Attenuation of inflammation and expansive remodeling by Valsartan alone or in combination with Simvastatin in high-risk coronary atherosclerotic plaques


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ABSTRACT

Aims: We investigated the role of Valsartan (V) alone or in combination with Simvastatin (S) on coronary atherosclerosis and vascular remodeling, and tested the hypothesis that V or V/S attenuate the pro-inflammatory effect of low endothelial shear stress (ESS).

Methods: Twenty-four diabetic, hyperlipidemic swine were allocated into Early (n = 12) and Late (n = 12) groups. In each group animals were treated with Placebo (n = 4), V (n = 4) and V/S (n = 4) and followed for 8 weeks in the Early group and 30 weeks in the Late group. Blood pressure, serum cholesterol and glucose were similar across the treatment subgroups. ESS was calculated in plaque-free subsegments of interest (n = 109) in the Late group at week 23. Coronary arteries of this group were harvested at week 30, and the subsegments of interest were identified, and analyzed histopathologically.

Results: V alone or with S reduced the severity of inflammation in high-risk plaques. Both regimens attenuated the severity of enzymatic degradation of the arterial wall, reducing the severity of expansive remodeling. V alone or with S attenuated the pro-inflammatory effect of low ESS.

Conclusions: V alone or with S exerts a beneficial effect of reducing and stabilizing high-risk plaque characteristics independent of a blood pressure- and lipid-lowering effect.

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1. Introduction

Therapies that reduce the adverse metabolic and proliferative milieu within an atherosclerotic lesion would be likely to reduce the progression of atherosclerosis. Angiotensin II receptor type 1 blocking agents (ARBs), like Valsartan (V), may enhance a vascular protective environment by normalizing endothelial function and reducing low density lipoprotein-cholesterol (LDL-C) oxidation and inflammatory cell infiltration [1,2]. Statins, like Simvastatin (S), in addition to their effects on lowering LDL-C, also exert a variety of pleiotropic effects on many components of atherosclerosis, including endothelial function and nitric oxide bioavailability, inflammatory cell migration, and plaque thrombogenicity, ultimately promoting plaque stability [3]. Since the mechanisms of action of V and S are different, it is possible that the antiatherosclerotic effects of these two agents will be complementary or even synergistic [4].

The nature and clinical significance of an atherosclerotic plaque is dependent not only on the formation and progression of atherosclerosis, but also on the vascular remodeling response to that atherosclerosis [5]. Expansive remodeling is associated with high-risk plaques, whereas constrictive remodeling is associated with stable fibrous plaques [5–7]. A dynamic process of extracellular matrix (ECM) protein synthesis and breakdown modulates the nature of the vascular remodeling response to plaque growth. The first two aims of the present work were to test the hypothesis that V either alone or in combination with S (a) exerts a plaque stabilizing effect by attenuating the high-risk plaque characteristics, and (b) reduces the severity of expansive remodeling by attenuating the inflammation and the ECM degradation within the arterial wall.

Low endothelial shear stress (ESS) is a major determinant of the localization and progression of atherosclerotic lesions [8,9]. Recent
histopathology studies have indicated that the magnitude of low ESS, as well as changes in ESS and the rate of change in ESS also determines the severity of plaque inflammation leading to formation of high-risk plaques [5–7,10]. The third aim of our work was to test the hypothesis that V alone or in combination with S attenuates the local pro-inflammatory effect of low ESS.

We utilized a well-established diabetic hyperlipidemic swine model capable of developing human-like atherosclerotic plaques [11]. Two complementary approaches were employed to assess the natural history of atherosclerosis and the effects of V and V/S on that natural history: (a) Serial in vivo vascular profiling, which is a methodology utilizing coronary angiography and intravascular ultrasound (IVUS) as described below. Vascular profiling enabled us to identify regions of interest with low baseline ESS, which provides an efficient and effective means to focus on areas where high-risk plaque will form. (b) Ex vivo histopathology of those regions of interest at the time of animal sacrifice enabled us to investigate the effect of the active agents on vascular inflammation and remodeling.

2. Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). A detailed description of the methods is presented in Online supplement. Briefly, 24 male Yorkshire swine were rendered diabetic and fed a high-fat diet supplemented with sucrose in quantities titrated to maintain serum total cholesterol (TC) and blood glucose (BG) levels between 500–700 and 150–350 mg/dl, respectively [11]. The pigs were allocated into two groups: Early Atherosclerosis group (n = 12) and Late Atherosclerosis group (n = 12) to study the early and late manifestations of atherosclerosis, respectively (Fig. I, Online Supplement). In each group the animals were assigned to one of three treatment subgroups: four were given placebo (P), four were treated with 320 mg Valsartan (V) daily, and four were treated with the combination of 320 mg Valsartan with 40 mg Simvastatin (V/S) daily. Intracoronary vascular profiling methods (i.e. IVUS and angiography) [8] were performed for assessment of local ESS along the lumen surface of 3D-reconstructed coronary arteries at weeks 4 and 8 after the induction of diabetes and initiation of high-fat diet in the Early group, and at weeks 23 and 30 in the Late group (Fig. I, Online Supplement). The progression of plaque burden and severity over time in the reconstructed arteries of each treatment subgroup were estimated by IVUS.

To investigate the effect of medication on lesion formation, progression, and differentiation to more advanced plaques, we focused on the Late group. In these arteries, we calculated ESS at baseline (week 23) to identify arterial regions where future plaque may develop and 3 mm long subsegments of interest from different ESS magnitude were selected. To be considered in the analysis, each subsegment of interest was required to be free of apparent atherosclerotic plaque at baseline, defined by IVUS as maximum intima–media thickness (IMT) ≤ 0.5 mm [5]. The animals were sacrificed immediately after the follow-up vascular profiling, and the epicardial portions of the major coronary arteries were harvested, snap frozen in liquid nitrogen and maintained at −80 °C. To locate the subsegments of interest on the harvested arteries two to three major and readily visible side branches were identified on the ESS and IMT maps, which were derived by the 3D-reconstructed arteries, and used as references [6]. The same branches were also identified on the harvested arteries by utilizing magnifying lenses (2×). Using these branches as landmarks the exact location of each subsegment of interest was then identified on the preserved coronary arteries. Subsegments of interest were frozen cut at the middle and cryosectioned (7 μm). Verhoeff’s elastin, oil red O, and picrosirius red staining, as well as CD45 immunostaining were performed in each cryosection for the assessment of intima-to-media ratio (IM), lipid accumulation, collagen content, and inflammatory cell infiltration, respectively. Oil red O, picrosirius red and CD45 positive intimal areas are presented either as absolute areas (mm²) or as percent of the intima. To investigate the effect of medication on the heterogeneity of the natural history of atherosclerotic lesions at follow-up (week 30), the lesions were histopathologically classified into three categories [6,12]: (a) minimal lesions with IM < 0.15, representing minimal depositions of lipids and inflammatory cells into the intima, (b) intermediate lesions, representing larger masses of lipid-laden inflammatory cells without fibrous cap and IM ≥ 0.15, and (c) FAs, representing severely inflamed atherosclerotic plaques with a thin fibrous cap covering a large necrotic lipid core. The integrity of the internal elastic lamina (IEL) beneath the atherosclerotic intima was assessed in Verhoeff’s elastin-stained sections and categorized into four grades [6] (grade 1: intact, well-organized IEL; grade 2: IEL with a few breaks; grade 3: IEL with many breaks but intact media; grade 4: IEL with severe fragmentation associated with underlying media degradation). Gene expression of the major ECM degrading enzymes (MMPs and cathepsins) and their inhibitors (TIMPs, cystatin C) was measured in each lesion by applying real-time PCR.

The nature of the remodeling response to plaque growth in each arterial subsegment of interest was assessed by comparing the local remodeling behavior of each individual subsegment with the global remodeling response of the entire artery, as previously described [6,13]. Three local remodeling patterns were defined: (a) excessive expansive remodeling, (b) compensatory expansive remodeling, and (c) inadequate remodeling.

2.1. Statistical analyses

All analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL) and Stata 10.0 (StataCorp LP, College Station, TX). Continuous variables with normal distribution are summarized as mean ± S.E.M., non-normally distributed variables as median and 25th and 75th percentiles, and categorical variables as actual numbers and percentages. To correct for systematic error introduced by the clustering of arterial subsegments within animals, several statistical methods were used. First, to investigate the association of continuous variables (e.g. histopathologic characteristic, normalized gene expression) with categorical variables (e.g. treatment subgroup, lesion category, remodeling pattern, IEL grade) mixed-effects ANOVA with the animal as random effect was used. Second, for analyses in which the dependent variable (e.g. IMT) was measured at baseline and follow-up, repeated-measures ANOVA was employed, and the animal and artery were specified as random effects. Finally, where the dependent variable was categorical (e.g. remodeling pattern), ordered logistic regression was implemented and the standard errors of the regression coefficient were adjusted for clustering of arterial subsegments within animals with the Huber White Sandwich Estimator. In all analyses, p values were adjusted for multiple comparisons of data using either the Scheffe or modified Bonferroni methods. Findings were considered to be statistically significant at the 0.05 level.

3. Experimental results

One pig in the Early group and one pig in the Late group died before the completion of follow-up. Two additional pigs in the Late group were excluded from the study because their plasma TC con-
centrations fell outside the 95% CI of TC distribution at follow-up. Thus, 11 pigs (4 on P, 3 on V and 4 on V/S) in the Early group, and 9 pigs (4 on P, 2 on V and 3 on V/S) in the Late group were studied. Thirty-two coronary arteries were profiled in the Early group (left anterior descending, LAD, \(n = 10\); left circumflex, LCx, \(n = 10\); obtuse marginal, OM, \(n = 1\); right coronary artery, RCA, \(n = 11\)), and 25 coronary arteries in the Late group (LAD, \(n = 8\); LCx, \(n = 8\); OM, \(n = 1\); RCA, \(n = 8\)).

Baseline and follow-up characteristics of the animals in each medication subgroup are shown in Table I (Online Supplement). There was no significant difference with regard to weight, TC, BG and systolic blood pressure across the medication subgroups within the Early and Late groups.

3.1. Effect of V and V/S on the progression of plaque burden and severity

Regardless of the medication assignment, the atherosclerotic burden by IVUS significantly increased after week 8 (Fig. 1a). Maximum IMT by IVUS was classified into four grades, and the percent length of the reconstructed artery occupied by each grade was estimated in each medication subgroup (Fig. 1b–d). Pigs on V and V/S developed very few grade 2 plaques, and no grade 3 plaques at weeks 23 and 30. In marked contrast, plaques in pigs treated with P progressed to more advanced grades after week 8, leading to marked heterogeneity in plaque severity at weeks 23 and 30.

3.2. Effect of V and V/S on plaque histopathologic characteristics

In the Late group 109 subsegments of interest (\(n = 46\) in P animals, \(n = 28\) in V animals, and \(n = 35\) in V/S animals) were identified at week 23 (approximately four to five subsegments per artery) and were cryosectioned and histopathologically stained at week 30. Compared to P animals, lesions in animals treated with the combination V/S were of comparable size (IM) but with significantly lower lipid deposition, plaque inflammation, and collagen content (Table II, Online Supplement). Compared to P-treated animals, animals treated with V only exhibited significantly less intimal inflammation, while no effect was observed on plaque size, lipid deposition, and collagen content (Table II, Online Supplement).

3.3. Effect of V and V/S on the development of inflamed high-risk plaques

Based on histopathology characteristics atherosclerotic lesions at follow-up were classified into three categories, representing distinct stages of their natural history: minimal lesions (\(n = 25, 22.9\%\)), intermediate lesions (\(n = 53, 48.6\%\)), and FAs (\(n = 31, 28.4\%\)). FAs that developed in animals treated with V or V/S had significantly less inflammation than those FAs that developed in P-treated animals, whereas there was no significant difference in terms of lipids and collagen content across the medication subgroups (Fig. 2). Accordingly, in the intermediate lesions there was no significant difference in plaque size, lipids, inflammation and collagen content across the medication subgroups (Fig. 2). These results suggest a stabilizing role of drugs especially in inflamed high-risk coronary plaques.

3.4. Effect of V and V/S on IEL integrity

The IEL integrity was classified into four grades and correlated with the severity of inflammation in each medication assignment. In plaques with severe IEL disintegration (i.e. grade 3) V and V/S significantly limited the inflammation compared to P (Fig. 3a).

3.5. Effect of V and V/S on vascular remodeling

Three local remodeling responses were identified in the subsegments of interest, i.e. excessive expansive remodeling (\(n = 23, 21.1\%\)), compensatory expansive remodeling (\(n = 56, 51.4\%\)), and inadequate remodeling (\(n = 30, 27.5\%\)), and were correlated with...
the histopathology in each medication subgroup. Ordered logistic regression analysis with remodeling pattern as the dependent variable showed that subsegments with excessive expansive remodeling were associated with more severe IEL disintegration than subsegments with inadequate remodeling ($p = 0.002$; Fig. 3b). In subsegments with excessive expansive remodeling V either alone or in combination with S reduced the severity of inflammation; in addition V/S only reduced the lipid and collagen content (Fig. 4a–d). Likewise, in subsegments with inadequate remodeling V and V/S reduced the severity of inflammation without, however, affecting the plaque size, and the lipid and collagen content (Fig. 4a–d). There was no significant effect of V and V/S on the histopathologic characteristics of subsegments that exhibited compensatory expansive remodeling (Fig. 4a–d). These results reveal a stabilizing role of V alone or in combination with S in subsegments with excessive expansive remodeling.

### 3.6. Effect of V and V/S on gene expression of ECM degrading enzymes

In subsegments with excessive expansive remodeling V either alone or in combination with S tended to attenuate the gene expression of MMP-9 (Fig. 5). This effect of medication on the expression of MMP-9 was associated with a significant reduction in the MMP/TIMP ratio, indicating that both regimens decrease the ECM degradation within excessively expanded subsegments (Fig. 5). No significant effect of V and V/S on gene expression of MMP-2, TIMP-1, TIMP-2, cathepsins K, L, and S, and cystatin C was observed in subsegments with excessive expansive remodeling. Likewise, there was no significant effect of V and V/S in gene expression of the ECM degrading enzymes and their inhibitors, as well as on the MMP/TIMP ratio in subsegments with compensatory expansive or inadequate remodeling (Fig. 5).
3.7. Attenuation of the pro-inflammatory effect of low ESS by V and V/S

Baseline ESS of the subsegments studied was 1.0 (0.7–1.4) Pa, limits: 0.0–3.5 Pa. To assess the role of medication on the pro-inflammatory effect of low ESS, local ESS was calculated in the subsegments of interest at baseline (week 23) and classified into two categories: low ESS < 1 Pa and moderate/higher ESS ≥ 1 Pa. The effect of medication on the severity of inflammation in lesions that developed in each ESS category was investigated.

In animals treated with P subsegments with low baseline ESS developed plaques with significantly more intense inflammation than subsegments with moderate/higher baseline ESS (Fig. 6). Low ESS subsegments in V and V/S subgroups exhibited lesions with significantly less inflammation than low ESS subsegments in P subgroup, whereas there was no difference in the severity of inflammation in low ESS subsegments between V and V/S animals (Fig. 6). These results indicate that V alone or in combination with S attenuates the pro-inflammatory effect of low ESS.

4. Discussion

In this study we explored in detail the effect of V alone and in combination with S on the early and late natural history of coronary atherosclerosis, as assessed by IVUS and histopathology. The diabetic, hyperlipidemic swine model we used in this study has proven to be an excellent model for studying coronary atherosclerosis in that swine develop regional atherosclerotic lesions similar to humans [11]. We observed, as anticipated, that atherosclerotic lesions are highly focal and develop independently of each other, driven by local hemodynamic factors, in particular low ESS. Using vascular profiling, a well-validated methodology for the assessment of local ESS in vivo [8], we were able to locate subsegments of interest with various severities of atherosclerotic lesions and then study the effect of medication on these lesions.

V alone, as well as in combination with S, decreased the severity and complexity of coronary plaques as identified by serial IVUS studies. Histopathologically, we observed a broad spectrum of atherosclerotic lesions after 30 weeks of diabetes and hyperlipidemia including minimal fatty streaks, intermediate plaques, and severe thin cap FAs. V alone or with S reduced the severity of plaque inflammation, particularly in the high-risk FAs. Also, V and V/S reduced the severity of inflammation and subsequently the enzymatic degradation of IEL and the arterial wall reducing the severity of excessive expansive remodeling.

V, either alone or with S attenuated the pro-inflammatory effect of low ESS, thereby preventing the escalation of the vicious cycle of low ESS, inflammation, excessive expansive remodeling and formation of high-risk plaque. Notably, the levels of TC, BG, and blood pressure across the medication subgroups were similar, suggesting that the anti-atherosclerotic effect of V alone or in combination with S was independent of the lipid- or blood pressure-lowering effect of the drugs.

4.1. Stabilizing role of V alone or in combination with S on high-risk plaques

We demonstrated that V alone or in combination with S reduced the recruitment of inflammatory cells into the intima. Although the anti-inflammatory action of ARBs has also been studied in atherosclerotic plaques of the aorta in the mouse [14–17], rabbit [18], and monkey [19,20], the present study is the first showing such an effect in the coronary arteries. Furthermore, the ability of our atherosclerotic model to develop high-risk coronary atherosclerotic plaque with intense inflammation, large necrotic cores and...
thin fibrous caps gave us the unique opportunity to focus on the role of V alone or in combination with S in the histopathologic characteristics of those plaques. We showed that V alone or in combination with S reduce the severity of inflammation in high-risk plaques, without affecting the plaque size, indicating that these drugs exert their stabilizing effect by altering the composition of the plaques. The anti-inflammatory effect of V is probably regulated by the blockade of the pro-inflammatory angiotensin II type 1 receptors (AT₁), as well as over-stimulation of the anti-inflammatory angiotensin II type 2 receptors (AT₂) by the uncoupled angiotensin II [15,21].

Another interesting finding in our study is that the combination of V with S reduce plaque lipid accumulation, whereas V alone did not exert such an effect. This effect is presumably attributable to the complementary pleiotropic effects of S, supporting the combination of ARBs with statins for more powerful anti-atherosclerotic effect [3,4,22,23].

4.2. Stabilizing role of V alone or in combination with S on vascular remodeling

To the best of our knowledge this is the first study investigating the effect of V alone or with S on IEL degradation and atherosclerotic wall remodeling in coronary arteries. V either alone or with S significantly reduced the severity of IEL disintegration, through reduction in severity of inflammation, suggesting a synergistic anti-inflammatory role of V and S [22,23]. Increased IEL disintegration provides the gateway for the extension of atherosclerosis into the media, where the inflammatory cells produce matrix degrading proteases, which degrade the medial extracellular matrix, leading to excessive expansive remodeling of the wall [6,24]. We demonstrated that V and V/S exert a stabilizing effect by reducing the infiltration of inflammatory cells through the disrupted IEL, thereby attenuating the severity of vascular wall degradation and subsequent excessive expansion.
The molecular mechanisms through which V alone or in combination with S attenuates expansive remodeling had not been previously studied. By performing transcriptional profiling of the major ECM degrading enzymes (MMPs and cathepsins) and their inhibitors (TIMPs and cystatin C) our study showed in vivo that V either alone or with S reduces the mRNA expression of MMP-9, which is actively involved in the ECM degradation [24,25], as well as the MMP/TIMP ratio, thereby shifting the ECM balance towards less degradation. However, V or V/S was not found to exert any effect on the amount of collagen in intermediate lesions or FAs. In vitro and in vivo investigations have demonstrated that ARBs exert an anti-growth and anti-fibrotic effect [1,26]. On the other hand ARBs and statins can reduce the collagen degradation through reduction in inflammation, which is a potent matrix degrading process. Furthermore, a recent study in aortas of atherosclerotic rabbits revealed that candesartan increases plaque collagen [18]. Therefore, the net effect of ARBs and statins on collagen content of a plaque is likely dependent on the balance between the anti-fibrotic effect through inhibition of growth factors and pro-fibrotic effect through inhibition of inflammation. In our atherosclerotic pig model the V- or V/S-mediated anti-fibrotic effect was not enough to destabilize the plaques since this may be counterbalanced by the anti-inflammatory (pro-fibrotic) effect of the drugs. Additional studies are needed to further clarify the true role of ARBs and statins on plaque collagen.

4.3. Attenuation of the pro-inflammatory effect of low ESS by V and V/S

Another novel finding in our study is the attenuation of the pro-inflammatory effect of local low ESS by V alone or in combination with S. Low ESS is a major determinant of the localization, formation and progression of atherosclerotic lesions. In addition, low ESS is associated with the localization and severity of high-risk plaques [5–7,10]. In the present study we observed that inflamed FAs preferentially developed in arterial regions with low ESS in animals treated with P, while in the same low ESS regions, V or V/S prevented this effect by reducing the severity of local plaque inflammation.

On the basis of these findings we propose the following mechanisms of interaction between local hemodynamic environment and development of high-risk plaque which can be perturbed or inhibited by the systemic intervention with V alone or in combination with a statin (Fig. 7): low ESS promotes endothelial dysfunction, lipid accumulation, production of oxidative stress, inflammatory cell recruitment into the intima and plaque development [5]. In the context of inflammation, the IEL underneath the plaque undergoes regional disruption and leads to local expansion of the arterial wall. The expansive wall remodeling perpetuates the initially low ESS environment, thereby fostering a self-perpetuating cycle among low ESS, inflammation and wall expansion, eventually transforming an early lesion to a high-risk plaque with proclivity to rupture [5–7]. As we demonstrate in the current study, V and V/S may block the abovementioned cycle by reducing lipid infiltration, oxidative stress, and inflammation [27], thereby preventing local IEL disruption and excessive expansive remodeling. At the vascular level the attenuation of the excessive local remodeling response by V and V/S may restore the initial pro-inflammatory low ESS stimulus to higher, more physiologic, levels, thereby attenuating the severity of inflammation and plaque rupture. Although the stabilizing effect of V alone or combined with S has not been investigated in human coronary atherosclerotic lesions, our findings may provide a pathophysiologic insight into the reduction of acute coronary syndromes that has been observed in large clinical trials [28].

4.4. Limitations

There are several limitations within the present study. A major limitation is that the independent role of S was not investigated in our model. However, the primary purpose of our study was to shed light into the independent role of V alone in coronary atherosclerosis, as well as into the potential synergism of V with S, and not to investigate the anti-atherosclerotic properties of S alone, which are well-documented in many experimental and clinical studies [3].

Our study was also limited by the small number of animals studied. However, the power of the study increased by investigating multiple subsegments in each coronary artery (average four to five subsegments per artery). In addition, despite the limited number of animals statistical significance was still achieved. Also, there was a selection bias since subsegments of interest were not randomly selected. This bias was limited by intentionally selecting subsegments of different ESS magnitude, so that the entire spectrum of ESS values and atherosclerotic plaques was represented.

Although the anti-atherosclerotic effect of V found in this study would be likely to exert an important clinical effect in man as well, our findings may not be directly applicable to human atherosclerosis; since our experimental model of atherosclerosis was based on induction of diabetes and intense hyperlipidemia to achieve very high levels of plaque inflammation.
In this study we measured only the mRNA expression of matrix degrading enzymes and their inhibitors. While gene expression is most often accompanied by protein expression and activation we are well aware that gene expression by PCR is not synonymous with protein expression. Some genes may not be translated to proteins or if translated the proteins may be inactivated or denatured without evident effect.

The results of the current study refer to V and could not be generalized on the entire class of ARBs. Although it is well accepted that all ARBs exhibit blood pressure-lowering action and vascular protective effects, different ARBs may exhibit different anti-atherosclerotic effects in various experimental models and clinical studies. In this study the experimental animals were treated with the highest recommended dose of V (320 mg daily) [2], and these results cannot necessarily be extrapolated to lower doses of the drug.

5. Conclusion

In this animal study we utilized in vivo vascular profiling to efficiently identify and follow-up coronary artery regions likely to develop high-risk plaques, and assessed the local impact of systemic anti-inflammatory pharmacologic interventions on the histopathologic characteristics of those plaques. We demonstrated that V alone, as well as in combination with S, independently of blood pressure- and lipid-lowering effects, reduces the initiation and progression of severe plaque, and exerts beneficial effects of reducing and stabilizing characteristics associated with high-risk plaque, even in atherosclerosis susceptible regions of the coronary tree with low ESS.

Conflicts of interest

Dr. Peter H. Stone and Charles L. Feldman receive research grants from Novartis Pharmaceuticals Inc. Dr. William Daley was prior employee of Novartis Pharmaceuticals Inc.

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Appendix A. Supplementary data


References