Biochemical Effects of Some Tyrosine Kinase Inhibitors on Pro-B Cells-Induced Apoptosis

CARMEN GENTIMIR[1], DIANA TATARCIUC[1], CATALINA ANDA ZAHARIA[1]*, LILIANA CHELARU[1], ANDREEA COSTIN[1], ALEXANDRA JITAREANU[1], COSTICA BEJINARIU[2,3]*, MARCEL COSTULEANU[1]

[1] Grigore T. Popa University of Medicine and Pharmacy, 16, University Str., 700115, Iasi, Romania

[2] Romanian Inventors Forum, 3 Sf. Petru Movila Str., 700089, Iasi, Romania

[3] Gheroghe Asachi Technical University of Iasi, Faculty of Science and Engineering Materials, 67 Dimitrie Mangeron Str., 700050, Iasi, Romania

The present studies aimed the effects of tyrphostin AG 494 and tyrphostin AG 1295 on apoptosis of mouse pro-B lymphocytes. The actual scientific literature lacks such data. Tyrphostin AG 494 is an inhibitor of epidermal growth factor receptor pathways and tyrphostin AG 1295 is an inhibitor of platelet-derived growth factor receptor pathways. Our obtained data demonstrated that tyrphostin AG 1295 was less effective in preventing the apoptosis of murine pro-B cells, triggered by the combination of Cytoporone B (NR4A1 agonist) and Cyclosporine A. In contrast, tyrphostin AG 494 had a stronger inhibitory effect on the apoptosis of the same cells, when administered for 24 h. Thus, when blocking the activation of epidermal growth factor receptor pathways, the inductive apoptotic effects of Cytoporone B and Cyclosporine A are reduced. Thus, we could conclude that such inhibition will increase the resistance to apoptosis of pro-B cells. Thus, such a resistance to apoptosis could be experimentally acquired by hematopoietic cells.

Keywords: tyrphostin AG 494, tyrphostin AG 1295, pro-B cells, apoptosis

Protein tyrosine kinase activity could be inhibited by some organic structures as tyrphostins. Among the first proven data there are mentioned the inhibitory effects of some tyrphostins on GTP-ase-associated activation of transducin and of other GTP-dependent biologically active proteins in retinal rod outer segments, including but not limited to guanylyl cyclase or fructose-6-phosphate kinase. On the other side, tyrphostins have no effects on e.g. hexokinase or rhodopsin kinase enzymes, which are dependent to be activated on ATP [1].

One tyrphostin compound, AG 494, was demonstrated to associate dual effects in intact cells. Thus, on one side, tyrphostin AG 494 failed to inhibit epidermal growth factor receptor (EGFR) kinase activity and activation. Other data series demonstrated that tyrphostin AG 494 was able to block proliferation of intact cells induced by epidermal growth factor or serum. These last effects have been strengthened by the observation that tyrphostin AG 495, as well as its congeners AG 490 and AG 555, all were able to inhibit activation of Cdk2. These results are astonishing since the more selective epidermal growth factor receptor kinase inhibitor tyrphostin AG 1478 had no effects in intact cells. Furthermore, the blocking effects of the above mentioned tyrphostins are intriguing, being efficient also when the compounds were administered 20 hours after the epidermal growth factor-induced cellular activation. The Cdk2 inhibitory effects were paralleled by a marked one on the DNA synthesis. The clear conclusion is that tyrphostins could target one or more intimate cellular mechanisms involved in the activation of Cdk2, interfering with the cell cycle machineries [2].

Overactivation and overfunctioning of protein tyrosine kinases represent the pathophysiological basis for proliferative diseases. Tyrphostins are actually known as potential and promising antiproliferative structures acting primarily as protein tyrosine kinase inhibitors. The selective protein tyrosine kinase inhibitors for epidermal growth factor receptor kinase are able to stop/alter the proliferation of cells, dependent primarily on epidermal growth factor [3]. Tyrphostin AG 494 is a well-known and potent blocker of EGF-R kinase (e.g., IC_{50} = 1 μM in HT-22 cells) [4].

Another compound with antiproliferative potential is tyrphostin AG 1295, demonstrated to drastically reduce the division of cells and DNA synthesis in the cultured aortic vascular smooth muscle cells of human type, stimulated by nicotine administration [5].

Another important member of tyrphostins family is tyrphostin AG 1295, selective permeable blocker for the tyrosine kinase activity and DNA-induced synthesis of platelet-derived growth factor receptor (e.g., IC_{50} =500 and IC_{50} = 2.5μM, respectively, in Swiss/3T3 cells). Tyrphostin AG 1295 was able to induce around 50% blocking effects on the development of neointima tissues in a balloon injury model, induced in the femoral arteries of pigs. These inhibitory effects are doubled by the reduction of isolated smooth muscle cells growth and autophosphorylation of platelet-derived growth factor receptor, being selective for platelet-derived growth factor receptor-BB and not for fibroblast growth factor-2 or epidermal growth factor effects [6].

Moreover, tyrphostin AG 1295 was demonstrated to have antiproliferative effects in a model of rabbit vitreoretinopathy development. Conjunctival fibroblasts of rabbit were treated with platelet-derived growth factor-AA and platelet-derived growth factor-BB in the presence of tyrphostin AG 1295 for 3 days. After that, the homologous cultured viable cells were injected intravitreally, followed by tyrphostin AG 1295 (100 microM). Tyrphostin effects were evaluated using tractional retinal detachment development. The reduced development of tractional retinal detachment was evident for at last 21 days after tyrphostin AG 1295 intravitreal injection, presenting no side effects by itself [7].

The present study aimed the effects of tyrphostin AG 494 and tyrphostin AG 1295 on apoptosis of mouse pro-B lymphocytes. Such data does not exist in literature this moment.

* email: catalina.avram@gmail.com; costica.bejinariu@yahoo.com

Experimental part

Pro-B cells of murine type in cultures were used for actual experiments, as previously described [8, 9]. Phase contrast photographic recordings were acquired in accordance with the anterior descriptions [10-12].

The apoptosis of pro-B cells was induced using a combination of Cytosporone B (50μM) and Cyclosporine A (1 μM), as previously established [12].

Tyrphostin AG 494 and tyrphostin AG 1295 were administered as treatment (10 μM) for 24 h , the same time as of Cytosporone B and Cyclosporine A, the inductors of apoptosis in our experiments. The absolute apoptosis control was represented by valinomycin (1 μM).

Results and discussions

The observed contrast phase morphology of pro-B cells was not significantly modified by tyrphostin AG 494 and tyrphostin AG 1295, when solely administered, suggesting that the tyrphostin themselves did not have significant effects on pro-B cells.

When assessed, the apoptotic morphologic alterations were found in a proportion of 59.92±7.41% of the cells in the case of tyrphostin AG 494 (10 μM) treatment (fig. 1), 75±8.69% in the case of tyrphostin AG 1295 (10 μM) treatment (fig. 2), as well as 99.22±0.65% for valinomycin (absolute apoptosis control).

Our obtained data demonstrated that tyrphostin AG 1295 was less effective in preventing the apoptosis of murine pro-B cells, triggered by the combination of Cytoporone B and Cyclosporine A [12]. In contrast, tyrphostin AG 494 had a stronger inhibitory effect on the apoptosis of the same cells, when administered for 24 hours.

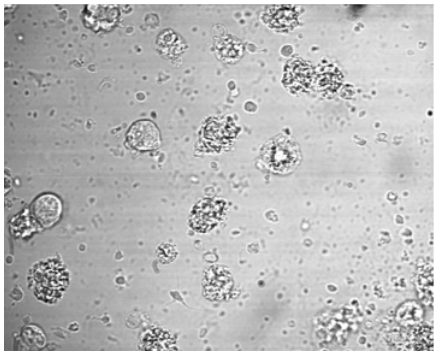

Fig. 1. When administered as treatment for 24 h, tyrphostin AG 494 (10 μM) significantly blocked pro-B cells apoptosis induction by Cytosporone B and Cyclosporine A (as control, 78.29±8.92%). The experiments were performed in triplicate (100x). We want to acknowledge the use of free ImageJ software for contrast phase images acquisition.

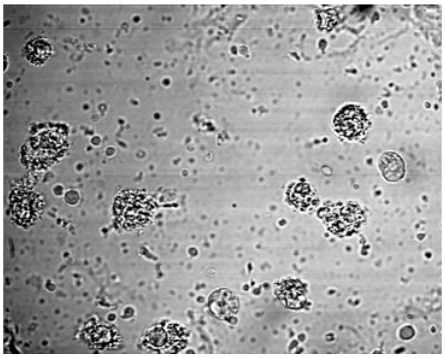

Fig. 2. The effects of treatment with tyrphostin AG 1295 were less evident when compared to those of tyrphostin AG 494 in the same inductive apoptotic conditions for 24 hours. The experiments were also performed in triplicate (100x).

A large number of studies were focusing on the roles played by the epidermal growth factor receptor in the development of tumor cells. On the other side, almost none studies were focusing on the possible effects of epidermal growth factor receptor on immune responses. The signaling pathway of the epidermal growth factor receptor could play a role in the modulation of T cells of regulatory type in patients with cancer. Amphiregulin is an epidermal growth factor receptor-like protein, largely associated with worsening of patients overall surviving. Furthermore, it was shown that up-regulation of Amphiregulin was almost entirely followed by an increased suppressive functioning of T cells of regulatory type. Amphiregulin regulation was involving the EGFR/GSK-3β / Foxp3 pathways either *in vitro* as well as *in vivo*. GSK-3β activity was restored when an inhibitor (gefitinib) of epidermal growth factor receptor was used. As a consequence, the functioning of T regulatory cells type was attenuated. Thus, it was demonstrated that Amphiregulin could regulate at the post-translational level the expression of Foxp3 in patients with cancer through the epidermal growth factor receptor/GSK-3β signaling pathway [13].

One of the most aggressive forms of cancer is the inflammatory breast cancer. The same time, one of the most important direction of treatment is represented by the attempt to develop monoclonal antibodies against epidermal growth factor receptors as well as against human epidermal growth factor receptor 2. Unfortunately, the most common result is pharmacologic and therapeutic resistance. Beside the interference with the oncogenic development pathways, such approach is intended to stimulate and amplify the antibody-dependent cellular cytotoxicity, allowing the activation of immune effector cells, which will become further capable to destroy the tumoral cells through the liberation of granzymes and activation of executioner caspases. The treatment resistance of inflammatory breast cancer cells could be induced by upregulation and overexpression of some anti-apoptotic molecules. X-linked inhibitor of apoptosis protein could be one of these overexpressed anti-apoptotic molecules in inflammatory breast cancer, being the basis for the resistance to antibody-dependent cellular cytotoxicity. In contrast, downregulation of X-linked inhibitor of apoptosis protein will enhance the tumoral cells susceptibility to antibody-dependent cellular cytotoxicity. The upregulated X-linked inhibitor of apoptosis protein effects were related to the inhibition of reactive oxygen species accumulation, a process independent this time of caspases activation. The enhancement of reactive oxygen species production is a normal process during antibody-dependent cellular cytotoxicity. All these biological effects of upregulated X-linked inhibitor of apoptosis protein were demonstrated by transcriptome analysis. Thus, the clear conclusion might be that X-linked inhibitor of apoptosis protein is a fine regulator of antibody-dependent cellular cytotoxicity, involving mechanisms dependent and independent on caspases [14].

Although there exists important progress concerning the administration of anti-EGFR antibodies for the cancers treatments, many unknowns remain uncovered. For example, the postulated mechanisms of immune effectors. Adaptive immunity is included and is involving the mechanisms primarily mediated by Fc region. It remains to clarify if epidermal growth factor receptor antibody, through the inhibition of oncogene, could have an important contribution to its vaccine effect. When administered in a tumoral mouse model, both an antibody anti-murine epidermal growth factor receptor (7A7) and an epidermal growth factor receptor-tyrosine kinase inhibitor (tyrphostin

AG 1478) showed strong antimetastatic potencies, only for the antibody was evident the involvement of CD4(+) and CD8(+) T cells. The link with adaptive immunity was revealed through the experiments *in vivo*, involving 7A7 F(ab')(2) antibodies. Interesting is the fact that subcutaneous injection of tumoral cells treated with 7A7 induced an unexpected antitumoral immune response. When administered, 7A7 blocked epidermal growth factor receptor activation and subsequently induced a pro-apoptotic effect on tumoral metastases as compared to the inhibitor tyrphostin AG 1478 of the same receptor (inhibitor of the intrinsic tyrosine kinase activity of epidermal growth factor receptor). These experiments very clear pointed out that the inhibition of the epidermal growth factor receptor mediated by 7A7 antibody is involving a strong immunogenic apoptosis component [15].

Epidermal growth factor receptor pathway blockade through quercetin could have as result the induction of natural killer group 2, member D (NKG2D) ligands on tumoral cells. The consequence is increased sensitivity of cancer cells to NK-cell-mediated killing. Erlotinib and gefitinib, inhibitors of epidermal growth factor receptor, augmented the expression at the surface of ULBP1, increasing subsequently the sensibility of cancer cells (pulmonary cancer cells A549, NCI-H23, and SW-900) to NK-92 cells. The effects of the inhibitors of epidermal growth factor receptor were reversed by phorbol 12-myristate 13-acetate. The intimate mechanism for the induction of ULBP1 and increased susceptibility of tumoral cells to NK cell-mediated cytotoxicity is represented by the inhibition of PKC pathways [16].

For the proliferation of many cells types, basically epithelial cells, the epidermal growth factor is an important trigger and stimulator. When the expression of the receptor for epidermal growth factor or of one of the members of its family is altered, there exists the conditions for tumoral cells transformation. Forming of the complex between the epidermal growth factor and its specific receptor will induce the activation of subsequent intracellular pathways, which will affect the cellular cycle progression and apoptosis. It is thought that epidermal growth factor receptor is not expressed or almost not expressed in hematopoietic cells. There are some data showing that the epidermal growth factor receptor exists and is functional in some murine hematopoietic cell lines, dependent on cytokines (e.g. FDC-P1, but not FL5.12, dependent on IL-3). The DNA synthesis and activation of ERK, induced by epidermal growth factor, was mild in FDC-P1 cell line. The same effects were induced by the addition of suboptimal IL-3 concentrations in FDC-P1 cell line. When administered, the tyrphostin inhibitor AG 1478 induced apoptosis in the cells type FDC-P1, positive for epidermal growth factor receptor. When epidermal growth factor receptor is activated, the subsequent expression of v-ERBB will induce a real transformation of both above mentioned cell lines, which will become independent on cytokines. They will be able to develop in cell cultures in the absence of autocrine growth factors from the medium (cytokines as IL-3). That is, such results underline the biological functional importance of endogenous signaling pathways as those mediated by epidermal growth factor in hematopoietic cell lines [17].

Physiological or pathological apoptosis is based on a mitochondrial execution phase, starting with the opening of the so-called mitochondrial permeability transition pore. Degenerative and hyperproliferative diseases are developing on altered mitochondrial functioning [18].

Photosensitizers initiate apoptosis in cancer cells also through the activation of mitochondria and mitochondrial permeability transition pore opening, increasing the level of released reactive oxygen species [19].

Some other anti-tumoral compounds as betulinic acid are able to enhance the OXPHOS state, which is equivalent to the enhancement of respiratory function, in mitochondria isolated from murine melanoma [20].

In carcinogenesis, there is demonstrated a lack of cellular growth control by growth factors. That means the appearance of a constitutive activation of receptors for cellular growth factors, although the cellular growth factors are not stimulating the cells. One of such factor involved in carcinogenesis is the epidermal growth factor receptor and its endogenous activated pathways, its overexpression or overfunctioning escaping from the radar of immune system. The tolerance of the immune system is thus enhanced. The activation and augmentation of epidermal growth factor endogenous pathways will reduce the tumoral antigen presentation, will upregulate PD-L1, will induce the expression and activation of transforming growth factor beta (an inhibitory molecule), and will modify the tumoral cells metabolic pathways, increasing aerobic glycolysis and the release of lactate, impairing as a result the natural killer and cytotoxic T lymphocytes recognition and functioning. Furthermore, the decipherment of detailed mechanisms covering the tumoral cells escape which is mediated by overenhanced epidermal growth factor receptor pathways will allow us to develop therapies based on specific monoclonal antibodies. Such specific monoclonal antibodies therapies are intended to reverse the tumoral cells metabolism, to reduce the signaling through oncogenes pathways and to enhance the cellular immune response charged to remove the tumors. The pharmacologic and clinical effects could be enhanced by the addition of other specific antibodies, able to target the PD-L1 or TGFβ suppressive pathways [21].

The most important actual challenge in cancer therapeutics and clinics is represented by acquired resistance. There are several investigations trying to find out if the acquired resistance of tumoral cells is naturally occurring or is the result of an increased plasticity of these cells. The experiments involved a checkpoint kinase (MPS1) and some of its inhibitors (AZ3146, NMS-P715, as well as CCT251455). These modelling studies identified point mutations (in number of five), located in the kinase domain of the protein, representing the basis for the resistance and inducing steric hindrance to the above mentioned inhibitors. All the described point mutations were found in non-treated tumoral cells, as well as in normal lymphoblasts and breast tissues. Furthermore, in tumoral cells and in normal tissues was described a natural mutation of epidermal growth factor receptor, namely p.T790M, the basis for the tumoral resistance to its inhibitor gefitinib [22].

In plastics industry the styrene compound is used on the large scales. It was demonstrated that styrene compound as well as its primary metabolite, namely styrene-7,8-oxide, have genotoxic and carcinogenic effects. Both chemical structures were able to experimentally induce tumours in mice and rats. In humans, the epidemiologic studies revealed higher rates of mortality or incidence of lympho-hematopoietic tumours (leukemia, lymphoma or both types) in plastics (styrene) workers. Concerning the active metabolite styrene-7,8-oxide yet there are no adequate studies involving humans, although it was clearly demonstrated that it forms DNA adducts [23].

The above mentioned studies are really important showing that plastics and their manufacturing compounds are inducing also hematopoietic tumours since the plastics

are scattered and wasted all around the human environment [24-31].

Conclusions

Our obtained data demonstrated that tyrphostin AG 1295 was less effective in preventing the apoptosis of murine pro-B cells, triggered by the combination of Cytoporone B and Cyclosporine A. In contrast, tyrphostin AG 494 had a stronger inhibitory effect on the apoptosis of the same cells, when administered for 24 h . These results demonstrate a powerful involvement, also in apoptosis, of epidermal growth factor receptor pathways activation as compared to platelet-derived growth factor receptor activation pathways in pro-B cells lines.

References

1.WOLBRING, G., HOLLENBERG, M.D., SCHNETKAMP, P.P., J. Biol, Chem., 269, **36**, 1994, p. 22470.

2.OSHEROV, N., LEVITZKI, A., FEBS Lett., 410, **2-3**, 1997, p. 187.

3.GAZIT, A., YAISH, P., GILON, C., LEVITZKI, A., J. Med. Chem., 32, **10**, 1989, p. 2344.

4.SAGARA, Y., ISHIGE, K., TSAI, C., MAHER, P., J. Biol. Chem., 277, **39**, 2002, p. 36204.

5.PESTANA, I.A., VAZQUEZ-PADRON R.I., AITOUCHE, A., PHAM, S.M., J. Cell. Biochem., 96, **5**, 2005, p. 986.

6.BANAI, S., WOLF, Y., GOLOMB, B., PEARLE, A., WALTENBERGER, J., FISHBEIN, I., SCHNEIDER, A., GAZIT, A., PEREZ, L., HUBER, L., LAZAROVICHI, G., RABINOVICH, L., LEVITZKI, A., GERTZ, S.D., Circulation, 97, **19**, 1999, p. 1960.

7.ZHENG, Y., IKUNO, Y., OHI, M., KUSAKA, S., JIANG, R., CEKIC, O., SAWA, M., TANO, Y., Jpn. J. Ophthalmol., 47, **2**, 2003, p. 158.

8.ZONDA, G.I., GORIUC, A., IANCU, R.I., COSTULEANU, M., ANAL. ªT. UNIV. Al. I CUZA IAªI. SECT. GEN. BIOL. MOLEC., **TOM XI**, 2010, p. 15.

9.BOISTEANU, O., ZONDA, G.I., AVRAM, C., CHELARU, L., GENTIMIR, C., ACATRINEI, D., COSTULEANU, M., Rev. Chim. (Bucharest), **66**, no. 9, 2015, p. 1452.

10.ZAHARIA (AVRAM), C., ACATRINEI, D., GENTIMIR (AMITITELOAIE), C., BOISTEANU, O., RADUCANU, C.O., CHELARU, L., VASINCU, D., BOGZA, G., COSTULEANU, M., Rev. Chim. (Bucharest), **66**, no. 12, 2015, p. 2040.

11.ACATRINEI, D., GENTIMIR (AMITITELOAIE), C., ZAHARIA (AVRAM), C., RADUCANU, C.O., BOGZA, G., CHELARU, L., VASINCU, D., BOISTEANU, O., COSTULEANU, M., Rev. Chim. (Bucharest), **67**, no. 1, 2016, p. 57.

12.GENTIMIR (AMITITELOAIE), C., ACATRINEI, D., ZAHARIA (AVRAM), C., BOISTEANU, O., RADUCANU, C.O., CHELARU, L., VASINCU, D., BOGZA, G., COSTULEANU, M., Rev. Chim. (Bucharest), **67**, no. 2, 2016, p. 353.

13.WANG, S., ZHANG, Y., WANG, Y., YE, P., LI, J., LI, H., DING, Q., XIA, J., J. Biol. Chem., 291, **40**, 2016, p. 21085.

14.EVANS, M.K., SAUER, S.J., NATH, S., ROBINSON, T.J., MORSE, M.A., DEVI, G.R., Cell Death Dis., **7**, 2016, e2073.

15.GARRIDO, G., RABASA, A., SANCHEZ, B., LOPEZ, M.V., BLANCO, R., LOPEZ, A., DERNANDEZ, D.R., PEREZ, R., FERNANDEZ, L.E., J. Immunol., 187, 10, 2011, p. 4954.

16.KIM, H., KIM, S.H., KIM, M.J., KIM, S.J., PARK, S.J., CHUNG, J.S., BAE, J.H., KANG, C.D., J. Immunother., 34, 4, 2011, p. 372.

17.SHELTON, J.G., STEELMAN, L.S., ABRAMS, S.L., WHITE, E.R., AKULA, S.M., BERTRAND, F.E., FRANKLIN, R.A., MCCUBREY, J.A., Cell Cycle, 4, **6**, 2005, p. 818.

18.KERTI, V., CORICOVAC, D., DUICU, O., DANILA, M., DEHELEAN, C., FAGADAR-COSMA, E., MUNTEAN, D., NOVEANU, L., Rev. Chim. (Bucharest), **65**, 2014, p. 447

19.TAMPA, M., MATEI, C., POPESCU, S., GEORGESCU, S.R., NEAGU, M., CONSTANTIN, C., ION, R.M., Rev. Chim. (Bucharest), **64**, 2013, p. 639

20.DUICU, O., CIURLEA, S., DEHELEAN, C., ARDELEAN, S.,, ANDRICA, F., MUNTEAN, D., SOICA, C., ANTAL, D., CORICOVAC, D., PINZARU, I., Rev. Chim. (Bucharest), **65**, 2014, p. 956

21.CONCHA-BENAVENTE, F., FERRIS, R.L., Front. Pharmacol., **8**, 2017, 332.

22.GURDEN, M.D., WESTWOOD, I.M., FAISAL, A., NAUD, S., CHEUNG, K.M., MCANDREW, C., WOOD, A., SCHMITT, J., BOXALL, L., WORKMAN, P., BURKE, R., HOELDER, S., BLAGG, J., VAN MONTFORT, R.L., LINARDOPOULOS, S., Cancer Res., 75, **16**, 2015, p.3340.

23.HUFF, J., INFANTE, P.F., Mutagenesis, 26, 5, 2011, p. 583.

24. PAHONIE, R.C., STEFAN, A., COSTULEANU, C.L., BOLDUREANU, D., ANDRUSEAC, G.G., Mat. Plast., **54**, no. 1, 2017, p. 155.

25.COSTULEANU, C.L., BOLDUREANU, G., ANDRUSEAC, G.G., Rev. Chim. (Bucharest), **68**, no. 5, 2017, p. 1058.

26.IGNAT, G., COLIBABA, C., COSTULEANU, C.L., BALAN, A., ROTARU, L., SANDU, I.C.A., Mat. Plast., **53**, no. 3, 2016, p. 367.

27. PAHONIE, R.C., STEFAN, A., ADOCHIEI, I.R., COSTULEANU, C.L., ANDRUSEAC, G.G., UNGUREANU, G., SARDARU, D.P., Mat. Plast., **54**, no. 2, 2017, p.396.

28.COSTULEANU, C.L., BREZULEANU S., IGNAT, G., BOLDUREANU, G., TOMA, M.C., ANDRUSEAC, G.G., Rev. Chim. (Bucharest), **67**, no. 10, 2016, p. 1990.

29.IGNAT, G., BALAN, G., SANDU, I., COSTULEANU, C.L., VILLE, S.T.S., Rev. Chim. (Bucharest), **67**, no. 8, 2016, p. 1560.

30.COSTULEANU, C.L., BOBITAN, N., DUMITRESCU, D., Environ. Eng. Manag. J., **14**, 6, 2015, p. 1423.

31. COSTULEANU, C.L., VINTU, C.R., ROBU, D.A., IGNAT, G., BREZULEANU, S., Rev. Chim. (Bucharest), **66**, no. 5, 2015, p. 743.

Manuscript received: 19.01.2017