Brave New Hope for Breast Cancer

Aminopyrazole derivates between rational design and clinical efficacy

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Breast cancer research is a priority nowadays because of the high burden the disease represents for health systems worldwide. New agents are intensively researched to overcome breast cancer biology. Our aminopyrazole derivatives showed promising results when tested in vitro on breast cancer cell lines, by inhibiting protein kinase pathways. Anti-proliferative effect of pyrazole compounds on different breast cancer cell lines was heterogeneous. Further research is necessary to design the optimal structure in terms of high antitumor efficacy and good safety profile. Ophthalmologist control is very important for patients with breast cancer because treatment with some drugs reported side ocular effects: cataract, maculopathy (crystals or drusen or yellowish spots), retinal hemorrhages or dry.

Keywords: aminopyrazole derivatives, Prediction of Activity Spectra for Substances (PASS), triple-negative breast cancer, protein kinases inhibitors

Breast cancer represents the most common cause of cancer in women and the second cause of mortality due to malignancy in women [1]. Breast cancer research represents a worldwide priority because of high prevalence and mortality and steady efforts are made to design new anticancer molecules with better efficacy and less side effects. Like all types of cancer, breast cancer represents an uncontrolled proliferation of abnormal breast cells. Genes regulate cell growth mechanisms. The malignant phenotype occurs through activation changes of oncogenes, followed by gain of function effects or through inactivation changes in tumor suppressor genes, which promote loss of function effects [2]. Blocking normal cell mechanisms of apoptosis, followed by uncontrolled proliferation, leads to the formation of breast cancer cells population. Depending on the biology of cancer cells, breast cancer can be divided into distinct subtypes with different prognostic and therapeutic implications. The routine evaluation of breast cancer patient consists in immunohistochemistry evaluation of the tumor to quantify the expression of estrogen receptor (ER), progesterone receptor (PgR) and amplification of human epidermal growth factor receptor 2 (HER-2/Neu) [3]. These markers allow classification of breast cancer tumors as hormone receptor positive tumors, HER-2/Neu non-amplified or negative tumors, with better prognosis, while breast cancer that do not express ER or PgR, and do not have HER-2/Neu amplification is referred to as triple-negative breast cancer (TNBC), based on the lack of these molecular markers [4]. TNBC represents approximately 10–15% of all breast cancers and has a poorer outcome compared to the other subtypes of breast cancer [5].

As selective inhibitors of PI3 kinase enzymes, pyrazole derivatives efficacy was evaluated in different medical fields, for example in the treatment of ophthalmic, autoimmune, cardiovascular, neurodegenerative or oncological pathology. Conditions with great impact on population worldwide, like glaucoma as one of the main causes of blindness, may benefit of new revolutionary treatment based on kinase inhibitors [6]. Ophthalmologist control is very important for patients with breast cancer because treatment with some drugs reported side ocular effects: cataract, maculopathy (crystals or drusen or yellowish spots), retinal hemorrhages or dry [7–9].

We focused our research efforts on the rational design of new anticancer drug molecules targeted as protein kinase inhibitors and developed, synthesized and assayed the biological properties of various pyrazole-based derivatives [10–14]. A series of pyrazole derivatives were analyzed in the Anticancer Drug Screening Program of the National Cancer Institute (NCI) and their growth inhibition effect was measured on a panel of 60 human tumor cell lines, representing nine tissue types (brain, blood and bone marrow, breast, colon, kidney, lung, ovary, prostate, and skin). A chemoinformatic study was performed on the most promising anti-proliferative compound, N-[5-(4-bromophenyl)-1*H*-pyrazol-3-yl]carbamothioyl] benzamide, and its anticancer profile was correlated with a number of standard drugs, functioning as inhibitors of various protein kinases connected with the PI3K/Akt/mTOR pathway, indicating the inhibition of this intracellular signaling pathway as the most probable pharmacological mechanism [15,16].

The objective of this research was to analyze the anti-proliferative effects of several pyrazole derivatives on breast cancer cells and to better understand the signaling pathways involved, in order to design new targeted molecules for triple negative breast cancer.

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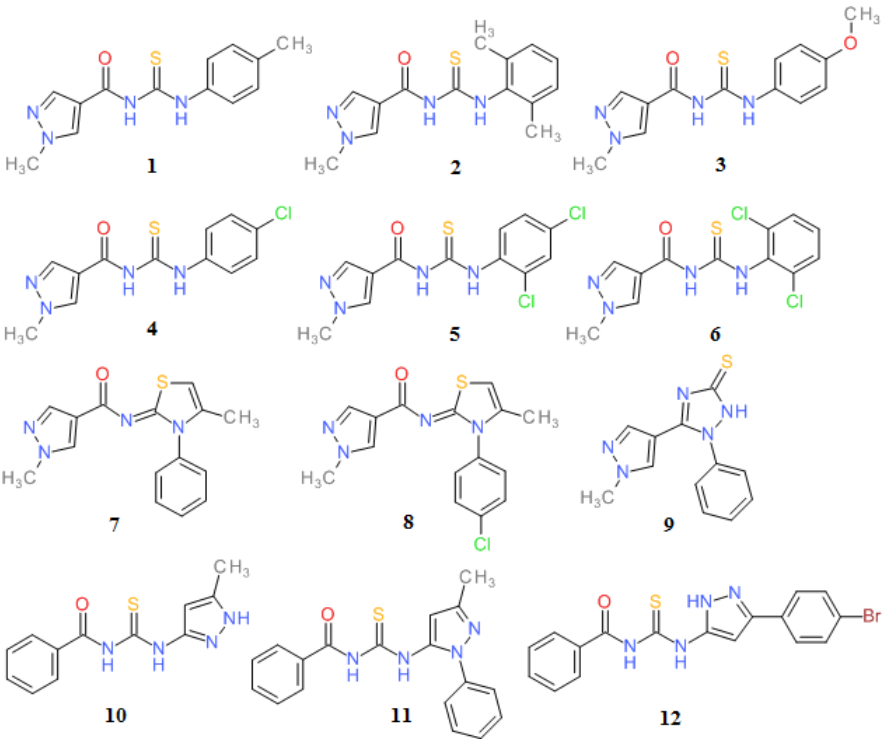

Fig. 1. Chemical structures of the pyrazole derivatives (**1-12**)

Experimental part

The small library of pyrazole derivatives was screened using the PASS application [17] to render each compound's biopharmaceutical profile and to evaluate the anti-proliferative mechanism on various tumor types, inhibiting protein kinases and to predict potential side effects. The compounds **1-12** were synthesized according to the procedure described in literature and their structures were analyzed by NMR and IR spectra [15]. The chemical structures of the compounds **1-12** are shown in figure 1.

The compounds (**1-12**) were tested at a dose of 10^{-5} M on various breast cancer cell lines (MCF7, T-47D, MDA-MB-231, HS 578T, BT-549 and MDA-MB-468), according to a previous disclosed procedure [18]. One of the major benefits of using cultured cell lines in cancer research is that they offer an infinite supply of a relatively homogeneous cell population that is capable of self-replication in standard cell culture medium [19].

PASS estimates the probable biological activity profile based on the structural formulae presented in mol file and uses the structure-activity relationships for more than 300,000 compounds with known biological activities. Average accuracy of prediction estimated in leave-one-out cross-validation procedure for the whole PASS training set is about 95% [20].

Results and discussions

The anti-proliferative effect results are reported as growth percentages compared with the untreated control cells after 48 h of exposure. The values under 100 indicate growth inhibitory effect, whereas those above 100 indicate growth stimulation (table 1).

The analysis of pyrazole derivatives effects on six lines of breast cancer cells revealed major variation depending on the cell subtype and its characteristics. The MCF7 is the most commonly used breast cancer cell line in the world,

Table 1

THE ANTI-PROLIFERATIVE EFFECT OF THE PYRAZOLE COMPOUNDS ON BREAST CANCER CELL LINES

	MCF7	T-47D	BT-549	HS 578T	MDA-MB-231	MDA-MB-468
1	98.3	98.5	-	109.2	101.2	112.9
2	90.9	98.3	102.2	116.7	98.6	132.9
3	102.3	87.9	-	-	117.8	111
4	69.7	56.9	-	97.9	100.8	53.4
5	97.2	85.2	83.1	100.1	97.1	116.4
6	93.6	-	101.9	99.8	98.9	117.9
7	49.8	97.3	85	104.7	92.3	72.6
8	48.4	97.8	110.9	106.1	82.8	79.9
9	99.3	108.4	103.2	105	110.6	115.7
10	103.2	97.7	-	113.6	-	107.5
11	87.3	84.9	-	92.8	95.7	79.2
12	59.5	80.5	-	76.5	56.2	116.3

Table 2
THE ESTIMATED ANTICANCER TARGETS COMPUTED FOR COMPOUNDS **1-12** (Pa VALUES)

	1	2	3	4	5	6	7	8	9	10	11	12
FAK1	0.43	0.43	0.43	0.44	0.43	0.44	-	-	-	0.45	-	-
FAK2	0.36	0.30	0.40	0.36	0.38	0.33	-	-	-	0.46	0.33	-
SIRT1	0.33	0.32	0.31	0.34	-	0.32	-	-	-	0.34	0.31	0.41
SIRT2	0.31	-	-	0.31	-	-	-	-	-	0.30	-	-
Hsp27	0.39	0.32	0.39	-	-	-	-	-	-	0.43	0.67	0.31
CDK2	-	-	-	-	-	-	-	-	-	0.46	-	0.66
CDK9	0.35	0.34	0.32	0.32	-	0.31	0.35	0.30	-	0.43	0.41	0.44
PDGFR	0.35		0.30	0.31	-	-	-	-	-	0.48	-	-
c-Fgr	-	-	-	-	0.32	0.33	-	-	-	-	0.34	-
EIF4E	-	-	-	-	-	-	0.31	0.33	-	-	-	-
Raf	-	-	-	-	-	-	-	-	0.39	-	-	-
MAP3K5	-	-	-	-	-	-	-	-	0.31	0.42	0.49	0.40
AurA	-	-	-	-	-	-	-	-	-	0.40	-	-
AurB	-	-	-	-	-	-	-	-	-	0.50	-	-
STAT3	-	-	-	-	-	-	-	-	-	0.39	0.43	0.51

FAK: Focal adhesion kinase, SIRT: Sirtuin, Hsp27: Heat shock protein 27, CDK2: CDK2/cyclin A, CDK9: CDK9/cyclin T1, PDGFR: Platelet-derived growth factor receptor, c-Fgr: Proto-oncogene tyrosine-protein kinase Fgr, EIF4E: eukaryotic translation initiation factor 4E, Raf: Raf kinase, MAP3K5: *mitogen-activated protein kinase kinase kinase 5, AurA: Aurora-A kinase, AurB: Aurora-B kinase, STAT3: signal transducer and activator of transcription 3*

being generated in 1973 at the Michigan Cancer Foundation [21]. They are breast cancer cells isolated from pleural effusion of patients with metastases with an aneuploid karyotype and tumorigenic. They are used to reproduce early stages, estrogen-dependent disease [22]. Besides estrogen receptors, the MCF7 cell line has receptors for glucocorticoids, synthetic progestogens and androgens. A test based on sucrose density gradients showed that MCF-7 cytosol contains approximately 100 fm/mg protein estradiol (E_2-^{3}H) receptor, more than 300 fm/mg protein progesterone receptor (measured with R5020-^{3}H), about 40 fm/mg protein 5α-dihydrotestosterone (5α-DHT-^{3}H) receptor, and 800 fm/mg glucocorticoid receptor (measured with dexamethasone-^{3}H). This demonstration of four classes of steroid receptors in human breast cancer means that MCF-7 may be an excellent *in vitro* model for studying the mechanism of tumor response to endocrine therapy, as well as the complex relationship between binding and biological actions of these hormones [23]. This is possibly the explanation for the increased sensibility to pyrazole compounds 7, 8 and 12, all producing a growth inhibition close to 50%.

The T-47D cell line has been established from the pleural effusion of a patient with metastatic breast carcinoma. These cells exhibit epithelial morphology and form monolayers in culture. The cultured cells exhibit an aneuploid karyotype with a mode of 66 chromosomes, including an extra-long subtelocentric chromosome. The cytosol of the T-47D cells contains specific high affinity receptors for estradiol, progesterone, glucocorticoid and androgen. Competition studies showed that steroid binding to estrogen and progesterone receptors was inhibited only by the homologous hormone, whereas binding of dexamethasone and dihydrotestosterone to their respective receptors was inhibited by certain other steroids as well [24]. MCF-7 and T-47D are the most representatives members of Luminal A breast cancer subgroup, made of cells stimulated by the presence of estrogen, therefore their growth is inhibited by anti-estrogen agents [19]. In spite of their similar functional profile, their response to pyrazole compounds varies considerable, the T-47D cells being inhibited only by compound 4.

The BT-549 cell line site of origin is primary tumor from Caucasians patients with invasive ductal carcinoma. The cells exhibit a karyotype with a mode of 74 chromosomes with three marker chromosomes [25]. According to its molecular classification, this cell line belongs to claudin-low subgroup. The claudin-low subgroup exhibits an immunoprofile that may reflect the clinical triple-negative tumor type [19,26]. This means that BT-549 cells does not contain specific receptors for estrogen (ER) and progesterone (PR), neither human epidermal growth factor receptor 2 (HER2). Another member of claudin-low subgroup is HS 578T cell line with origins in epithelial cells derived from a breast carcinosarcoma. The cells exhibit an aneuploid karyotype with chromosome number ranging from 50–77 and chr17 is missing. It is used to model metaplastic disease [22]. MDA-MB-231 cell line is also a claudin-low member. These cells form loosely cohesive grape-like or stellate structures consistent with the more invasive phenotype they reproduce *in vitro* [26]. They are regarded as invasive *in vitro* and remain relatively poorly metastatic [27]. In this group of cells, only MDA-MB-231 responded to the tested compounds, with the best anti-proliferative effect produced by compound 12.

The MDA-MB-468 cell line belong to the basal subgroup and is characterized by lack of estrogen receptors (ER-), progesterone receptors (PR-) and human epidermal growth factor receptor 2 (HER2-) [19]. These characteristics make MDA-MB-468 cells resistant to the classical antitumor agents. However, the compound 4 has promising anti-proliferative effects and by careful drug, design could provide new potent solutions for triple negative breast cancer.

In order to better understand the structure-activity relationship, a virtual screening of the small pyrazole library was performed using the computer program PASS (*Prediction of Activity Spectra for Substances*), a software product designed for evaluating the general biological potential of an organic drug-like molecule. PASS provides simultaneous predictions of many types of biological activity based on the structure of organic compounds and can be used to estimate the biological activity profiles for virtual molecules and is a useful tool for selecting compounds with a pre-defined biological activity profile including desirable pharmaco-therapeutic effects and biochemical mechanisms. The output data is represented

by the probability of a compound to interact with a particular target (Pa) or not (Pi). This method provides a good biological interpretation regarding the underlying molecular mechanisms of the anti-proliferative effects on breast cancer cells and it offers valuable information for the rational drug design of new safer anticancer therapies.

The analysis of Pa value for each compound and the corresponding target, presented in table 2, indicates that differences in structure are translated in differences in anti-proliferative efficacy. The compound 4 demonstrated good anti-proliferative effects on MCF-7, T-47D and MDA-MB-468 cell lines, but no effect was observed on the MDA-MB-231 and HS 578T cells. The predicted data indicates the inhibition of the focal adhesion kinases or of the histone deacetylase SIRT1 and SIRT2. Another lead molecule is the structure 12, compound that produced a significant growth inhibition on MCF-7, MDA-MB-231 and HS 578T cell lines, but with stimulating effect on MDA-MB-468. The predicted mechanism is based on CDK2/cyclin A inhibition or the inhibition of the transcription factor STAT3.

The drug development process, from structure design to clinical use, is long and costly. Rigorous processes are used to protect the safety of clinical study participants and also ensure that documentation of adverse events is complete. *In silico* prediction of drug side-effects in early stage of drug development is becoming essential, not only reducing the time of drug development, but also the costs [28]. PASS provides a tool for excluding structures associated with undesirable adverse or toxic effects. Colchicine and phenazone were used as positive controls. The PASS analysis revealed a number of 19 distinct possible side effects with a Pa value over the 0.3 threshold. An analysis of adverse events incidence was performed and revealed neutrophilic dermatitis (Sweet's syndrome), nail discoloration and inflammation as most frequent side effects. Colchicine is known to have retinal neurons toxicity, greatly impacting the safety profile of the drug [29]. Odds are used to describe the chance of an event occurring and they can be computed by the ratio of the probability in favor (Pa) and of the probability (Pi). We analyzed the effects with an odd value over 10. This operation restricted the number of possible adverse effects only to inflammation, with 6 compounds flagged as potential risks. The analysis of structure-activity relationships found a strong correlation between the inflammation risk and the N-(1-methyl-1*H*-pyrazole-4-carbonyl)-thiourea scaffold.

Conclusions

Aminopyrazole derivatives are a class of substances with promising results against breast cancer. However, cytotoxic effect of aminopyrazole derivatives *in vitro* was heterogeneous, depending on the structure used and the profile of breast cancer cell line. The toxicity of these agents has to be carefully analyzed and the structure with best safety profile selected. PASS assessment is a useful tool to select the structure with a certain biological activity and to anticipate associated side effects. It provides a good biological interpretation regarding the underlying molecular mechanisms of the anti-proliferative effects and offers information for the rational drug design of new safer anticancer therapies. Ophthalmologist control is very important for patients with breast cancer because treatment with some drugs reported side ocular effects: cataract, maculopathy (crystals or drusen or yellowish spots), retinal hemorrhages or dry.

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References

1. *** U.S. CANCER STATISTICS WORKING GROUP. Department of Health and Human Services, Centers for Disease Control and Prevention and National Cancer Institute; 2013. Available at: http://www.cdc.gov/uscs.
2. OSBORNE, C., WILSON, P., TRIPATHY, D**.,** Oncologist, **9**, 4, 2004, p. 361-377.
3. BRENTON, J. D., CAREY, L. A., AHMED, A., CALDAS, C. J. Clin. Oncol., **23**, 29, 2005, p. 7350-7370.
4. CHACON, R. D., COSTANZO, M. V., Breast Cancer Res., **12**, Suppl 2, 2010, p. S3.
5. ANDERS, C. K., CAREY, L. A. Clin. Breast Cancer, **9**, Suppl 2, 2009, p. S73.
6. CHOLKAR, K., TRINH, H.M., PAL, D., MITRA, A.K., Expert Opin Drug Discov, **10**, 3, 2015, p. 293-313.
7. BOUCHTON B., CHALAM K.V., EINER A., FRAUNFELDER R., Clinical Update, 2013, p.27-29.
8. PARKKARI, M., PAAKKALA, A.M., SALMINEN, L., HOLLI, K., Acta Ophthalmol. Scand., **81**, 2003, p. 495-499.
9. NAYFIELD, S.G., GORIN, M.B., J Clin Oncol., **14**, 1996, p. 1018-1026.
10. NITULESCU, G. M., MATEI, L., ALDEA, I. M., DRAGHICI, C., OLARU, O. T., BLEOTU, C. Arab. J. Chem., 2015.
11. ANUTA, V., NITULESCU, G. M., DINU-PIRVU, C. E., OLARU, O. T. Molecules, **19**, 10, 2014, p. 16381.
12. NITULESCU, G. M., DRAGHICI, C., OLARU, O. T., MATEI, L., IOANA, A., DRAGU, L. D., BLEOTU, C. Bioorg. Med. Chem., **23**, 17, 2015, p. 5799.
13. NIÞULESCU, G. M., PAUNESCU, H., DRAGHICI, C., MISSIR, A.-V., COMAN, O. A., FULGA, I. Farmacia, **58**, 2, 2010, p. 190.
14. NIÞULESCU, G. M., NEDELCU, G., BUZESCU, A., VELESCU, B. [a]., OLARU, O. T. Stud. Univ. Vasile Goldis Arad, Ser. Stiint. Vietii, **25**, 2, 2015, p. 79.
15. NITULESCU, G. M., SORIGA, S. G., SOCEA, L. I., OLARU, O. T., PLESU, V.., Rev. Chim.(Bucharest), **67**, no. 1, 2016, p. 162
16. NITULESCU, G. M., MARGINA, D., JUZENAS, P., PENG, Q., OLARU, O. T., SALOUSTROS, E., FENGA, C., SPANDIDOS, D. A., LIBRA, M., TSATSAKIS, A. M. Int. J. Oncol., **48**, 3, 2016, p. 869-885.
17. FILIMONOV, D. A., LAGUNIN, A. A., GLORIOZOVA, T. A., RUDIK, A. V, DRUZHILOVSKII, D. S., POGODIN, P. V, POROIKOV, V. V Chem. Heterocycl. Compd., **50**, 3, 2014, p. 444.
18. NITULESCU, G. M., DRAGHICI, C., MISSIR, A. V. Eur. J. Med. Chem., **45**, 11, 2010, p. 4914.
19. HOLLIDAY, D. L., SPEIRS, V. Breast Cancer Res., **13**, 2011, p. 215.
20. LAGUNIN, A., STEPANCHIKOVA, A., FILIMONOV, D., POROIKOV, V. Bioinformatics, **16**, nr. 8, 2000, p. 747-748.
21. SOULE, H. D., VAZGUEZ, J., LONG, A., ALBERT, S., BRENNAN, M. J Natl Cancer Inst, **51**, 5, 1973, p. 1409-1416.
22. BEAUDIN, S. G., ROBILOTTO, S., WELSH, J. J. Steroid Biochem. Mol. Biol., **148**, 2015, 96-102.
23. HORWITZ, K. B., COSTLOW, M. E., MCGUIRE, W. L. Steroids, **26**, nr. 6, 1975, 785-795.
24. KEYDAR, I., CHEN, L., KARBY, S., WEISS, F. R., DELAREA, J., RADU, M., CHAITCIK, S., BRENNER, H. J. Eur. J. Cancer, **15**, 5, 1979, p. 659-670.
25. KYTOLA, S., RUMMUKAINEN, J., NORDGREN, A., KARHU, R., FARNEBO, F., ISOLA, J., LARSSON, C. Genes Chromosom. Cancer, **28**, 3, 2000, p. 308-317.
26. NEVE, R. M., CHIN, K., FRIDLYAND, J., YEH, J., BAEHNER, F. L., FEVR, T., CLARK, L., BAYANI, N., COPPE, J. P., TONG, F., SPEED, T., SPELLMAN, P. T., DEVRIES, S., LAPUK, A., WANG, N. J., KUO, W. L., STILWELL, J. L., PINKEL, D., ALBERTSON, D. G., WALDMAN, F. M., MCCORMICK, F., DICKSON, R. B., JOHNSON, M. D., LIPPMAN, M., ETHIER, S., GAZDAR, A., GRAY, J. W. Cancer Cell, **10**, 6, 2006, p. 515-527.
27. KANG, Y., SIEGEL, P. M., SHU, W., DROBNJAK, M., KAKONEN, S. M., CORDON-CARDO, C., GUISE, T. A., MASSAGU, J. Cancer Cell, **3**, 6, 2003, p. 537-549.
28. JAHID, M. J., RUAN, J. Proceedings. (IEEE. Int. Conf. Bioinformatics Biomed)., 2013, p. 440.
29. PENHA, F.M., RODRIGUES, E.B., MAIA, M., FURLANI, B.A., REGATIERI, C., MELO, G.B., MAGALHAES, O. Jr., MANZANO, R., FARAH, M.E., Ophthalmic Res., **44**, 4, 2010, p. 205-224.

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