Abstract # 1317

Antiprogestin Drug Development: In Vitro Validation of a Potential Clinical Biomarker

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Abstract:

Antihormonal agents (AH) are some of the most clinically useful anticancer drugs. AH resistance and the availability of alternative treatments have made patient selection increasingly important for AH drug development. We hypothesized that the progesterone receptor (PR) may be constitutively active because of the aberrant biological pathways and that selecting patients with baseline tumor PR activation would predict for antiprogestin activity. Activated PR (APR) can be visualized in nuclei as fluorescent aggregates via fluorescent green protein engineered PR cell lines in vitro and by immunofluorescence (IF) in tissues. The detection of PR foci has been associated with transcription and gene activation in vitro. PR phosphorylation (phos) is induced by a variety of pathways. We have developed a IHC method that allows APR determination in tumor biopsies on a routine basis (SABCS 2012). The predictive nature of the observation of APR by IHC in vitro has been tested with onapristone (ONA) for cell survival and proliferation. Methods: 8 human breast cancer (BT-474, CAMA-1, EVSA-T, HCC-1954, MCF-7, MDA-MB-231, T-47D, ZR-75-1) and 2 human endometrial cancer (Ishikawa, HEC-1-A) cell lines were studied. Cytoblocks for APR analysis were made for each time point and experimental condition, baseline ER/PR expression was determined by IHC and PR phos was analyzed with specific antibodies (pSER162, 190, 294, 400, 554) by Western Blotting and IHC. Culture conditions: normal and stripped FBS, +/- ONA and growth factor/hormone exposure (EGF, FGF2, E2 and P4) in triplicate. Analysis was performed at baseline (BL), 6h, 96h and 7 days. Cell viability was assessed by MTS assay, proliferation was studied by Ki67 analysis. APR foci were determined by IHC and read by a pathologist blinded to the experimental conditions. Results: At BL only two cell lines were PR pos/APR pos (T47D, CAMA-1) and all other cell lines where PR pos/APR neg (BT-474, EVSA-T, HCC-1954, MCF-7) or PR neg/APR neg (Ishikawa, HEC-1-A, MDA-MB-231, ZR-75-1). ONA exerted inhibitory effect only in APR pos cell lines. In stripped FBS at 6h, T47D APR pos status was converted to APR neg by ONA except with E2 stimulation which required longer treatment. The CAMA-1 data are not yet available. No APR neg cell lines were converted into APR pos except in one experiment after protracted exposure to EGF. ONA generally decreased phosphorylation and did not correlate to growth inhibition. Phos status determined by IHC and Ki67 proliferation assessment are currently in process and will be presented with the confirmatory data. Conclusions: Baseline APR positivity is predictive of ONA activity in vitro. ONA converts APR pos cells to APR neg within 6h in most cases, a time frame consistent with PR biology. The growth inhibitory effects of ONA were not correlated to PR phos in the cell lines and conditions tested. The APR IHC methodology may be applicable in the clinical setting.

Background:

The determination of steroid hormone receptor status is routine in the treatment of certain hormone-dependent cancers. However, numerous patients fail to respond to existing hormone treatments and no factors have consistently been associated with clinical treatment failure. There are two forms of the PR: PR A which is thought to be the main regulator in endometrial tissue and PR B which is necessary for normal breast development. The two receptors PR A and PR B have a balanced expression in normal human tissues. However, in malignant tissues there is a dysregulation of the PR and some cancers may express only PR A or PR B. Advances in research techniques has enabled the identification of the functional or activated PR. Figure 1 is normal endometrium in the first phase of the menstrual cycle; in the absence of Pg, nuclei have a diffuse fluorescent staining for PR. Figure 2, in the presence of Pg, the PRs migrate to form subnuclear aggregates or nuclear foci, with a mottled heterogeneous pattern, indicative of activated PR (APR).

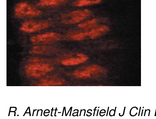


Fig.1

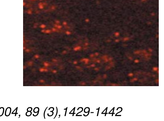


Fig.2

R. Arnett-Mansfield J Clin Endo & Met, 2004, 89 (3),1429-1442

Our hypothesis is that the PRs are constitutively active in a large proportion of PR positive tumors, and that this activation is driven by multiple mechanisms. Various kinase and growth factor pathways phosphorylate the PR, thereby modulating PR ligand affinity, turnover and sumoylation. While the observation of APR foci indicates that an antiprogestin may effectively antagonize activated PR in these cells, in contrast, the expression of PR in a non-functional form is indicative of a non-activated PR pathway and therefore antiprogestins may not have a therapeutic effect. The outlined experiments were conducted to demonstrate that the activity of ONA is associated with the APR status. The IHC methodology as described in SABCS 2012, Abstract # 723, Poster # P1-07-11 was used to determine the APR status of the tested cancer cell lines and to determine the APR status of tumor samples (Fig 3&4). Cytoblocks were prepared from the cell lines, then processed as biopsies and analyzed by IHC. Several cell lines were used in order to have different baseline APR status and different biologic characteristics when exposed to a variety of "growth" factors and culture conditions. ONA was used in this experiment, as the APR biomarker is being developed as a potential companion diagnostic for this type 1 antiprogestin.

PR IHC in cancer biopsies: Three APR nuclear morphology patterns are observed; 1. a diffuse (D) pattern of PR throughout the nucleus, indicative of non-activated PR, 2. a pattern of large and heterogeneous aggregates (A) of PR in the nucleus indicative of activated PR, 3. a mixture of A and D cells, or A cells with smaller aggregates heterogeneously expressed and sometimes with some diffuse staining component. These PR nuclear morphological patterns were observed with standard high powered bright field microscopy.

Immunochemistry with anti-PR antibodies:

Fig.3 shows breast cancer cells with a diffuse PR staining (APR negative).

Fig.4 shows an heterogeneous PR nuclear staining (APR positive).

Fig.3

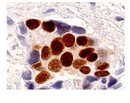
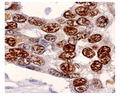


Fig.4



Material and Methods:

(Detailed method on request) Cell lines characteristics are described in Table **1** . Tumor cells were grown as monolayer in various conditions as listed on Tables 2 and 3. Samples were processed for cytoblock evaluation of the PR nuclear foci, (75 cm^2 flasks), WB (25 cm^2 flasks) and cytotoxic assays (6-well plates), samples were treated with "growth" factors and ONA. Collection/analysis times were 6 h, 4 days, and 7 days. Cell line viability was determined using a MTS assay at day 7. IHC cytoblocks were prepared in a modified fashion using Cytoblock® Kit (Thermo Fisher Scientific). IHC was conducted for PR A, PR B and ERα with specific commercially available antibodies (Novocastra). PR and APR analysis were made using a Zeiss Axioscope at x100 magnification. All cytoblocks were stained by HES in order to estimate to cellular density. Staining of cytoblocks to observe PR nuclear morphological patterns was done without background staining. The percent of PR stained cells was assessed relative to "ghost" i.e. PR negative cells. PR positive stained cells were then described as having a diffuse or aggregated pattern and their relative percentage was recorded. Antibodies directed at PR-phosphorylated sites (pSER190, 294, 400 and 554) were studied by WB, these antibodies were not found usable for cytoblocks and were tested in tissue biopsies with background staining to characterize their possible utility. Cytoblocks were also stained for ki67.

Table 1: Determination of ER and PR Positivity by standard IHC

		Reported Phenotype		Observed Phenotype		
Cell Line	Origin	ER	PR	ER	PR A	PR B
EVSA-T	Breast Cancer	Neg.	Pos.	Pos.	Pos.	Pos.
HCC1954	Breast Cancer	Pos.	Pos.	Pos.	Pos.	Neg.
MCF7	Breast Cancer	Pos.	Pos.	Pos.	Pos.	Pos.
MDA-MB-231	Breast Cancer	Neg.	Neg.	Neg.	Neg.	Neg.
T47D	Breast Cancer	Pos.	Pos.	Pos.	Pos.	Pos.
BT-747c	Breast Cancer	Pos.	Pos.	Neg.	Neg.	Pos.
CAMA-1	Breast Cancer	Pos.	Pos.	Pos.	Pos.	Pos.
HEC-1-A	Endometrial Cancer	Neg.	Neg.	Pos.	Neg.	Neg.
Ishikawa	Endometrial Cancer	Neg.	Neg.	Neg.	Neg.	Neg.
ZT-75	Breast Cancer	Neg.	Neg.	Neg.	Neg.	Neg.

Results:

The cell line growth +/- ONA, for EGF, FGF2, E2 and Pg and two controls (vehicle EtOH, RPMI) are presented on Fig 5 and Table 2 (stripped FBS); in two separate experiments (exp 1, exp 2). In charcoal stripped FBS, T47D and CAMA-1 were stimulated by Pg and E2. ONA had a strong inhibitory effect in the T47D cell line when stimulated by E2 and Pg. At the 100 nM concentration ONA also an inhibitory effect in the second experiment in the CAMA-1 cell line stimulated with Pg but no effect on E2 stimulated proliferation. Table 1 shows the ER and PR phenotype of the cell lines by conventional IHC. Generally, a decrease of PR phosphorylation was associated with ONA treatment (Table 4). Cytoblocks processed for IHC determination of APR were performed in parallel in experiment 1 for every condition and each time point. The APR biomarker results are shown on Table 3 for representative cell lines.T47D, CAMA1 and MCF7 cell lines have been fully analyzed at the 6 h and 4 d time points. Other cell lines were either (i.) PR negative or (ii.) PR positive and of D APR phenotype; and their status did not change. PR and APR assessments were always characterized separately with anti-PR A and anti-PR B antibodies. Fig 6 shows examples of MCF7, which has a non-functional PR determined by IHC and was not inhibited by ONA, and T47D which has the APR biomarker, and is inhibited by ONA. With T47D, the APR biomarker significantly decreases after 6-hours of exposure to ONA.

In the tested T47D cell lines, the cellular density of the cytoblocks was scored from 1 to 3 (by HES). There is a decrease in cellular density scores from 2 to 1 (i.e. decreased by approximately 1/3), when comparing cytoblocks not exposed to ONA (all score 2) or exposed to ONA (2/6 blocks score 2, p = 0.01) at the 6 hour time point (data not shown).

Table 3 shows for the 6 hour and 4 day time point, the percent of PR positive cells, and for the PR positive cells the proportion with the A or D pattern PR nuclear morphological pattern. There was no relationship between PR positivity % and ONA exposure.
The actual % of observed APR was obtained by multiplying the percent of APR cells/PR Positive cells X the percent of PR stained cells/ total cells.

Table 2: Ratio treated ONA / Control

		Serum DCC	
		Exp 1	Exp 2
MCF-7	EGF 10 ng/ml	2,10	3,35
	FGF2 5 ng/ml	2,02	2,11
	E2 10 nM	1,46	1,20
	Prog. 30 ng/ml	1,30	1,41
MDA-MB-231	EGF 10 ng/ml	1,07	1,00
	FGF2 5 ng/ml	0,75	0,82
	E2 10 nM	1,13	0,90
	Prog. 30 ng/ml	0,96	0,98
T47-D	EGF 10 ng/ml	2,04	2,36
	FGF2 5 ng/ml	3,01	2,19
	E2 10 nM	4,80	2,15
	Prog. 30 ng/ml	9,36	2,26
BT-474	EGF 10 ng/ml	2,61	2,54
	FGF2 5 ng/ml	1,99	1,64
	E2 10 nM	1,60	1,99
	Prog. 30 ng/ml	1,40	1,57
CAMA-1	EGF 10 ng/ml	2,51	1,74
	FGF2 5 ng/ml	2,43	3,40
	E2 10 nM	4,52	4,26
	Prog. 30 ng/ml	3,83	3,57

Table 3: APR Biomarker status is determined by sub nuclear PR foci detection as D (Diffuse or Non Functional) or AD (Mixed of Aggregated and Diffuse pattern, or Activated). Baseline, 6 Hours and 4 Days after "growth factor" stimulation .

	6 Hours												Day 4											
	PR B						PR A						PRB						PR A					
	Control			Onapristone			Control			Onapristone			Control			Onapristone			Control			Onapristone		
	Pos.(+)	A	D		A	D	Pos.(+)	A	D	Pos.(+)	A	D	Pos.(+)	A	D	Pos.(+)	A	D	Pos.(+)	A	D	Pos.(+)	A	D
CAMA1	20	A	D	No Cell			0			0	0		1			D	<1		0	<1		100	1	
	<5		D	5		D	0			0	0		1			D	0		0	<1		100	<1	
	No Cell			No Cell			0			0	0		1			D	5		D	<1		100	<1	
	<5		D	0		D	0			0	0		0			0	<1		D	<1		100	<1	
	0	0		<5		D	0			0	0		<1			D	0		0	<1		100	0	
	No Cell			<5		D	0			0	0		0			0	<1		D	<1		100	0	
T47D	90	30	70	90	0	100	90	60	40	60	40	60	30	0	100	90	0	100	40	10	80	90	0	100
	90	40	60	80	0	100	90	70	30	60	70	30	55	0	100	70	0	100	30	0	100	60	0	100
	90	30	70	90	20	80	90	70	30	80	80	20	80	0	100	20	0	100	70	30	60	70	0	100
	100	50	50	70	0	100	90	80	20	60	55	45	90	0	100	80	0	100	90	0	100	90	0	100
	80	30	70	90	30	70	60	70	30	70	80	20	60	0	100	50	0	100	80	0	100	50	0	100
	80	10	90	80	60	40	80	70	30	75	70	30	40	0	100	60	0	100	30	0	100	60	0	100
MCF7	10	0	100	0	0	0	0	0	0	0	0	0	5	0	100	0	0	0	0	0	0	0	0	0
	10	0	100	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	10	0	100	10	0	100	0	0	0	0	0	0	50	0	100	40	0	100	0	0	0	40	0	100
	10	0	100	0	0	0	0	0	0	0	0	0	20	0	100	20	0	100	0	0	0	0	0	0
	15	0	100	5	0	100	0	0	0	0	0	0	5	0	100	0	0	0	0	0	0	0	0	0
	5	0	100	0	0	0	0	0	0	0	0	0	0	0	0	5	0	100	0	0	0	0	0	0

In addition to the decreased cellularity at 6 hours, there was a decrease of actual APR % rate associated with ONA exposure for both PR A and B (p=0.05). (Data was paired by factor treated vs. not treated with ONA). At day 4, cellular density was poor (all score = 1), all APR status were D (12/12 cytoblocks) for PR B. For PR A ONA treatment resulted in a APR D phenotype, and 2/6 controls were APR A phenotype.

In a separate data set, Ki67 was not correlated to total PR positivity, total ER positivity, cellularity score, activated PR B rate but was correlated to activated PR A rate (p=0.07).

For CAMA1, cytoblock cellularity was poor and were PR A negative and weakly PR B (≤ 20%). APR was observed in 2/6 control cytoblocks at 6 hours, and 1/6 control cytoblocks at day 4 day.

The MCF7 cell was s either PR positive and D PR nuclear morphology pattern; or PR negative. All other PR positive cell lines were of the D PR nuclear morphology pattern and insensitive to ONA.

In breast cancer tumor biopsies, the pSER190 antibody stained tumor and normal epithelial cells and appeared similar to standard PR IHC staining. The pSER400 antibody rarely stained epithelial cells (normal or malignant cells) and stromal fibroblasts. pSER554 had the same profile as pSER400 and weakly stained endothelial cells. pSER294 antibody staining was negative.

Fig.5: Stimulation of cell lines by Growth factors and Hormones (Ratio of Growth Factor Effect/Control)

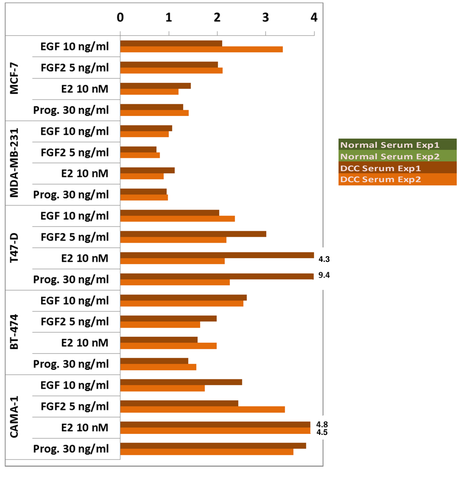
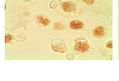
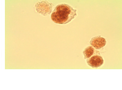


Fig. 6: The MCF7 cell line has no APR biomarker at baseline (Pic.1) or after 6h of Pg (Pic. 2). In contrast, T47D has the APR biomarker at baseline (Pic. 3), after 6h of EGF and ONA, the mottled PR nuclear pattern disappears (APR negative, Pic. 4).

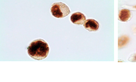
Pic. 1



Pic. 2



Pic. 3



Pic. 4

Table 4: Western Blot analysis of T47D and MCF-7 cell lines at different time points. The expression of the two PR forms A and B were analyzed together with their phosphorylation status. The tables show the difference of levels observed in the treated conditions compared to vehicle only (blue = increased, red = decreased phosphorylation).

	Serum	T47D										MCF7									
	Induction	Veh.		Prog		E2		EGF		FGF2		Veh.		Prog		E2		EGF		FGF2	
	Onapristone	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM	0	1 μM
H6	Total PR	--	--	--	-	--	-	-	-	--	--	++						+	+	+	
	Form B PR		-							--	--	+	--			--	--	--	--	--	--
	pS190		-				-	-	-	-	--										
	pS400			+	+															+	+
	pS554	--	--	--	--	--	--	--	--	--	--			-	-	-	-	-	-	-	-
D4	Total PR	--	--	--	--	--	--	--	--	--	--	--	--	+	-	+	--	--	--	--	--
	Form B PR	--	--			--	--	--	--	--	--	--	--	+	--	+	--	--	--	--	--
	pS190	--	--	-	-	--	--	--	--	--	--	--	--	--	--	-	-	--	--	--	--
	pS400	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	pS554					-	-											+	+		
D7	Total PR	--	--	--	--	-	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
	Form B PR	--	--	--	--	-	--	--	--	--	--	--	--					--	--	--	--
	pS190	--	--					--	--	--	--							--	--	--	--
	pS400	--	--	--	--			--	--	--	--										
	pS554	--	--	--	--			--	--	--	--	--	--	-	--	-	-	-	-	--	--

++ Increased PR Phosphorylation
+ Slight Increase
No Change
- Slight Decrease PR Phosphorylation
-- Decrease
--- Very Low Expression
Uninterpretable

Discussion:

In stripped FBS, the APR biomarker was positive at baseline in two cell lines, T47D and CAMA-1. BT-474 was APR positive in one control but not in two others (Table 3). All other PR positive cell lines (EVSA-T, MCF7, HCC-1954) were APR negative. In stripped PBS, ONA demonstrated an anti-proliferative and/or cytotoxic effect in all APR positive cell lines and was inactive in all APR negative cell lines and PR negative cell lines (Tables 1 & 3). In stripped FBS conditions, where exposure to growth factors is standardized, ONA demonstrated an anti-proliferative effect with E2 and Pg stimulated cell lines which were APR positive (T47D and CAMA-1), no such effect of ONA was observed in the stimulated cell lines which were APR negative. Baseline APR is predictive of ONA's anti-proliferative activity. At the 6h time point, ONA decreased the cellular density and the APR expression of the APR positive/negative mixed T47D phenotype. For the T47D cell line, at the day 4 time point, ONA treatment resulted in the APR negative phenotype in all experimental conditions. For all cell lines which were APR negative at baseline, no change in APR status was observed at 6 hours. These short-term changes in APR status are consistent with the time of transcription activation or inhibition in vitro, and support the notion that APR status reflects a biological process. In parallel, modification of PR phosphorylation was influenced by several growth factors and conditions.

Conclusion:

The presence of the APR biomarker was identified in different cell lines and the hypothesis that APR positivity is predictive of ONA activity is supported by the data. ONA had activity in all APR positive cell lines, while none of the cell lines without APR positivity were associated with ONA activity. Furthermore, the APR positivity was converted into a non-functional APR by ONA supporting its biological relevance. The APR testing may be predictive for the efficacy of ONA in patients with APR positive tumors.