BOC2 (N-TERT-BUTOXYCARBONYL-PHE-LEU-PHE-LEU-PHE): A NOVEL MULTITARGET-ANGIOGENIC GROWTH FACTOR INHIBITOR.

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Abstract- Angiogenesis is a process by which new vascular networks develop from preexisting vessels. The angiogenesis process is a result of the simultaneous actions of different angiogenic growth factors, including the members of vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families. The N-tert-butyloxycarbonyl-Phe-Leu-Phe-Leu-Phe (BOC2) peptide is a widely used antagonist of formyl peptide receptors (FPRs). Extensive investigations have been performed by using BOC2 as an FPR1/FPR2 antagonist to assess the role of FPRs in the regulation of inflammation and angiogenesis. Here we assessed the possibility that BOC2 may exert also a direct inhibitory effect on the angiogenic activity of heparin-binding growth factors independent of its FPR antagonist activity. Our data demonstrate that BOC2 inhibits the angiogenic activity of VEGF-A and FGF2. In endothelial cell-based bioassays, BOC2 prevents VEGF-A and FGF2-mediated sprouting of human umbilical vein endothelial cell spheroids embedded in a 3D fibrin gel and endothelial cell proliferation. Accordingly, BOC2 inhibits VEGFR2 and FGFR1 phosphorylation and activation of the downstream secondary signaling mediator AKT in endothelial cells. In keeping with these observations, BOC2 suppresses the angiogenic potential of the vitreous fluid obtained from patients affected by proliferative diabetic retinopathy and that exerted by highly metastatic human melanoma A2058 cells and human prostate cancer DU145 cells. Thus, BOC2 appears to act as a novel multitarget-angiogenic growth factor antagonist. These findings caution about the interpretation of FPR-focusing experimental data obtained with this compound and set the basis for the design of novel BOC2-derived, FPR independent multi-target angiogenesis inhibitors.

Index Terms- BOC2, Angiogenesis, VEGF-A, FGF2, FPRs.

I. INTRODUCTION

Angiogenesis is a vital process during embryonic development, wound healing, and tissue regeneration [1]. However, when dysregulated, uncontrolled angiogenesis becomes a hallmark feature of several pathogenic conditions, including immune disorders, malignant tumors, and proliferative diabetic retinopathy (PDR) [1]. Pathological angiogenesis is regulated by the balance between negative and positive regulators that eventually initiate the cascade of signal transduction pathways to promote endothelial cell migration, proliferation, and vessel maturation. Suppression of any of these steps would inhibit the formation of new vessels and will ameliorate abnormal neovascularization [2].

Various angiogenic growth factors have been identified that activate cognate signaling tyrosine kinase (TK) receptors expressed by endothelial cells. They include, among others, members of the VEGF and FGF families, their prototypic members being represented by VEGF-A165 (hereafter named VEGF-A) and FGF2 that activate the endothelial pro-angiogenic TK receptors VEGFR2 and FGFR1, respectively [3, 4].

Anti-VEGF therapies showed promise in clinical trials and agents like anti-VEGF antibodies and small molecule VEGFR TK inhibitors have been approved by FDA for treatment of several cancers [5, 6]. Yet, the increase in overall survival of patients treated with these drugs has been modest. Moreover, their use has been associated with severe side effects including gastro-intestinal toxicity, neurotoxicity, impaired wound healing, hypothyroidism, proteinuria, coagulation disorders, and excessive chaotic neovascularization [7].

When compared to mono-therapies, the use of multiple anti-angiogenic agents or combined therapies may be more efficient in sustained inhibition of angiogenesis [8]. This calls for the identification of multitarget inhibitors able to inhibit simultaneously different angiogenic growth factors.

Formyl peptide receptors (FPRs) are G protein-coupled receptors implicated in the regulation of innate immune responses, inflammation, tissue repair and angiogenesis [9]. Three FPRs have been identified in humans (FPR1–FPR3) and the peptide N-tert-butyloxycarbonyl (BOC)-Phe-Leu-Phe-Leu-Phe (BOC-FLFLF in single letter code, BOC2) acts as an FPR1/FPR2 antagonist. Extensive investigations have been performed using BOC2 as a tool to assess the role of FPRs in physiological and pathological conditions (see for instance [9, 10] and references therein). However, no data are available to assess the possibility that BOC2 may exert also a direct anti-angiogenic effect independent of its FPR antagonist activity.

In this study, we evaluated the capacity of BOC2 to neutralize the biological activity of the prototypic angiogenic growth factors VEGF-A and FGF2. In

addition, BOC2 was assessed for its capacity to hamper the angiogenic response exerted by the vitreous humor obtained after pars plana vitrectomy from patients affected by PDR, a major complication of diabetes mellitus and the leading cause of visual impairment [11]. Indeed, numerous angiogenic factors and cytokines related to retinal neovascularization are elevated in the ocular fluid of PDR patients, including VEGF-A [12]. Finally, BOC2 was evaluated for its capacity to inhibit the angiogenic activity of different human tumor cell lines.

Our data demonstrate that BOC2 exerts a significant inhibitory activity against VEGF-A and FGF2 and suppresses the angiogenic potential of PDR vitreous fluid and of highly metastatic human tumor cell lines that co-express both angiogenic mediators. These findings set the basis for the design of novel BOC2-derived multi-target angiogenesis inhibitors and caution about the interpretation of FPR-focusing experimental data obtained with this compound.

II. MATERIAL AND METHODS

2.1 Reagents

BOC2 was from Phoenix Pharmaceuticals Inc. (Burlingame, CA); porcine gelatin, porcine heparin, endothelial cell growth factor and anti-α-tubulin were from Sigma-Aldrich (St. Louis, MO); M199, DMEM, RPMI 1640 media, and FCS were from Gibco Life Technologies (Grand Island, NY); VEGF-A was kindly provided by K. Ballmer-Hofer (PSI, Villigen, Switzerland); FGF2 was from Tecnogen (Caserta, Italy); anti-pVEGFR2 and anti-pAKT antibodies were from Cell Signaling Technology (Beverly, MA); anti-FGFR1 and anti-Focal Adhesion Kinase (FAK) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords and used at early (I–IV) passages. Cells were grown on culture plate coated with porcine gelatin in M199 medium supplemented with 20% FCS, endothelial cell growth factor (10 µg/mL), and porcine heparin (100 µg/mL).

FGFR1-overexpressing fetal bovine aortic endothelial GM7373-flg cells [13] and human melanoma A2058 cells (provided by R. Giavazzi, Istituto Ricerche Farmacologiche Mario Negri, Milano, Italy) were grown in DMEM medium supplemented with 10% FCS. Human prostate cancer DU145 cells were obtained from ATCC repository and cultured in RPMI plus 10% FCS. Serum-free conditioned media (CM) were prepared by incubating confluent A2058 and DU145 cell cultures with DMEM for 48 h. Conditioned media were collected and filtered to remove cell debris. Filtered CM was tested for their capacity to induce HUVEC proliferation and sprouting.

All cells were maintained in a humidified 5% CO_2 incubator at 37°C, with medium replaced every 2-3 days until cells reached confluency. Cells were maintained at low passage, returning to original frozen stocks every 3 to 4 months, and tested regularly for Mycoplasma negativity.

2.3 Human vitreous fluid samples

Vitreous fluid samples (0.3–0.6 ml) were obtained from patients with PDR during pars plana vitrectomy at the Clinics of Ophthalmology (University of Brescia, Italy). Collection and analysis of human samples were approved by the internal review board of the Spedali Civili of Brescia and followed the principles of the Declaration of Helsinki. Informed consent was obtained from all participants. Samples were stored at −80°C. All assays were performed on vitreous samples pooled from 4-5 patients. Data are representative of at least three independent pools of vitreous fluid that provided similar results.

2.4 HUVEC sprouting assay

HUVEC aggregates (800 cells/spheroid) were embedded in fibrin gel and stimulated with VEGF-A (30 ng/mL), or FGF2 (30 ng/mL), or PDR vitreous fluid (1:4 vol:vol) or CM from human tumor cell line (1:4 vol:vol) plus increasing concentrations of BOC2. Sprouts were counted after 24 h and data were expressed as a percentage of activity by an optimal dose of stimulus in the absence of any competitor.

2.5 Endothelial cell proliferation assays

HUVECs were seeded at $17x10^3$ cells/cm^2 and serum-starved overnight. Then, cells were stimulated with 30 ng/mL VEGF-A, and increasing concentrations of BOC2 in the presence of 2.5% FCS. FGFR1-overexpressing endothelial GM7373-flg cells [13] were seeded at $15x10^3$ cells/cm^2 and treated with 30 ng/mL of FGF2 and increasing concentrations of BOC2 in the presence of 1.25% FCS. After 48 h, cells were counted with a MACSQuant cytofluorimeter and cell proliferation was expressed as a percentage of growth factor activity in the absence of any competitor.

2.6 Western blot analysis

HUVECs and GM7373-flg treated with VEGF or FGF2 were probed by Western blotting with anti-pVEGFR2, anti-pFGFR1 and anti-pAKT antibodies (1:1,000). Images were acquired (ChemiDoc Touch; Bio-Rad Laboratories, Inc.) and band intensity was evaluated (Image Lab 3.0 software; Bio-Rad Laboratories, Inc.). Data were normalized to the levels of FAK, or α-tubulin as appropriate.

2.7 Chick Embryo Chorioallantoic Membrane (CAM) Assay

Alginate beads (4 µL), containing 2 µL of vitreous fluid or $2x10^4$ human tumor cells added or not with 60 µM BOC2, were placed on the top of the CAM of fertilized white Leghorn chicken eggs at day 11 of

incubation (7 eggs per experimental group) [14]. After 3 days, blood vessels converging toward the implant were counted under a stereomicroscope (STEMI-SR, x2/0.12; Zeiss) at x5 magnification.

2.9 Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 (San Diego, CA, USA) using one-way ANOVA followed by Bonferroni multiple comparison post-test or Student's t-test. All data are the mean ± SEM of 3 experiments in triplicate unless specified otherwise and P values < 0.05 were considered statistically significant.

III. RESULTS AND DISCUSSION

In the first set of experiments, BOC2 was assessed for its capacity to affect the activity of VEGF-A and FGF2 in endothelial cells. As shown in Fig. 1a-b, BOC2 inhibits both VEGF-A and FGF2-mediated sprouting of fibrin-embedded HUVEC spheroids (Fig. 1a) and endothelial cell proliferation (Fig. 1b). The above observations prompted us to assess whether BOC2 is able to inhibit VEGF-A or FGF2-induced activation of the cognate endothelial pro-angiogenic TK receptors VEGFR2 and FGFR1, respectively. As shown in Fig. 1c-d, BOC2 inhibits VEGF-A-induced VEGFR2 phosphorylation and activation of the downstream secondary signaling mediator AKT in HUVECs in a dose-dependent manner. Accordingly, BOC2 inhibits FGF2-stimulated FGFR1 phosphorylation and AKT activation in FGFR1-overexpressing bovine endothelial GM7373-flg cells (Fig. 1c-d).

Heparan sulfate proteoglycans (HSPGs) present on the cell surface and extracellular matrix are known to mediate the binding of VEGF-A to its signaling receptor VEGFR2, leading to the formation of bioactive HSPG/VEGF-A/VEGFR2 ternary complexes [15]. Similarly, FGF2-initiated FGFR signaling requires the assistance of heparin/heparan sulfate. The formation of HSPG/growth factor/TK receptor ternary complexes stabilizes the growth factor in an active conformation, prevents its degradation, and greatly enhances the angiogenic response in endothelial cells [16]. Therefore, the interaction between HSPG, growth factor, and TK receptor, in order to form active ternary complexes, represent an appropriate therapeutic target for inhibiting pathological angiogenesis [17].

Previous observations have shown that thrombospondin-derived small molecule FGF2 antagonists that engage the heparin-binding domain (HBD) of FGF2 can induce long-range dynamics perturbations along the FGF2/FGFR1 interface [18, 19]. It seems possible to hypothesize that BOC2 will exert a similar effect by binding to the HBD of VEGF-A and FGF2, resulting in the loss of their ability to form bioactive HSPG/growth factor/TK receptor ternary complexes. Further studies are required to elucidate the underlying binding interaction of BOC2 with multiple of angiogenic growth factor.

Proliferative diabetic retinopathy is a disease the damages the blood vessels in the retina (the light-sensitive tissue that lines the back of the eye) and is a major cause of blindness among working adults worldwide. In the pathogenesis of PDR, the growing body of evidence suggests that hyperglycemia causes the activation of neurovascular cells to release numerous locally acting factors, particularly heparin-binding cytokines, and angiogenic growth factors, into vitreous body. Vitreous can thus form an overwhelming "reservoir" of pathological paracrine signals evolving angio-inflammatory mediators like VEGF and others. Accordingly, our previous observations have shown that vitreous fluid from PDR patients is able to induce a series of angiogenic/inflammatory responses in endothelial cells in vitro and in vivo [20, 21]. Thus, the biological activity exerted by PDR vitreous fluid may represent a useful tool to investigate the anti-angiogenic activity of potential novel drug candidates in preclinical experimental models.

On this basis, we assessed the capacity of BOC2 to hamper the angiogenic response exerted by the vitreous fluid from patients affected by PDR. As anticipated, BOC2 suppresses the angiogenic potential of PDR vitreous fluid in vitro and in vivo (Fig 2). Indeed, BOC2 inhibits PDR vitreous fluid-mediated sprouting of HUVEC spheroids embedded in a 3D fibrin gel (Fig. 2a-b) and suppresses neovessel formation induced in vivo by vitreous fluid delivered on the top of the chick embryo CAM (Fig. 2c-d). Altogether, these observations suggest that BOC2 may act as a multi-heparin binding growth factor inhibitor. Indeed pan-heparin-binding growth factor antagonists exert a significant inhibitory activity on the angiogenic responses induced by heparin-binding VEGF-A and PDR vitreous in pre-clinical settings [20].

Several studies have indicated that angiogenic activators play an important role in tumor growth and spreading, their levels representing a prognostic parameter of several tumor types, including melanoma [22] and prostate cancer [23].

These observations prompted us to assess the capacity of BOC2 to inhibit the angiogenic activity exerted by highly metastatic human melanoma A2058 cells and human prostate cancer DU145 cells. These tumor cell lines express high levels of various angio-inflammatory mediators, including VEGF and FGF2, and their conditioned media are endowed with a potent angiogenic activity. As shown in Fig. 3, BOC2 was able to suppress the sprouting of HUVEC spheroids induced by the conditioned media of the two cell lines as well as the activity exerted by the co-administration of optimal concentrations of VEGF-A plus FGF2. In keeping with these observations, BOC2 inhibits neovessel formation induced in vivo

by A2058 and DU145 tumor cells grafted on the top of the chick embryo CAM (Fig. 3a-b).

In conclusion, our data demonstrate that BOC2 is endowed with a significant anti-angiogenic activity that appears to be independent of its FPR antagonist potential. Thus, the capacity of BOC2 to act as a multi-growth factor antagonist advocates caution about the interpretation of FPR-focusing experimental data obtained with this compound whose use has implicated FPRs in a variety of physiological and pathological processes [9, 10]. Due to its multi-growth factor trapping capacity, BOC2 may represent the basis for the design of novel derivatives with potentially interesting therapeutic profiles (e.g. higher potency and lack of anti-FPR activity) for the development of novel multi-target therapies of angiogenesis-dependent diseases, including cancer.

Figures legends

Figure 1: BOC2 inhibits the angiogenic activity of VEGF-A and FGF2. a HUVEC sprouting assay. HUVEC spheroids embedded in fibrin gel were incubated with VEGF-A (black circles) or FGF2 (black square) in the presence of increasing concentrations of BOC2. After 24 h, the number of radially growing sprouts was counted. b Endothelial cell proliferation assay. HUVECs and GM7373-flg cells were stimulated with VEGF-A (black circles) or FGF2 (black square), respectively, in the presence of increasing concentration of BOC2. After 48 h, cells were counted. c, d Western blot analysis. HUVECs and GM7373-flg cells were stimulated for 10 min with vehicle (untreated) or with VEGF-A (black circles) or FGF2 (black squares), respectively, in the presence of increasing concentration of BOC2. Then, cell extracts were probed with anti-pVEGFR2, anti-pFGFR1, and anti-pAKT antibodies. c, densitometric analysis of pVEGFR2 (black circles) and anti-pFGFR1 (black squares) bands normalized to the expression levels of FAK or□□-tubulin. d densitometric analysis of pAKT immunoreactive bands following VEGF-A (black circles) or FGF2 (black squares) treatment normalized to the expression levels of □-tubulin. All data are the mean ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. VEGF-A or FGF2 (ANOVA).

Figure 2: BOC2 inhibits the angiogenic activity of human PDR vitreous fluid. a, b HUVEC sprouting assay. HUVEC spheroids embedded in fibrin gel were incubated with vitreous fluid in the presence of increasing concentrations of BOC2. After 24 h, the number of radially growing sprouts were counted and data (mean ± SEM of 3 independent experiments) represented as a percentage of angiogenic activity of PDR vitreous. b Representative images of vitreous fluid-treated spheroids in the absence (upper panel) or in the presence (lower panel) of 60 µM BOC2. c, d Chick embryo CAM assay. Number of neovessels/implant (n = 14) infiltrating the CAM mesenchyme (c) induced by PDR vitreous fluid in the absence or in the presence BOC2. d Representative images of CAMs grafted with PDR vitreous in the absence (upper panel) or in the presence (lower panel) of 60 µM BOC2. Data are the mean ± SEM of 3 experiments. **P < 0.01 and ***P < 0.001 vs. vitreous treatment (ANOVA).

Figure 3: BOC2 inhibits the angiogenic activity of human cancer cells. a HUVEC sprouting assay. HUVEC spheroids embedded in fibrin gel were incubated with conditioned media (CM) from A2058 or DU145 cells or with fresh medium added with 30 ng/mL VEGF-A plus 30 ng/mL FGF2 in the absence or in the presence of 60 µM BOC2. Radially growing sprouts were counted after 24 h. Data, normalized to the activity of CM without BOC2, are the mean ± SEM of 3 independent experiments. b Chick embryo CAM assay. Number of neovessels/implant (n = 14) infiltrating the CAM mesenchyme induced by A2058 or DU145 cancer cells in the absence or in the presence 60 µM BOC2. Data are the mean ± SEM of 3 experiments. ***P < 0.001 vs. untreated (ANOVA).

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Figure 1:

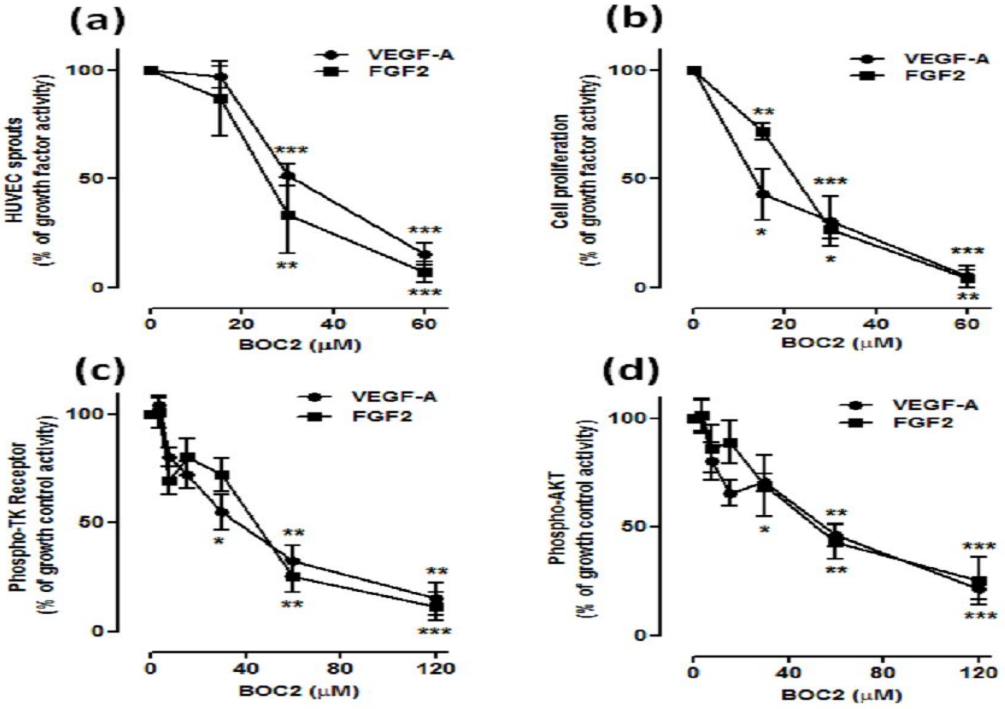


Figure 2:

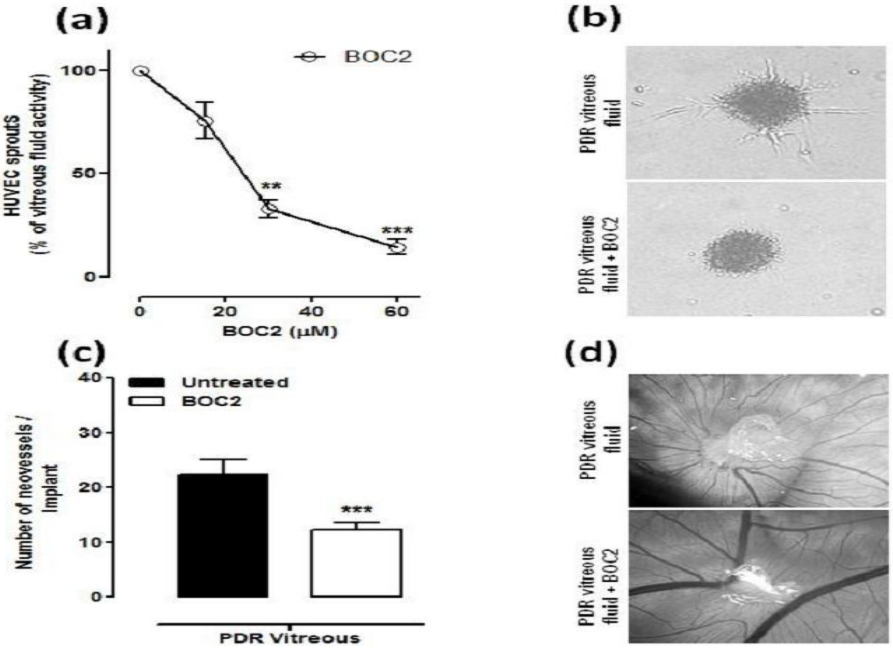
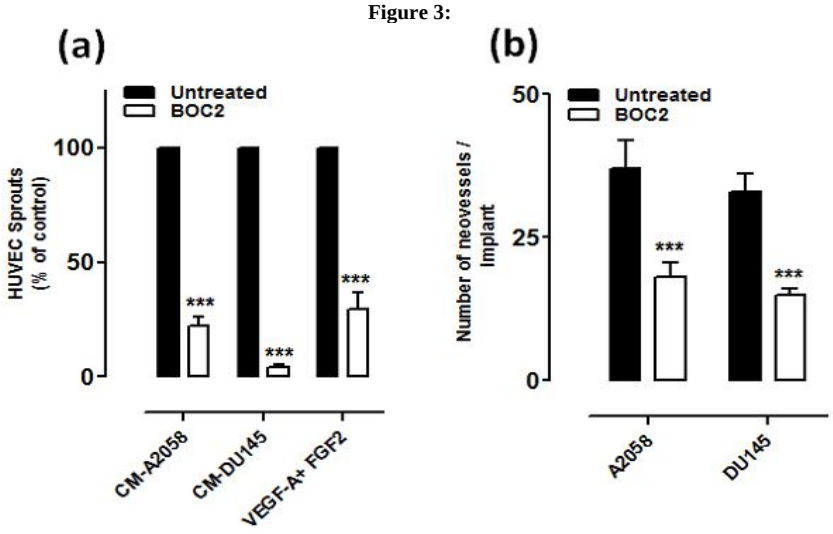


Figure 3:


(a)
Untreated
BOC2
HUVEC Sprouts (% of control)
100
50
0
***
***
***
CM-A2058
CM-DU145
VEGF-A+ FGF2
(b)
Untreated
BOC2
Number of neovessels / Implant
50
25
0
***
***
A2058
DU145

★★★