in response to treatment in immunodeficient mice Balb/c-nude (nu/u) subcutaneously implanted with human PC line LNCaP. Figure shows the average tumor sizes in control and experimental groups of animals in each study day.

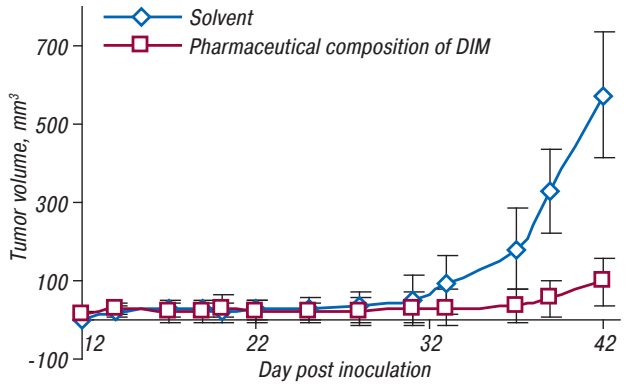

Figure. Dynamics of tumor growth in PC3 xenograft mouse model in experimental (after intragastrically administration of the DIM formulation for 33 days at a dose of 130 mg/kg/day) and control groups

The average tumor volumes of the treated mice were not significantly different (ns) from that of the control group till the 33-rd day post tumor cell inoculation. Subsequently, however, the antitumor effect of injected drug has become more evident. Statistically significant size reduction of the tumors treated with the formulation compared with the control tumors have registered since 37 day. As a result within 42 days post inoculation the average volume of full-grown xenograft tumors in control group reached ~ 600 mm^3, by comparison only ~100 mm^3 in experimental group (animals treated with the formulation). Therapeutic effect lasts for 6 days till study ends after the cessation of the formulation administration (36^{th} day after cell inoculation). We suggest that this is result of stable positive changes, caused by multiple targeted action of the active substance on the molecular intracellular mechanisms that mediate tumor growth.

According to the data, the highest antitumor effect of the DIM formulation was observed on the 39^{th} day of experiment (37^{th} day of observation post inoculation), when the T/C% ratio, which was calculated by dividing the average tumor volumes of experimental animals by the control values, reached 16.8%. In the same day of the experiment the value of inhibition ratio (D%) was 83.2%. The values of these parameters remained unchanged until the end of experiment.

Besides, we've registered dynamics of animals' weight. It was shown that animals in both groups had weight loss after the drug/solvent administration, however weight and general condition of animals became stable through about a week.

Thus the average volume of xenograft tumors in experimental group which was treated with the formulation was 6-fold less than in group treated with original DIM and T/C ratio reached 16.8% on day 42.

It should be noted that anticancer activity of crystalline DIM, and bioresponse-DIM (BR-DIM) formulation ("BioResponse" LLC, USA), which contains DIM combined with pegylated vitamin E and phosphatidyl choline, has been successfully studied by different authors in xenograft model of PC [2, 13–15], and other cancers [3, 16–19]. However, bioavailability of DIM in BR-DIM formulation compared with crystalline DIM was enhanced only in 1,5- to 2-fold, which demonstrate that administration of BR-DIM formulation (*per os*) doesn't ensure peak concentrations of the active substance (DIM) and, consequently, desired therapeutic effect. This could help to explain why no significant clinical effect was achieved with BR-DIM formulation in treatment of cervical dysplasia [20].

However, animal *in vivo* experiments revealed that crystalline DIM and BR-DIM formulation — by various routes of administration — demonstrate significant dose-dependent antitumor effect. They induced a reduction in tumor volume which was established by implantation of PC3 cells, and/or reduce the amount of newly formed metastases. According to the study in a SKOV-3 xenograft tumor model of ovarian cancer T/C ratio was 47.2%, in other study this value was 51.63% (PC3 xenograft tumor model of PC) (T/C values were calculated according to the original experimental data reported in these publications).

Our findings confirm inhibitory effect of the new formulation containing DIM, cod liver oil, polysorbate 80 and α-tocopherol acetate (vitamin E) on tumor development in the xenograft animal model and complement previously obtained experimental data.

ACKNOWLEDGMENTS

Research and experimental studies were carried out at the FGBOU VPO Peoples' Friendship University of Russia in the context of Project "Production of drugs based on biotechnology for the treatment of socially significant diseases", financed by Presidential Grant of The Russian Ministry of Education and Science in compliance with Russian Government's decree № 218.

REFERENCES

1. Banerjee S, Kong D, Wang Z, ***et al***. Attenuation of multi-targeted proliferation-linked signaling by 3,3′-diindolylmethane (DIM): from bench to clinic. Mutat Res 2011; **728**: 47–66.

2. Fares F, Azzam N, Appel B, ***et al***. The potential efficacy of 3,3′-diindolylmethane in prevention of prostate cancer development. Eur J Cancer Prev 2010; **19**: 199–203.

3. Chang X, Tou JC, Hong C, ***et al***. 3,3′-Diindolylmethane inhibits angiogenesis and the growth of transplantable human breast carcinoma in athymic mice. Carcinogenesis 2005; **26**: 771–8.

4. Kong D, Banerjee S, Huang W, ***et al***. Mammalian target of rapamycin repression by 3,3′-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells. Cancer Res 2008; **68**: 1927–34.

5. Semov A, Iourtchenko L, Lin Fang Liu, ***et al***. Diindolylmethane (DIM) selectively inhibits cancer stem cells. Biochem. Biophys Res Commun 2012; **424**: 45–51.

6. Anderton MJ, Manson MM, Verschoyle R, ***et al***. Physiological modeling of formulated and crystalline Diindolylmethane pharmacokinetics following oral administration in mice. Drug Metab Dispos 2004; **32**: 632–8.

7. Reed GA, Arneson DW, Putnam WC, ***et al***. Single-dose and multiple-dose administration of indole-3-carbinol to women: pharmacokinetics based on 3,3′-diindolylmethane. Cancer Epidemiol. Biomarkers Prev 2006; **15**: 2477–81.

8. Kiselev VI. Diindolylmethane-based drug for the treatment of hyperplastic and inflammatory diseases. WO 2011/136691 A1. 2011.

9. Paltsev M, Kiselev V, Muyzhnek E, ***et al***. Comparative preclinical pharmacokinetics study of 3,3′-diindolylmethane formulations: is personalized treatment and targeted chemoprevention in the horizon? EPMA J 2013; **4**: 25.

10. Karan D, Lin MF, Johansson SL, ***et al***. Current status of the molecular genetics of human prostatic adenocarcinomas. Int J Cancer 2003; **103**: 285–93.

11. Kaisary A, Tyrrell C, Peeling W, ***et al***. Comparison of LHRH analogue (Zoladex) with orchiectomy in patients with metastatic prostatic carcinoma. Br J Urol 1992; **67**: 502–8.

12. Gittes RF. Carcinoma of the prostate. N Engl J Med 1991; **324**: 236–45.

13. Cho HJ, Park SY, Kim EJ, ***et al***. 3,3-Diindolylmethane inhibits prostate cancer development in the transgenic adenocarcinoma mouse prostate model. Mol Carcinog 2011; **50**: 100–12.

14. Nachshon-Kedmi M, Fares FA, Yannai S. Therapeutic activity of 3,3-diindolylmethane on prostate cancer in an *in vivo* model. Prostate 2004; **61**: 153–60.

15. Singh-Gupta V, Banerjee S, Yunker CK, ***et al***. B-DIM impairs radiation-induced survival pathways independently of androgen receptor expression and augments radiation efficacy in prostate cancer. Cancer Lett 2012; **318**: 86–92.

16. Kandala PK, Srivastava SK. Diindolylmethane suppresses ovarian cancer growth and potentiates the effect of cisplatin in tumor mouse model by targeting signal transducer and activator of transcription 3 (STAT3). BMC Med 2012; **10**: 9.

17. Kandala PK, Srivastava SK. Regulation of macroautophagy in ovarian cancer cells *in vitro* and *in vivo* by controlling glucose regulatory protein 78 and AMPK. Oncotarget 2012; **3**: 435–49.

18. Kim EJ, Shin M, Park H, ***et al***. Oral administration of 3,3-diindolylmethane inhibits lung metastasis of 4T1 murine mammary carcinoma cells in BALB/c mice. J Nutr 2009; **139**: 2373–9.

19. Sepkovic DW, Stein J, Carlisle AD, ***et al***. Diindolylmethane inhibits cervical dysplasia, alters estrogen metabolism, and enhances immune response in the K14-HPV16 transgenic mouse model. Cancer Epidemiol Biomarkers Prev 2009; **18**: 2957–64.

20. Castañon A, Tristram A, Mesher D, ***et al***. Effect of diindolylmethane supplementation on low-grade cervical cytological abnormalities: double-blind, randomised, controlled trial. Br J Cancer 2012; **106**: 45–52.